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CEREBROVASCULAR DISEASE RELATED TO HYPERTENSION: EFFECTS OF ANTIOXIDANT AND CHOLINERGIC PRECURSOR MOLECULES

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ABSTRACT

Background. A major risk factor for cerebral vascular disease and cognitive impairment is hypertension. Increased arterial wall thickness and elevated blood pressure cause hypertension. Moreover, many studies have shown that cerebrovascular alterations may cause disease in hypertensive individuals' brains because of an increase in reactive oxygen species, neuroinflammation, and cholinergic dysfunction. The effects of hypertension on the brain lead to cognitive decline and dementia. Increasing reactive oxygen species and impairment of cholinergic pathways are the mechanisms of hypertension-induced cerebrovascular alterations. In spontaneously hypertensive rats as a model of essential hypertension and brain injury, neural alterations, blood-brain barrier dysfunction, and gliosis were observed.

Aim. The study aimed to determine whether choline alphoscerate (GPC) and (+) thioctic acid [(+) TIO], either alone or in combination, were investigated for their potential neuroprotective properties. GPC is a choline-containing phospholipid known for its ability to enhance cholinergic neurotransmission. The (+) TIO eutomer has been shown to exhibit greater antioxidant and anti-inflammatory activity than its racemic counterpart.

Methods. Hypertensive rats aged 24 weeks were treated with GPC and (+) TIO individually or in combination. Normotensive age matched Wistar Kyoto rats were used as control. To study the oxidative status, 4-hydroxynonenal (4-HNE) concentration, protein oxidation was measured in the brain sample. For the assessment of the inflammatory process, the frontal cortex and the hippocampus were collected for western blot and immunohistochemistry investigations of glial, BBB and neuroinflammatory and the cholinergic markers.

Results. In hypertensive rats, the blood pressure was higher than in normotensive ones. After four weeks of treatment with GPC and (+) TIO, slight reductions in systolic blood pressure were observed. OxyBlot in the brain showed an increase of oxidative state proteins in SHR. Based on Western blot and immunohistochemistry studies, GPC alone was able to restore the protein levels in neuronal nuclei. There was no difference between the two compounds regarding the downregulation of synaptic proteins in hypertensive rats. However, GPC and (+) TIO alone or in combination reduced astrogliosis, microglial activation, and decreased levels of the proinflammatory cytokine tumor necrosis factor alpha. It was observed that treatments partially restored the modulation of the blood-brain barrier markers aquaporin-4 and glucose transporter-1 in hypertensive rats. Because of the cholinergic neurotransmission mechanism of GPC, it was able to increase the expression level of vesicular acetylcholine transporter in brain areas of SHR. The increase expression of nAChRα7 with the GPC and the GPC and (+) TIO may suggest a protective effect mediated by the cholinergic anti-inflammatory pathway.

Conclusion. Our findings can contribute to better defining the role of the inflammatory processes of neurovascular unit in brain disorders characterized by vascular impairment. Based on the evidence, treatment with GPC alone and GPC plus (+)TIO attenuates the glial reaction and the neuroinflammation in the two brain areas, providing neuroprotection by the stimulation of the cholinergic pathways. Furthermore, that may be a therapeutic strategy worth exploring in further preclinical and clinical research.

1.INTRODUCTION

1.1 Hypertension

Hypertension, which is caused by an elevation of blood pressure (BP) and increased arterial wall thickness, represents a risk factor for the development of cerebrovascular disease and cognitive impairment. Evidence suggests that hypertension leads to neuroinflammation, which significantly contributes to the physiopathology of cerebrovascular alterations due to the increasing production of reactive oxygen species and cholinergic pathways dysfunction [Kearney et al, 2005]. Hypertension is a disease that develops silently as a result of the interaction of a genotype and specific phenotypes, which modulate cellular signaling pathways, such as central obesity, insulin resistance, or intracellular homeostasis abnormalities, and evolves progressively over time ranging from 10 to 30 years [Piskorz et al., 2020]. According to the study of non-communicable diseases (NCD) risk factor collaboration, the number of adults with high BP increased from 594 million in 1975 to 1.13 billion in 2015 [NCD-RisC., 2020]. According to this survey, hypertension affects more than 40% of adults over the age of 25. Moreover, according to earlier studies 1.56 billion people will be affected by hypertension by 2025, with the prevalence expected to rise by 60% [Kearney et al.,2005]. Due to its frequent lack of overt symptomatology, it is frequently referred to as "the silent killer". Wherein approximately 50% of patients still lack a definitive diagnosis of hypertension [O'Shea et al., 2017].

As blood circulates through arteries, the walls of the major vessels in the body, exerts force on the walls. Typically, chronically high systemic arterial pressure that is above a particular threshold is what defines hypertension [Giles et al., 2009]. BP is represented by two values. The first which refer as systolic - represents the force of the heart's contraction or beat in the blood vessels. The second value called diastolic, measures the pressure in the blood vessels between heartbeats. In accordance with the World Health Organization (WHO), hypertension can be diagnosed if at least two separate blood pressure measurements indicate a systolic blood pressure greater than 140 mmHg and/or a diastolic blood pressure greater than 90 mmHg [Hypertension, WHO.,2021]. Additionally, the American Heart Association (AHA) divided blood pressure into five categories (Figure 1):

Normal: 120 mmHg systolic and 80 mmHg diastolic.

Prehypertension: 120-139 mmHg systolic, 80-90 mmHg diastolic.

Stage 1 hypertension: 140-159 mmHg systolic and 90-99 mmHg diastolic.

Stage 2 hypertension: 160 mmHg or higher systolic and 100 mmHg or higher diastolic.

Stage Emergency (Hypertensive crisis): 180 mmHg or higher systolic and 110 mmHg or higher diastolic.

Furthermore, hypertension can be classified as essential or secondary. In essential hypertension, the cause of high BP cannot be identified, whereas, in secondary hypertension, direct causes such as endocrine disturbances, high-salt intake, obesity, diabetes are identifiable. Approximately 90% to 95% of arterial hypertension is caused by unknown factors, so most cases are "essential", while only 5–10% of patients suffer from secondary forms [Rimoldi et al., 2014].

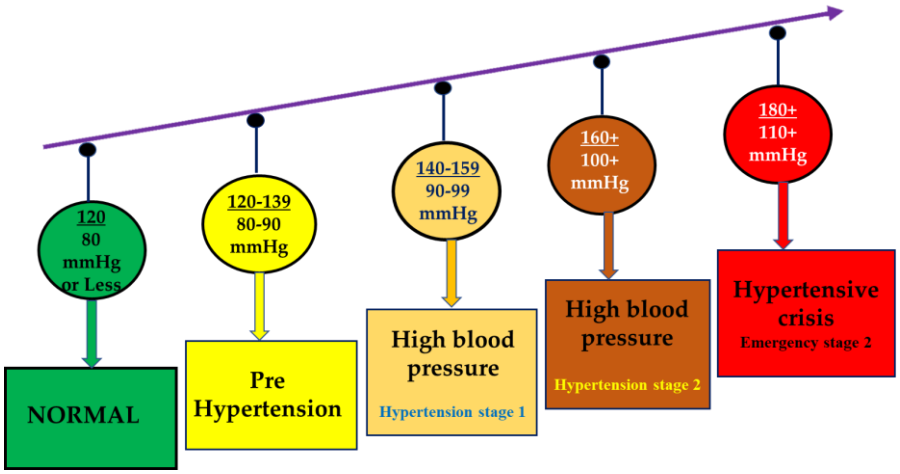

***Figure 1**: Blood pressure levels displayed in different colored with their health risk factor (modified from – Muntner p et al., 2019).*

A specific remediable cause of hypertension can be found in approximately 10% of adults with hypertension, termed secondary hypertension (Table 1). If the cause can be accurately diagnosed and treated, patients with secondary hypertension can achieve normalization of BP or marked improvement in BP control, with concomitant reduction in cardiovascular disease risk. The

majority of patients with secondary hypertension have primary aldosteronism or renal parenchymal or renal vascular disease, whereas the remainder may have more unusual endocrine disorders or drug- or alcohol-induced hypertension [Carey et al., 2018].

Table 1. Cause of secondary hypertension

Disorder	Major Clinical Findings	Prevalence Estimated percent prevalence among adults with hypertension
Primary aldosteronism	Resistant hypertension; hypertension with hypokalemia; hypertension with muscle cramps or weakness; hypertension and incidentally discovered adrenal mass; hypertension and family history of early-onset hypertension or stroke	8–20
Renovascular disease	Resistant hypertension: hypertension with abrupt onset, worsening or increasingly difficult to control; flash pulmonary edema (atherosclerotic cardiovascular disease); early-onset hypertension, especially in women (fibromuscular hyperplasia)	5–34
Renal parenchymal disease	Urinary tract infections; obstruction; hematuria; urinary frequency/nocturia; analgesic abuse; family history of polycystic kidney disease; increased serum creatinine; abnormal urinalysis	1–2
Drug- or alcohol-induced	Sodium-containing antacids; nicotine (smoking); alcohol; nonsteroidal anti-inflammatory drugs; OCs; cyclosporine or	2–4

	tacrolimus; sympathomimetic agents; cocaine, amphetamines, other illicit drugs; neuropsychiatric agents; erythropoietin-stimulating agents; clonidine withdrawal	
Pheochromocytoma/paraganglioma	Resistant hypertension: paroxysmal hypertension or crisis superimposed on sustained hypertension; "spells," blood pressure lability; headache, sweating, palpitations, pallor; family history of pheochromocytoma/paraganglioma; incidentally discovered adrenal mass	0.1–0.6
Cushing's syndrome	Rapid weight gain with central distribution; proximal muscle weakness; depression; hyperglycemia	<0.1
Hypothyroidism	Dry skin, cold intolerance, constipation, hoarseness, weight gain	<1
Hyperthyroidism	Warm, moist skin; heat intolerance; nervousness; tremulousness; insomnia; weight loss; diarrhea; proximal muscle weakness	<1
Coarctation of the aorta	Young patient with hypertension (<30 years of age)	0.1

Modified from: *Carey, R. M. Journal of the American College of Cardiology 2018. 72(11).*

1.1.1 The global epidemiology and risk factor of hypertension

Multiple factors make it crucial to understand epidemiology, not the least, which is the fact that high blood pressure is the most common risk factor for early death and ill health. In addition,

hypertension, which accounts for nearly 10% of all worldwide healthcare spending, has a considerable impact on healthcare expenses. It imposes an enormous financial impact on individuals, households, and healthcare systems [Gaziano et al., 2009]. The prevalence of hypertension in the world is estimated at one billion, and it is the leading cause of death worldwide. The prevalence of hypertension increases with age. In the USA, hypertension affects more than half of people aged 60-69 and over three-fourths of those aged 70 and older. In the ageing population, systolic BP increases and leads to hypertension. In approximately 90% of cases, people who are not hypertensive at 55 or 65 years of age will have hypertension by 80 or 85 years of age [Vasan et al., 2002]. Over time, health patterns around the world are changing due to differences in lifestyles and preventive measures. Therefore, hypertension epidemiology is changing around the world. To analyze high BP prevalence trends, the Noncommunicable Disease Risk Factor Collaboration recently conducted and analyzed 1201 studies. This analysis was performed in over 200 countries involving 104 million participants aged 30–79 years, between 1990 and 2019 [NCD-RisC, 2022]. According to this the global age-standardized prevalence of hypertension was 32% in women and 34% in men in 2019 (Figure 2), the same as in 1990 (32% in women and 32% in men). Global prevalence was stable due to a decrease in high-income countries and in central and eastern Europe for women, but an increase in some low-income and middle-income countries. In 2019, men and women in Canada and Peru had the lowest prevalence of hypertension, followed by Taiwan, South Korea, Japan, and some countries in western Europe for women, and some countries in low- and middle-income categories for men. In all these countries, the age-standardized prevalence for women was under 24% and for men, it was under 25%. The prevalence of hypertension was highest in Central and Eastern Europe, Central Asia, Oceania, Southern Africa, and some Latin American and Caribbean countries (Figure 2).

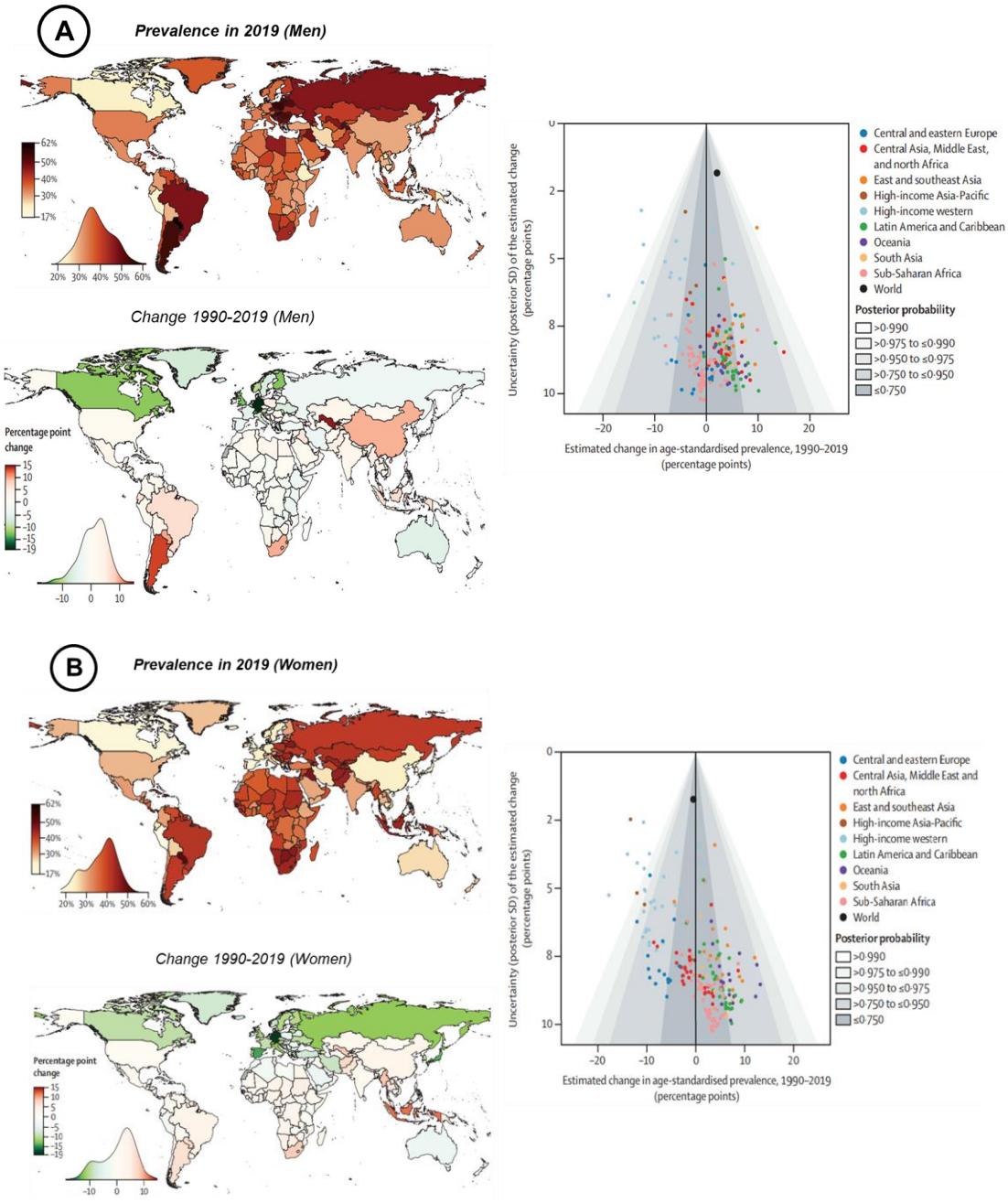

***Figure 2**: Prevalence of hypertension in 2019 and change from 1990 to 2019 in male(A) and Female(B) (modified from -NCD Risk Factor Collaboration (NCD-RisC 2022)*

Despite stable prevalence, the absolute number of people with hypertension aged 30–79 years has doubled from 331 million women and 317 million men in 1990 to 626 million women and 652 million men in 2019. As a result of the large increase in the number of people with hypertension, more people failed to achieve effective control in 2019 than in 1990 despite improvements in

detection, treatment, and control rates (Figure 3). In 2019, 82% of all people with hypertension lived in low- and middle-income countries [NCD-RisC, 2022]. There was a significant increase in this number in comparison to 1990 because the prevalence remained unchanged or increased and the population grew and aged [NCD-RisC, 2022].

The high incidence of hypertension can be attributed to several factors. A few examples include obesity, the consumption of excess sodium in foods, a decrease in physical activity, inadequate intake of fruits, vegetables, and potassium, as well as excessive alcohol consumption [Whelton PK et al., 2002]. Compared to 1980, obesity prevalence had almost doubled by 2008. By the year 2008, the prevalence of obesity has increased from 5% to 10% in men and from 8% to 14% in women. In the 21st century, people intake more processed foods, which are heavily salted, so ultimately people are consuming more sodium [Hruby and Hu., 2015]. Moreover, declining physical activity increases the risk of many NCD including hypertension. A study suggests that in the absence of physical inactivity, the world's population's life expectancy would increase by 0.68 years [Lee IM et al., 2012].

Recently WHO estimated that in 2016, more than 1.9 billion adults aged 18 years and older were overweight. Of these over 650 million adults were obese. In 2016, 39% of adults aged 18 years and over (39% of men and 40% of women) were overweight. The worldwide prevalence of obesity nearly tripled between 1975 and 2016. Overall, about 13% of the world's adult population (11% of men and 15% of women) were obese in 2016.

In 2019, an estimated 38.2 million children under the age of 5 years were overweight or obese. Overweight and obesity are now on the rise in low- and middle-income countries, particularly in urban settings. In Africa, the number of overweight children under 5 has increased by nearly 24% percent since 2000 [WHO, www.who.int/news-room/fact-sheets/detail/obesity-and-overweight, 2023]

Primary prevention can be achieved by taking measures to reduce or minimize the causal factors that contribute to hypertension in the population. Particularly, prehypertensive individuals should be aware of this. Prevention and control of hypertension can be achieved through directed and/or population-based strategies. For control of hypertension, the targeted strategy involves interventions to increase awareness, treatment, and control in individuals. Corresponding population-based strategies involve interventions designed to achieve a small reduction of BP in

the population. Having a usual source of care, optimizing adherence, and minimizing therapeutic inertia are associated with higher rates of BP control. The Chronic Care Model, a collaborative partnership among the patient, provider, and health system, incorporates a multilevel approach for control of hypertension.

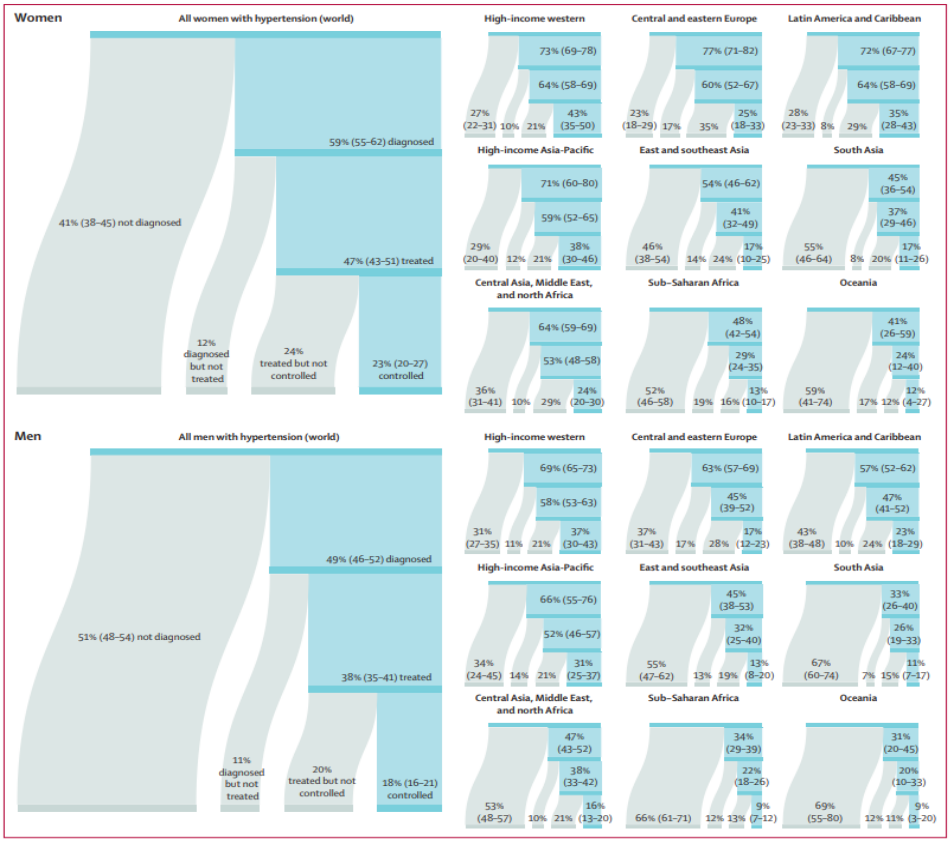

***Figure 3**: Hypertension treatment cascade in 2019, for women and men globally and by region (modified from -[NCD-RisC, 2021](#))*

1.2 Hypertension and cerebrovascular disease

Hypertension contributes significantly to the global disease burden, with its harmful effects on the brain being one of its most significant effects [Forouzanfar et al., 2017]. There is a strong correlation between hypertension and brain damage, which can either manifest itself as an acute cause of strokes, such as thrombotic, embolic, or haemorrhagic stroke, or in a chronic form, such

as vascular dementia and cognitive dysfunction. Additionally, hypertension-related small vessel disease has been linked to lacunar infarction, white matter changes, as well as intracerebral hemorrhagic events, reducing the threshold at which Alzheimer's symptoms and signs occur as well as influencing the development of vascular dementia (VAD) [Meissner, 2016]. Therefore, hypertension is increasingly recognized as a "**global neurological problem**". According to Amenta et al. (2003) in high BP, the vessel wall is overstressed, causing it to thicken and undergo regressive changes. As a result, cerebral hemorrhage or infarction can occur, leading to damage to the brain [Amenta et al., 2003]. Also, different types of hypertensions, including isolated systolic or diastolic hypertension, and combined systolic and diastolic hypertension, increase the risk of stroke. The relationship between stroke and BP, however, is stronger with systolic pressure than diastolic pressure. The pathogenesis of stroke in hypertension may be influenced by changes in the cerebral circulation, such as vascular remodeling, inflammation, oxidative stress, baroreflex dysfunction (Figure 4).

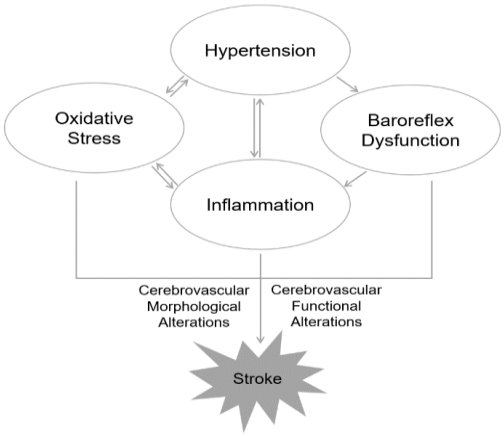

***Figure 4**: Pathogenetic links between hypertension and stroke (modified from – Yu et al., 2011)*

As a result of arterial hypertension, there is an increased risk of cerebral hypoperfusion, ischemia, and a decrease in brain oxygen supply related to cerebrovascular diseases associated with cognitive impairment and VaD [Sharp et al., 2011]. The etiology of VaD may be cerebral small vessel disease (cSVD) resulting in extensive leukoencephalopathy, a lacune development or strategically located infarcts in large vessels. It may also result from hypoperfusion of the entire brain, intracerebral hemorrhage, or other factors such as vasculitis. Moreover, in humans, hypertension is associated with damage to white matter. In patients with long-term hypertension, white matter

lesions are found in the periventricular area and the subcortical area. The treatment of hypertension effectively prevents the development of white matter lesions and the impairment of cognitive function that they lead to [Amenta et al., 2003]. Nuclear magnetic resonance (NMR) imaging studies showed pathological brain and cerebrovascular changes in at least 50% of hypertensive patients, whereas only 10%–30% showed neurological signs and symptoms [Karla M et al., 1994]. Furthermore, other studies highlighted that hypertension and older age is constant risk factors for cerebral white-matter lesions (WML). There is a higher rate and extent of cerebral WML in hypertensive patients than in normotensive patients, and uncontrolled hypertension patients have a higher rate of WML than those who are under control. Studies have shown that cerebral WML in hypertensive patients can be viewed as an early marker of brain damage. Furthermore, in essential hypertensives, the total volume of cerebrospinal fluid, which makes up approximately 15% of lateral ventricular volume, is significantly elevated and associated with brain atrophy. Progressive research findings in neurovascular regulation and the pathobiology of hypertension have led to a more comprehensive understanding of how hypertension disrupts the cerebral blood supply [Sierra et al., 2006].

1.2.1 Pathogenesis of hypertensive brain damage

The most common risk factor associated with stroke is hypertension. An ischemic stroke or an intracerebral hemorrhage may occur because of pathologic changes to the walls of small arteries and arterioles (diameter x 300 microns). A major contributor to stroke prevention is the reduction of blood pressure through diuretics, beta-blockers, calcium channel blockers, and angiotensin-converting enzyme inhibitors (ACE inhibitors). In hypertensive emergencies, blood pressure exceeds 180/120 mmHg accompanied by chest pain, shortness of breath, encephalopathy, or focal neurologic deficits. It is believed that hypertensive encephalopathy is caused by an acute failure of cerebrovascular autoregulation [Price et al., 2014].

When there is chronic high intra-luminal pressure is observed the growth of smooth muscle cells in the arterial resistance vessels and enhanced media thickness has been observed over a large range of arterial sizes in the intracranial vascular bed. Chronic hypertension alters the autoregulation curve to the right towards higher-pressure values. The upper limit of autoregulation may be raised by up to 30 mmHg. The lower limit at which adequate cerebral blood flow (CBF)

can be maintained is shifted to the right with the consequence that symptoms of cerebral hypoperfusion develop at a correspondingly higher level of arterial pressure than in normotensives (Figure 5) [Andrea,et al 2003].

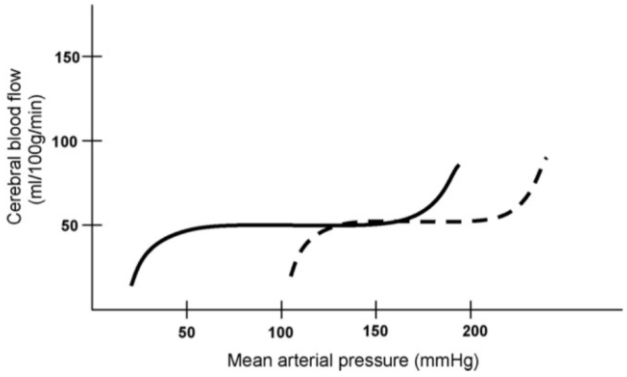

***Figure 5**: Cerebral blood flow autoregulation. From: (Veglio et al., 2009).*

A rise in mean arterial pressure (MAP) in normotensive people (the complete line) causes cerebral arteriolar vasoconstriction (mechanical autoregulation), maintaining a steady cerebral blood flow and an intact blood-brain barrier. Mechanical autoregulation contributes to a greater value of MAP in hypertensive patients.

Moreover, intracranial arterial vasculature is dilated in the presence of hemodynamic compromise. This baseline vasodilation restricts the brain's capacity to respond to new ischemia events with more vasodilation, increasing the chance of accidents in the future. Cerebral autoregulation response (it has both myogenic and neurogenic components) is believed to occur primarily at the level of small artery and arteriole. When systemic blood pressure exceeds the upper limits of the normal physiological range, cerebral autoregulation appears to be predominantly caused by myogenic autoregulation, which takes place in the smaller arterioles. Perivascular sympathetic autonomic nerves that travel in the adventitia of the vascular wall are responsible for neurogenic autoregulation at the level of both big and small arteries. It has been found that myogenic autoregulation is diminished or eliminated in cerebral arteries subjected to experimental endothelial damage. Nitric oxide synthase is upregulated in endothelial dysfunction, which leads to an excess of nitric oxide (NO). When intravascular flow rates are high, NO causes vasodilation, which tends to counteract the protective benefits of vasoconstriction. Additionally, there is some evidence to suggest that NO may improve cerebral vascular permeability through a cyclic-GMP regulated mechanism. Therefore, endothelial damage or dysfunction might promote breakthrough

of autoregulation in those small vessels that are downstream from arteries with less sympathetic innervation. This explains the tendency for edema to collect primarily in the posterior cerebral regions [Schwartz et al., 2002].

1.2.2 Hypertensive encephalopathy

When arterial pressure is high, resistance vessels are forced to dilate. Therefore, excessive pressure loads are placed on the distal small vessels, while passively increasing blood flow increases cerebral perfusion. It should be noted, however, that high arterial pressure disrupts the blood-brain barrier and affects plasma proteins, including fibrin, which results in a leak in the interstitial space, resulting in the destruction of smooth muscle cells (fibrinoid necrosis) in small arteries. It is possible that hemorrhages may occur if the endothelial damage has been severe enough to allow extravasation of erythrocytes. Vascular pathology results in generalized vasodilation, cerebral edema, and papilledema, both of which cause neurological deficits and cognitive impairment. [Miller et al., 2018].

1.2.3 Hypertension Induced large and small vessel atherothrombosis.

Atherosclerosis is the cause of all the complications associated with large vessel diseases or atherothrombotic infarctions. The process of atherosclerosis is accelerated by hypertension. In addition to dyslipidemia, free radicals, and cigarette smoke, hypertension negatively impacts endothelial function, resulting in severe damage to the cells (injury hypothesis of atherosclerosis) [Rovira et al; 2005].

Endothelial injury allows excessive egress of lipids in the intima initiating the atherosclerotic process that evolves from fatty streaks to fibrous and complicated plaques. Plaques are usually located in specific sites such as large vessel bifurcations and curves where the laminar flow is disturbed. Their small size and their point of origin, proximal to the arterial network, means that these vessels are exposed to significant forces. Longitudinal animal and human experimental studies indicate that leakage of plasma constituents into the vessel wall is the first step in the development of hypertensive degenerative vascular changes [Lammie et al 1997]. Plasma constituents in the surrounding tissue are not detectable whereas increased pericytes contain an

increased number of phagocytic inclusions. At a later stage, smooth muscle cells in the tunica media degenerate and fibrinoid material that is mainly composed of plasma proteins leaks through the damaged blood brain barrier into the vessel wall (fibrinoid change). Later phagocytes and fibroblasts produce increased amounts of collagen into the vessel wall (fibrosis or hyalinosis). This results in an increased thickness of the media and so, we refer to the term “arteriolosclerosis”.

Hypertensive brain lesions due to small vessel disease may appear in two neuropathological:

-focal ischemic lesions, named lacunar infarction

-Diffuse white matter disease, named leucoaraiosis.

Ischemic lesions—lacunar infarction most lacunar infarctions occur in the territories of the “perforating arteries”. Lacunar infarctions (LI) are frequently a consequence of small vessel occlusion or of non-occlusive post-stenotic hypoperfusion. The lack of collateral results in a small infarct that spreads distally from the point of occlusion through the entire territory of the affected vessel. In most cases, arterial occlusion occurs during the first half of a penetrating vessel's course. Most LI are asymptomatic. Symptoms are related to size and location of the lesion (usually lesions in the basal ganglia tend to be silent). LI may appear with focal neurological deficits, the so-called lacunar syndrome: pure motor hemiparesis, pure sensory stroke, sensorimotor stroke, ataxic hemiparesis and dysarthria-clumsy hand syndrome [Kato et al., 2002].

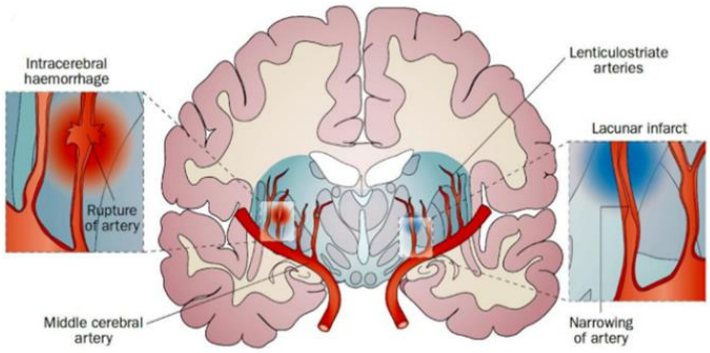

Figure 6*. Pathophysiology of lacunar infarction and hemorrhagic stroke. (Spence JD et al 2019)*

1.2.4 How does hypertension cause cerebrovascular alteration?

A sustained increase in BP causes adaptations in cerebral blood vessels designed to reduce the mechanical stress on arterial walls and protect micro vessels from pulsatile pressure. Hypertension-

mediated adaptive alterations in the systemic and cerebral arteries are referred to as hypertrophy and eutrophic remodeling. In hypertrophic remodeling, smooth muscle cells were involved hypertrophy or hyperplasia and expand inward, pushing into the arterial lumen. Consequently, the media/lumen ratio and media cross-sectional area increased. In this case vascular smooth muscle cell size increases and extracellular matrix proteins accumulate in the vessel wall, such as collagen and fibronectin. On the contrary, eutrophic remodeling is characterized by a rearrangement of smooth muscle cells that reduces the artery lumen without altering the total vascular mass or wall thickness. In this scenario, the change in vascular smooth muscle cell size is modest, and the lumen reduction is due to a reorganization of cellular and acellular material in the vascular wall, coupled by increased apoptosis in the blood vessel's outer regions (Figure 6A) [Faraco and Iadecola, 2013; Iadecola and Davisson, 2008]. Hypertension also causes vascular stiffening, which increases collagen content and arterial wall rigidity. As a result, during vascular remodeling, vessels' lumen is reduced and vascular resistance increases, and this alteration increases the likelihood of cardiovascular events and cerebrovascular disease. Moreover, recent studies reveal that, in hypertensive individuals, arterial stiffness is linked to clinically silent brain lesions and is a reliable indicator of stroke risk and cognitive deterioration [Henskens et al., 2008].

Moreover, high BP is a major risk factor for atherosclerosis. The likelihood of developing complicated aortic atherosclerosis (protruding atheroma, ulcerated plaques, and mobile debris), which is highly predictive of ischemic strokes, increases by 43% with every 10 mm Hg increase in arterial pressure. Additionally, atherosclerotic lesions are seen in cerebral arteries less commonly and at areas of turbulent flow like the vertebrobasilar system and the carotid bifurcation (Figure 7B). A possible explanation is vascular shear stress at such places, which results in the development of innate immunity receptors on macrophages/monocytes and inflammation [Sakamoto et al., 2001]. Atherosclerotic plaques can result in stroke by rupturing and hemorrhaging, which causes acute cerebrovascular occlusions, or by releasing fragments that cause artery-to-artery embolism.

Hypertension also causes unique changes in the small arteries and arterioles that supply the deep hemispheric white matter and basal ganglia, resulting in a disorder known as small vessel disease (SVD; Figure 7B). This vessel's susceptibility to SVD may be related to its short linear path from the base of the brain, which makes it more vulnerable to hypertensive mechanical stresses [Sörös

et al., 2013]. Surprisingly atherosclerosis is the most common pathological substrate for SVD associated with hypertension. Arteriolosclerosis is characterized by the loss of smooth muscle cells in the tunica media, the deposit of fibro-hyaline material, the narrowing of the vessel's lumen, and the thickening of the walls of the vessels (Figure 7 A and B).

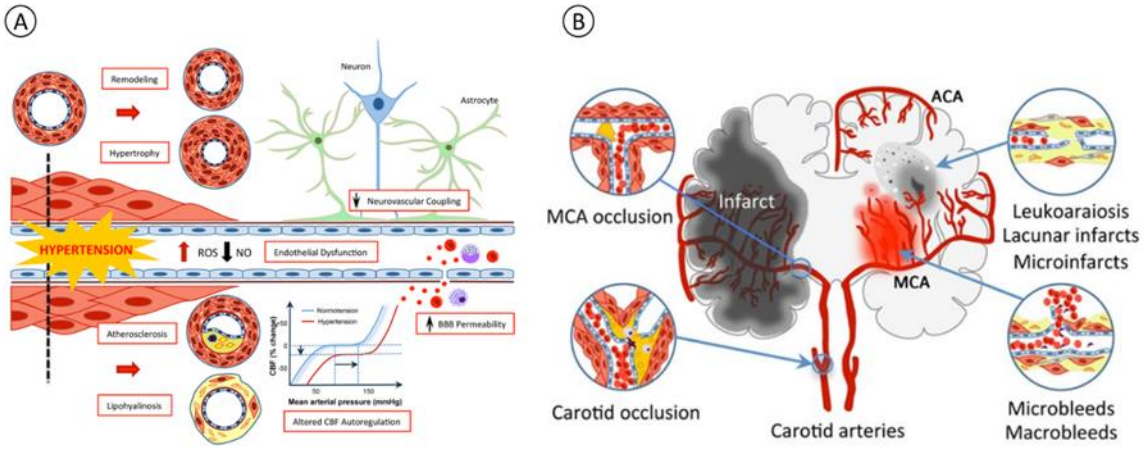

***Figure 7**. (A) Effect of hypertension on cerebral blood vessels. BB: blood–brain barrier; CBF: cerebral blood flow; ROS: reactive oxygen species. (B) Ischemic brain lesions associated with hypertension (modified from - Faraco and Iadecola, 2013)*

Additionally, hypertension alters cerebrovascular autoregulation, shifting the pressure-flow curve to the right [Iadecola and Davisson, 2008]. Due to this, higher perfusion pressures are required to maintain the same CBF level in hypertension. The alteration in autoregulation is connected to the rise in myogenic tone brought on by myocytes' increased Ca^{2+} sensitivity. Remodeling and hypertrophy, which reduce arterial lumen and increase cerebrovascular resistance, also contribute to the shift in autoregulation. When blood pressure declines, the brain is more vulnerable to cerebral ischemia due to changes in autoregulation because the cerebral blood arteries are unable to compensate up for the decrease in perfusion pressure [Immink et al., 2004]. Hypertension also shifts the functional mechanism of endothelium cells in the neurovascular unit and the functional hyperemia. Previous studies reveal that, in cerebral blood vessels, hypertension leads to reactive oxygen species (ROS) production, resulting in functional hyperemia and endothelial dysfunction, particularly in the BBB. Moreover, evidence is accumulating that oxidative stress is associated with hypertension's harmful effects. According to Touyz (2004), in humans, fundamental markers of oxidative stress are upregulated in preeclampsia, renovascular hypertension, essential

hypertension, and malignant hypertension [Touyz, 2004]. Because hypertension and its cerebrovascular effects are largely driven by oxidative stress in the brain and cerebral blood vessels. In summary, hypertension results in functional hyperemia, autoregulation, and endothelial dysfunction, which act together with structural changes of cerebral blood vessels to lower the compensatory capacity of the cerebral circulation and make the brain more susceptible to vascular insufficiency [Iadecola and Davisson, 2008].

1.2.5 Hypertension mediated vascular cognitive impairment and dementia.

Dementia is characterized by a progressive and often irreversible decline in cognitive function, which is most common in older adults. It is one of the most common neurological diseases, affecting approximately 30–40 million people worldwide, and the number is expected to triple by 2050. Alzheimer's disease (AD) and cerebrovascular diseases are the leading causes of cognitive dysfunction, accounting for nearly 80% of cases, and patients commonly have a pairing of both pathologies [Prince et al., 2013]. Among vascular risk factors, chronic arterial hypertension is a key contributor to cognitive impairment since the brain is one of the primary target organs damaged by hypertension. Because hypertension poses a potential threat to cerebrovascular pathology leading to stroke and consequently forming dementia. Vascular cognitive impairment and dementia (VCID) is a severe cognitive dysfunction syndrome caused by ischemia and hypoxia injury resulting from chronic low perfusion in the whole brain or a specific brain region [Tian et al., 2022].

Although one-third of stroke patients have cognitive impairment within three months, and having a stroke doubles the risk of dementia, VCI can occur in patients without a history of stroke who have brain infarcts (silent infarcts) on imaging. Despite this, cerebral small vessel disease (CSVD) remains one of the most common causes of dementia, contributing to 45% of cases [Gorelick et al., 2011]. There are many theories related to the pathophysiological mechanisms behind hypertension-related brain dysfunction. A central part of these theories is impaired cerebrovascular autoregulation in hypertension and chronic maladaptive changes in cerebral vasculature in hypertensive individuals [Singh and Kaur, 2021]. For instance, CSVD is closely interconnected with white matter damage or leukoaraiosis (LA), indicating a reduction in white matter density. High systolic BP precedes the onset of LA and lowering BP slows its progression. LA, which is

frequently found in the periventricular white matter, may be caused by hypoxia-hypoperfusion. Because it is located at the boundary between separate arterial territories, the periventricular white matter is thought to be more susceptible to hypoperfusion (Figure 8B) [Faraco and Iadecola, 2013]. As mentioned in the previous section, hypertension alters the CBF autoregulation which ultimately causes hypoperfusion. Moreover, due to the impairment of autoregulation, it is common for patients with vascular cognitive dysfunction to experience increased permeability of the blood-brain barrier (BBB), vascular remodeling, parenchymal oedema, oxidative stress, inflammation, and neuronal degeneration [Singh and Kaur, 2021]. Further hypertension induced changes at the Neuronal Vascular Unit (NVU) that progressively impaired communication between cells, altering the normal crosstalk into the pathological onset. Because alteration in NVU leads to glial inflammation (i.e., astrogliosis and microgliosis) and this process creates more BBB disruption and excessive oxidative stress. Consequently, these deleterious processes diminish the stability of the NVU and impair neuronal function, eventually leading to neurodegeneration and cognitive decline (Figure 8) [Presa et al., 2020].

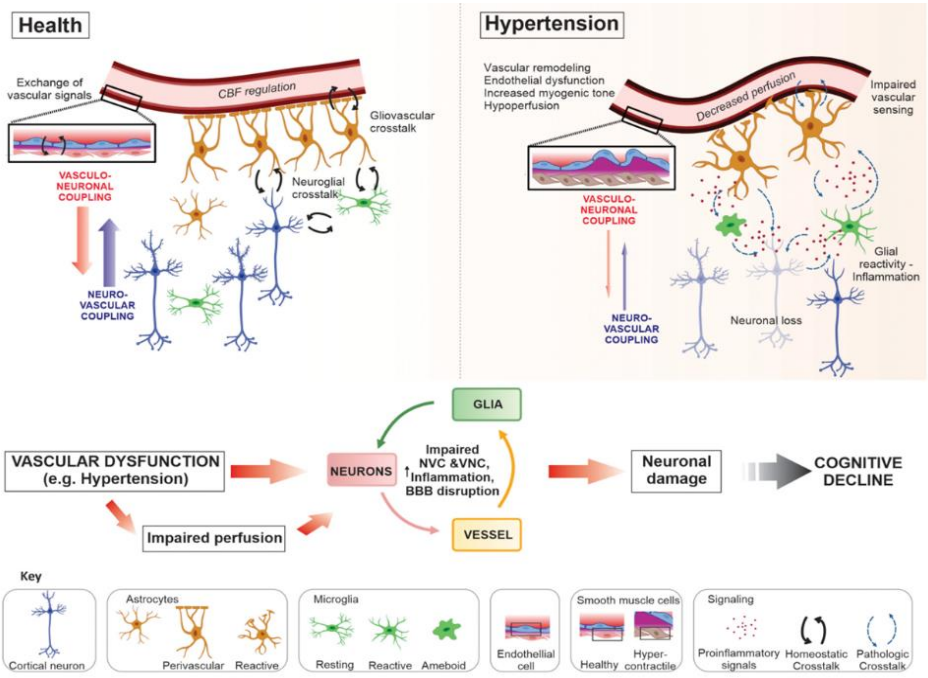

***Figure 8**: Hypertension mediated cerebrovascular alteration and subsequent crosstalk between vessels, neuron & glia (modified from – Presa et al., 2020].*

Additionally, despite being formerly regarded as independent pathologies, increasing evidence indicates that VCI and AD share similar pathogenetic processes [Faraco and Iadecola, 2013]. For instance, cerebral vascular pathology is typically found at considerably higher rates in autopsy-confirmed cases of AD than in age-matched control cases. The neurovascular hypothesis of AD proposes that cerebrovascular dysregulation, including the effect of systemic hypertension on the brain, disrupts amyloid and tau protein homeostasis, resulting in neuronal injury and cognitive impairment. At the same time, neurovascular coupling can be disrupted by amyloid and tau-mediated injury. The NVU, which is maintained in part by astrocytes and microglia, is central to this bidirectional hypothesis. Both glial cell types exhibit significant physiological responses to hypertension and amyloid. Finally, the recent SPRINT-MIND trial found that the intensive blood pressure control group had a lower incidence of cognitive impairment than the standard treatment group, indicating a direct relationship between neurodegenerative disease and elevated blood pressure [Levit et al., 2020].

1.2.6 Hypertension and cholinergic system alterations related to cognitive impairment.

There are sympathetic adrenergic nerves that innervate the majority of the body's arteries and veins and release norepinephrine (NE) as a neurotransmitter. Parasympathetic and sympathetic cholinergic nerves innervate some blood vessels, both of which release acetylcholine (ACh) as their primary neurotransmitter. The brain's adrenergic and cholinergic receptors are activated by neurotransmitters, which lead to changes in vascular function as a result of signal transduction pathways. It appears that NE preferentially binds to alpha-adrenoceptors, causing smooth muscle contractions and vasoconstriction to occur [Roy et al 2014].

Similar responses are also observed when NE binds to postjunctional α2-adrenoceptors on some blood vessels. Furthermore, NE binds weakly to postjunctional beta-adrenoceptors and causes vasodilation (this effect can be observed during alpha adrenoceptor blockade)[Fujii et al 2017], although the effect is relatively minor and overshadowed by vasoconstriction triggered by beta-adrenoceptors. In certain parts of the body, parasympathetic cholinergic fibers are responsible for innervating blood vessels (such as coronary arteries). As a result, these nerves release ACh, which binds to muscarinic M3 receptors found in smooth muscle and/or endothelium. Nitric oxide (NO)

is produced by endothelial M3 receptors, which causes smooth muscle relaxation [Fujii et al., 2020].

Cholinergic systems are known to be involved in the central regulation of blood pressure (BP) in several species, including humans. In clinical trials with acetylcholinesterase (AChE) inhibitors, several case reports have described pressor responses. The properties of AChE inhibitors can be attributed to their ability to prevent acetylcholine (ACh) from being rapidly inactivated. This prolongs and enhances the effects of ACh as a neurotransmitter released from cholinergic terminals. Several studies have shown that choline acetyl-transferase-positive neurons and muscarinic receptors are located within or near the brainstem area that controls blood pressure and heart rate (HR). The administration of ACh or cholinomimetic drugs causes an increase in blood pressure, caused by an increase in sympathetic tone and the release of vasopressin (AVP) [Lazartigues et al., 1999].

Cholinergic precursors have represented one of the first approaches attempting to relief cognitive impairment in dementia related disorders. In cerebrovascular pathologies, ischemic or hemorrhagic processes may cause tissue damage and disrupt cholinergic pathways. As a result of ischemic processes, areas of varying sizes can be affected by territorial infarctions, watershed infarctions, deficiencies, and white matter demyelination. By considering the routes of the cholinergic pathways and the vascular territory, it is possible to locate the points where the lesions may interrupt these pathways [Parnetti et al., 2007].

In experimental rodent models, such as the spontaneously hypertensive stroke prone rat there is a significant reduction in cholinergic markers including acetylcholine (ACh) in the neocortex. White matter infarction in rodent models results in substantial decreases in cholinergic markers, presumably through an impact on cholinergic projection fibers. In human disease there is a reported loss of cholinergic neurons in 70% of AD cases and in 40% of VaD patients examined neuropathologically, and reduced ACh activity in the cortex, hippocampus, striatum and CSF [Timo Erkinjuntti et al., 2004].

Circumstantial evidence also suggests that cholinergic deficiency is causally related to cognitive impairment in VaD. First, the basal forebrain is one of the regions in which ischaemic injuries are preferably caused by amnesty.

Second, AChE inhibitors dopezil and rivastigmine have demonstrated long-term efficacy in the treatment of VaD. Galantamine, another AChE inhibitor that modulates allosterically.

Presynaptic nicotinic receptors, thus amplifying the ACh response, had a beneficial effect in probable VaD. These effects in VaD may be due to the retrograde degeneration of the cholinergic neurons in nBM, although coexisting AD pathology may also account for the beneficial effects of the AChE inhibitor [Tomimoto et al.,2005].

The precise mechanisms underlying vascular cognitive impairment (VCI), the second common cause of dementia, remain unclear. Several lines of evidence suggest a central role for cholinergic deficiency. First, both cholinergic neuronal deficits and cholinergic denervation have been identified in patients with cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), a purely genetic form of vascular dementia (VaD). Secondly, significantly decreased cerebrospinal fluid acetylcholine concentrations were observed in patients with Binswanger or multi-infarct dementia [Liu et al., 2017].

The vesicular acetylcholine transporter (VAChT) facilitates the loading of ACh into secretory organelles in neurons, thereby allowing ACh to be released. Thus, impairment of cholinergic anti-inflammatory pathways contributes to the development of EOD during hypertension. [Lepori et al., 2001]. Moreover, choline supplementation activated the a7 non-neuronal nicotinic Ach receptors (nAChRs) and served as a key function in regulating blood vessels. Thus, choline can be protective against hypoxia induced endothelial dysfunction [Zhang et al., 2017]. There have been several proposals for using the alpha7 nicotinic acetylcholine receptor (α7nAChR) as a therapeutic target for inflammatory disorders. Accordingly, we investigated the role of α7nAChRs in inflammation.

1.3 Neurovascular Unit and cerebral blood flow regulation.

Brains are highly vascularized organs, with each neuron located within 15μm of a blood vessel. Due to this proximity, nutrients and waste products can be exchanged quickly, enabling the brain to have a high metabolic rate despite its limited intrinsic energy capacity [Kaplan et al., 2020]. A vascular network in the brain connects surface (pial) arteries to penetrating (parenchymal) arterioles, along with a vast network of capillaries and ultimately drains back to the surface through

post-capillary venules. Within the brain, perfusion is a highly controlled process that is achieved by the operation of activity-dependent and constitutive mechanisms. This group of mechanisms works together to ensure brain homeostasis and optimal performance [Presa et al., 2020]. The brain is a complex functional and structural organization. NVU represents the smallest level of cellular interactions which establishes brain perfusion. The NVU consists at its simplest neuronal, astrocyte, endothelial, vascular smooth muscle and/or pericyte interaction as showed in Figure 9 and Table 2 [Iadecola., 2017].

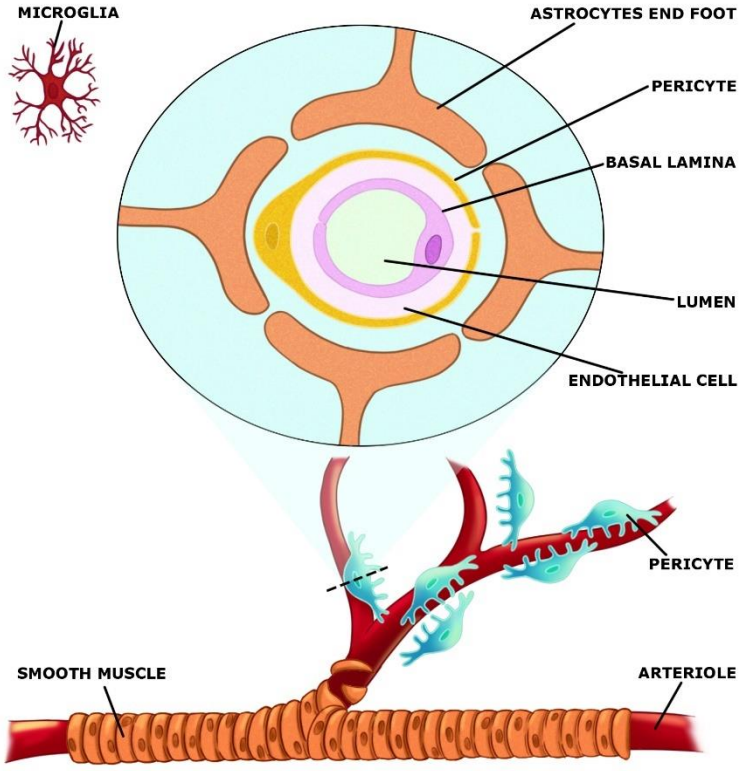

Figure 9. *Elements of the neurovascular unit (NVU).*

Table 2. *Various components that constitute the Neurovascular Unit*

NVU Components	Classification	Receptors and subtypes	NVU Antibodies/Markers
Microglia	Ramified microglia	Complement type 3 receptor (CR3)	OX-42, MAC-1, CD11b/CD18, OX-6, OX-6, OX-17 and OX-3
	Activated microglia	Major histocompatibility complex class II (MHC II)	ED-1 (rat CD68), Iba1
	Phagocytic or amoeboid	Major histocompatibility complex class I (MHCI), CD4 receptor	OX42, F4/80, ED1 and ED2, OX-18, OX-6, OX-17, OX-1, OX-35
	Perivascular microglia	Major histocompatibility complex class II (MHC II)	Iba-1, ED-1, ED-2, OX-17, OX-18 and F4/80, OX-42
	Ramified microglia		OX-6
Astrocytes			Glial fibrillary acidic protein (GFAP)
Neurons	Mature neurons	N-methyl-d-aspartate (NMDA) 1 glutamate receptors	Neuronal antigen nuclei (NeuN), HuC/D RNA-binding proteins.
Pericytes		The tyrosine-kinase receptor PDGFRβ	Platelet-derived growth factor receptor β (PDGFRβ)
			α-Smooth muscle actin (α-SMA)
			Neuron-glial 2 (NG2)
			Desmin (cytoskeleton)
Endothelial cells	TJPs proteins	Claudins	Claudin-1, Claudin-3, Claudin-5, Claudin-12
		Occluding	Zonula occludens (ZO)-1 and ZO-2, ZO-3
		Junctional adhesion molecule (JAM)	VE-cadherin, β-catenin, Caveolin-1, Plasma lemma vesicle-associated protein (PLVAP), Platelet-endothelial cell adhesion molecule (PECAM-1), Intercellular adhesion molecule 1 (ICAM-1), Vascular endothelial cell adhesion molecule-1 (VECAM-1)
Basement membrane			Bulins1 (or BM90) and 2; thrombospondin Laminin, Collagen IV, Agrin, Fibronectin
Other blood brain barrier marker		Aquaporins (AQPs)	AQP1, AQP4 and AQP9

		β-Dystroglycan	Dystrophin-glycoprotein complex (DGC), laminin 2
		Matrix metalloproteinases (MMPs)	collagenases (MMP-1, MMP 8, MMP-13, MMP-18), gelatinases (MMP-2, MMP-9), stromelysins (MMP-3, MMP-10, MMP-11), matrilysins (MMP-7, MMP-26) and membrane type MMPs (MMP-14, MMP-15, MMP-16, MMP-17, MMP-24, MMP-25).

Modified from. Roy et al., 2023

As report in our published paper:

"The neurovascular unit (NVU) (Figure 2), which is made up of both neurons, glial and vascular cells is responsible for integrating changes in blood supply to increases or decreases in neuronal activity (Kim and Filosa 2012). Vascular components of NVU include endothelial cells, pericytes, and vascular smooth muscle cells, whereas glial cells include astrocytes, microglia, and oligodendroglia. Acetylcholine as well as CCPs play an active role in the regulation of NVU. These molecules display protective effects on neuronal death, microglial activation, blood brain barrier (BBB) disruption and consequent neurological injury (Inazu et al., 2019). NVU is a relevant structure in the maintenance of brain homeostasis. The concept of NVU was introduced by Harder (Harder et al., 2002) as a brain structure formed by neurons, interneurons, astrocytes, basal lamina covered with smooth muscular cells and pericytes, endothelial cells and extracellular matrix (Muoio et al., 2014). Each element is intimately and reciprocally interconnected, establishing a comprehensive anatomical and functional system of cerebral blood flow control (Abbott and Friedman 2012).

In the central nervous system (CNS), astrocytes are the most common glial cells. Astrocytes are essential for molecular transport and the integrity of the BBB and through their end-feet, astrocytes connect with endothelial cells in the NVU (Yamazaki et al.,2017). Neurovascular coupling is a process in which astrocytes link neural activity with blood arteries. They react to neural activity and send out signals to control cerebral blood flow (CBF) (Gordon et al., 2011). Astrocytes are formed in the last stages of neurogenesis, along with neurons and oligodendrocytes. As multifunctional cells, astrocytes are strongly connected with neurons and blood vessels, and communicate with neuronal pre-and post-synaptic terminals to help control synaptic transmission by releasing glutamate, D-serine, and ATP (López-Bayghen et al., 2011; Santello et al., 2012). Astrocytes can be largely coupled into syncytial structures of up to 100 units by gap junctions. Extend end-feet process can modulate CBF or the BBB and high levels of aquaporin-4 water channel proteins enhance perivascular clearance by the newly identified "glymphatic system" (Plog et al., 2018).

Microglia cells are derived from the yolk sac and seed in the brain as the first glial cells. During development they grow as highly flexible cells with mobility alongside neurons (Pont-Lezica et al., 2011). Through their interaction in the NVU, active microglia and astrocytes may reach a condition of immunological "optimization". Microglia cells, which are found throughout the brain, differentiate early in embryonic development or as the first response of the central nervous system to neuroinflammation (Klein et al., 2017).

In the brain, endothelial cells constitute the tubes represented by capillaries. The BBB is formed of continuous endothelial cells with tight junctions, basement membrane, and astrocyte end-feet in the NVU. BBB controls through chemical and/or anatomic barriers substances entering the brain,and removes dangerous proteins from the brain parenchyma into the bloodstream (Yamazaki and Kanekyio, 2017). Endothelial cells are joint by tight junctions and adherent junctions, and tight junctions reduce BBB paracellular permeability. All the elements, when combined with neurons, constitute the NVU (Zlokovic, 2008; Zlokovic 2011).

The NVU maintains the optimal functioning of the brain microenvironment, contributing to neuronal survival, and information processing by regulating BBB permeability and CBF. Vascular dysfunction has been implicated in various neurodegenerative disorders. The NVU may be damaged, causing BBB malfunction and a drop in CBF, which may concur to the pathophysiology of neurodegenerative diseases. When the NVU is desegregated and CBF drops, the supply of oxygen and nutrients to the brain is reduced as well as the clearance of neurotoxic substances (Sweeney et al., 2018). Claudins, that occlude junctional adhesion molecule, and zonula occludens-1 are all transmembrane proteins involved in the formation of tight junctions (Bell et al., 2010; Yamazaki and Kanekiyo, 2017). The main cadherin that forms the adherent's junction and mediates intercellular adhesion is vascular endothelial cadherin (Zenaro et al., 2017). Tight and adherent junctions are critical in controlling endothelial permeability and help to support normal brain physiological function by limiting the entry of macromolecules, toxins, wastes and perilous blood-borne pathogens (Daneman et al 2015). Tight junctions restrict protein diffusion and seal the paracellular cleft between endothelial cells, whereas adherents' junctions are important for cell-to-cell interaction and cell growth (Tietz et al 2015).

*Pericytes cover the abluminal surface of capillaries and adjust capillary diameter to regulate blood flow. BBB permeability, CBF, immunological trafficking, trans endothelial fluid transport, and vascular integrity are all regulated by pericytes (Brown et al., 2019). Pericytes are also involved in the establishment of tight junctions and remove harmful proteins to keep the CNS stable (Daneman et al., 2010; Sagare et al., 2013). Furthermore, for the development, maintenance, and effective functioning of the BBB, interaction between endothelial cells and pericytes is essential (Geranmayeh et al., 2019)" [**Roy et al., 2022 Appendix 1**]*

The components of NVU communicate dynamically, regulating arterial and capillary blood flow as well as providing neurons with energy substrates and removing waste products. However, it has also been observed for more than a century that molecules cannot move freely across this dense vascular system in either direction. As the result of the concerted action of the several cell types that form the NVU, these emergent properties - the blood-brain barrier (BBB) and neurovascular coupling (NVC) - are evident [Kaplan et al., 2020]. There is a significant variation in neurovascular association across the cerebrovascular network. The cerebral vessels run along the brain surface within the subarachnoid space (pial arteries and arterioles), forming a highly collateralized network in the subarachnoid space (Figure 5). According to De Silva and Faraci (2016), the internal carotid artery penetrates the skull and joins with vertebral artery branches to form the circle of Willis around the base of the brain [De Silva and Faraci, 2016]. An extensive region of the cerebral cortex is supplied by the middle cerebral artery, which originates in the circle of Willis. From the middle cerebral artery pial arteries and arterioles originate which run on the surface of the brain, forming a highly interconnected network from which penetrating arterioles emerge. A network of capillaries feeds into the venous system, which returns blood to the heart via penetrating arterioles. Each cerebrovascular segment has a component (percentage) of the total vascular resistance, which represents its ability to control blood flow. As 60% of the resistance comes from vessels outside the brain, and 40% from vessels within the brain.

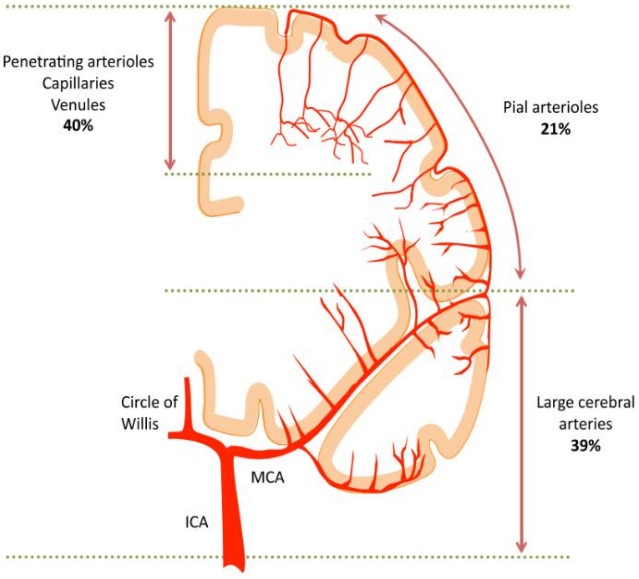

***Figure 10**: Anatomy of the cerebrovascular tree and segmental vascular resistance (modified from – Iadecola, 2017)*

A key component of the cerebrovascular network, NVU is crucial for stabilizing the brain's environment. NVU signaling plays an important role in health and disease as an interface between the periphery and the central nervous system (CNS). Figure 11 presents the microanatomical architecture of NVU which consists of endothelial cell layers, pericytes, smooth muscle cells and glial cells including astrocytes, microglia, and oligodendrocytes. In the broad sense, capillary tubes in the brain are constructed by endothelial cells. A pericyte and an astrocyte end-feet surround the outside of the endothelial tube. Furthermore, the extracellular matrix that forms the basement membrane surrounds endothelial tubes. Together with neurons, all these components make up the NVU. In the NVU, the BBB is formed by the tight junctions, adhesion junctions, and end-feet of astrocytes that comprise the endothelium (Figure 11A). In tight junctions, several transmembrane proteins are involved, including claudin, occludin, junctional adhesion molecule, and zonula Occludens-1 (ZO-1). The primary cadherin that constructs the adherens junction and regulates intercellular adhesion is vascular endothelial (VE) cadherin [Zlokovic, 2011]. When it comes to regulating endothelial permeability, tight and adherens junctions are crucial. Additionally, pericytes safeguard the abluminal surface of capillaries and manage capillary diameter to control blood flow. Likewise, pericytes play a crucial function in the creation of tight junctions and eliminate harmful proteins to preserve the stability of the CNS [Yu et al., 2020]. Besides the abundance of astrocytes as glial cells in the CNS, they also act as a conveyor belt in the NVU. The end-feet of astrocytes in the NVU serve as a means of communication with endothelial cells. Astrocytes combine neuronal activity with blood vessels through a process known as neurovascular coupling (NVC) (Figure 11C). Their function is to regulate Cerebral Blood Flow (CBF) in response to neuronal activity [Gordon et al., 2011]. Moreover, astrocytes are essential for BBB integrity and molecular transport.

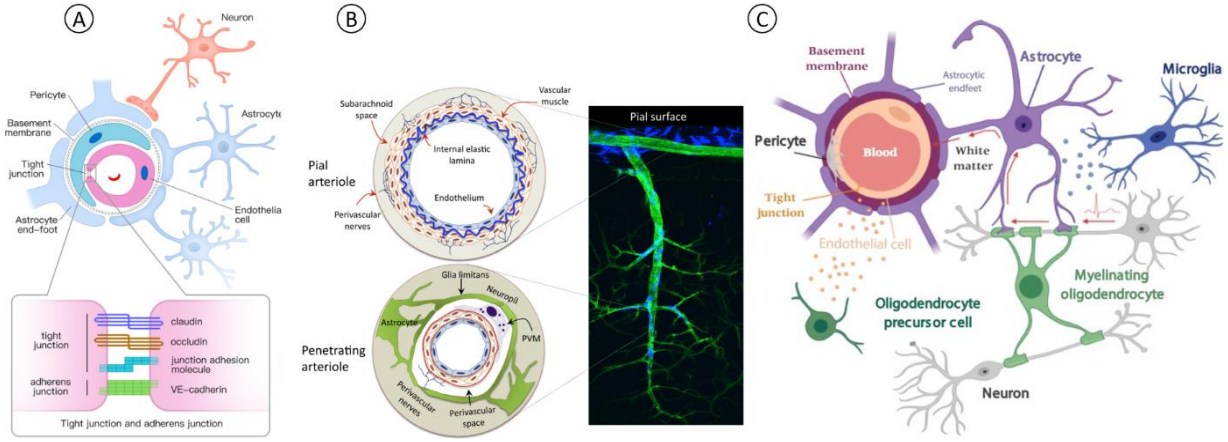

Figure 11: *(A) Structural diagram of the neurovascular unit (NVU) and the composition of tight junctions and adherens junctions; (B) Neurovascular associations along the cerebrovascular tree: pial arteries and penetrating arterioles; (C) The Neuro vascular Coupling (NVC): A Dynamic Connection between the Blood, the White Matter, and Neurons Responsive to Energy Demands (modified from - Yu et al., 2020 ; Iadecola, 2017 ; Quick et al., 2021)*

According to previous studies, the brain consumes almost 20% of the body's resting energy though it comprises only 2% of the body's weight. NVU-mediated mechanisms ensure constitutive perfusion in the brain and therefore maintain a constant demand for energy [Howarth et al., 2012]. CBF is driven by cerebral perfusion pressure, which is the difference between intracranial pressure and mean arterial pressure. CBF is essentially constant in the absence of neural stimulation, but it is susceptible to variations in cardiac output, vascular resistance, and intravascular and intracranial pressure gradients. The dynamic mechanisms that control basal CBF are primarily governed by the delicate coordination of vasodilatory and vasoconstrictive signaling pathways, as well as by the integration of reflex autonomic pathways and local regulatory processes, such as cerebral autoregulation [Hill-Eubanks et al., 2014]. There are several mechanisms underlying cerebral autoregulation, including pressure-induced vasoconstriction (myogenic response), metabolic signaling, and humoral signaling. The myogenic response is the best studied of these cellular pathways. This involves the activation of mechanosensitive Ca^{2+} - permeable ion channels and the subsequent increase in Ca^{2+} influx into vascular smooth muscle cells (VSMCs). This process is dependent on both pressure and flow/wall shear stress in cerebral vessels. Under physiological circumstances, these dynamic mechanisms work within a mean arterial pressure range of 50 to 160 mmHg and change the diameter of arterioles to keep CBF roughly constant. However, there are

variations in the actual systemic arterial pressure range associated with this plateau phase, with considerably significant variations observed among people. Additionally, chronic conditions including cerebral ischemia and hypertension can alter this autoregulatory range. Due to these consequences, impaired autoregulatory mechanisms can increase microcirculation vulnerability, and therefore NVU. There is a decrease in cerebral reactivity and an increase in large-artery stiffness in hypertension, both of which are associated with cognitive decline [Hill et al., 2006; Chirinos et al., 2019].

1.4 Causal link between hypertension and oxidative stress

The pathophysiology of hypertension is extremely complex and multifactorial, and its long-term complications are often accompanied by oxidative stress and inflammation. Evidence strongly suggests that oxidative stress, inflammation, and hypertension are involved in a self-perpetuating vicious cycle, which if not interrupted, results in progressive injury and dysfunction of target organs [Vaziri, 2008]. For linking hypertension and oxidative stress, it's possible to consider three different elements: first, oxidative stress has been associated with hypertension in nearly all forms of acquired and hereditary hypertension in experimental animals. As an example, animals with hypertension have been shown to exhibit oxidative stress due to chronic lead exposure, chronic kidney disease, deoxycorticosterone acetate-salt administration, coarctation of the aorta, diabetes, metabolic syndrome, inhibition of nitric oxide synthase (NOS), and salt consumption [Roberts et al., 2006]. Secondly, pharmacological doses of several antioxidants have been shown to reduce blood pressure in hypertensive animals, but not in normotensive animals [Vaziri, 2004]. Third, the observations cited above provide indirect evidence that oxidative stress plays a role in hypertension pathogenesis. There is direct evidence for oxidative stress's causal role in diseases. For instance, it has been shown that genetically normal, otherwise intact animals develop hypertension after oxidative stress is induced [Vaziri et al., 2000]. So, it is commonly believed that hypertension-mediated vascular shear stress upregulates oxidative stress and this causes endothelial dysfunction, which consequently induces vascular inflammation, and vice versa. And this interplay between oxidative stress, inflammation and endothelial dysfunction is commonly refereed as vascular health triad (Figure 12) [Ranadive et al., 2021]. Additionally, several studies have demonstrated

circulating leukocyte activation in hypertensive humans and animals. Inflammation in the vascular system is caused by the activation nuclear factor-κB (NF-κB), which is a transcription factor responsible for many pro-inflammatory cytokines, chemokines, and adhesion molecules. Similarly, animal studies that have shown amelioration of hypertension with interventions such as NF-κB activation inhibitors have provided evidence that inflammation is causally related to hypertension pathogenesis [Rodríguez-Iturbe et al., 2005].

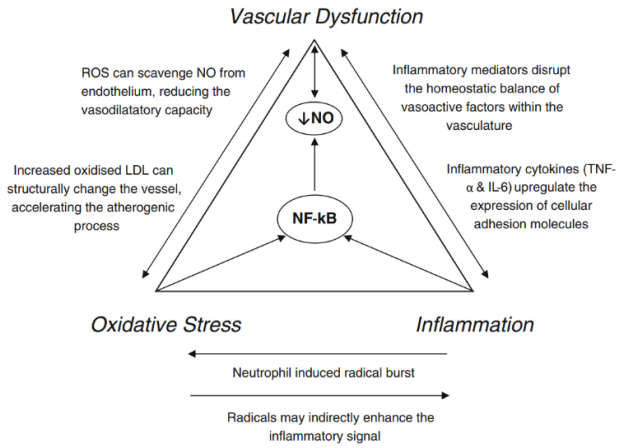

Figure 12: *The Vascular Health Triad. The relationships between oxidative stress, inflammation, and vascular health (modified from – Wadley et al., 2013).*

1.4.1 Oxidative stress

Oxidative stress results from an imbalance between the production and accumulation of Reactive Oxygen Species (ROS) in cells and tissues and the ability of the body to detoxify them. ROS include superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH), and singlet oxygen ($^1O_2$); they are metabolic by-products created by biological processes [Pizzino et al., 2017]. As ROS play an important role in maintaining the vascular wall's homeostasis, they could also contribute to hypertension. For instance, in previous studies patients with essential, renovascular, or malignant hypertension have been found to have elevated blood pressure and oxidative stress [Lip et al., 2002]. Due to the mechanical forces on the vascular wall, in hypertension, ROS production is also upregulated simultaneous manner, which led to structural and functional alteration [González et al., 2014]. For example, previous research has shown that hypertension induces endothelial dysfunction and VSMC hypertrophy. Endothelium is a single layer of cells that lines the luminal surface of blood vessels. In addition to serving as a protective barrier between

tissues and circulating blood, it also controls vascular tone and structure. Hence, oxidative stress could alter vascular function and tone by oxidizing proteins and nucleic acids. A key component of the endothelial system's ability to maintain vascular tone is the production of endothelial-derived nitric oxide (NO), which acts as a vasodilator. Among the major mechanisms that oxidative stress impacts vascular tone is its reduction in nitric oxide (NO) bioavailability, resulting in endothelial dysfunction (Figure 13A). It has been demonstrated that ROS play a role in inflammation, dysfunction of the endothelium, cell proliferation, migration, and activation, as well as extracellular matrix deposition and fibrosis [Schulz et al., 2011].

In the broad sense NO is synthesized from its precursor L-arginine by an endothelial form of nitric oxide synthase (eNOS). This activity is dependent on the presence of cofactors such as Tetrahydrobiopterin (BH4, THB). After synthesis, NO diffuses easily from the endothelium to activate soluble guanylyl cyclase in VSMCs, increasing the production of cyclic guanosine monophosphate and resulting in the relaxation of these cells [Flammer and Lüscher, 2010].Also, NO suppresses local wall inflammation by inhibiting thrombosis, proliferating cells, and leukocyte adhesion. Consequently, any loss of NO bioavailability or function is detrimental to endothelial health. It has been shown that mice lacking the endothelial isoform of NOS are hypertensive with endothelial dysfunction [Napoli and Ignarro, 2009].

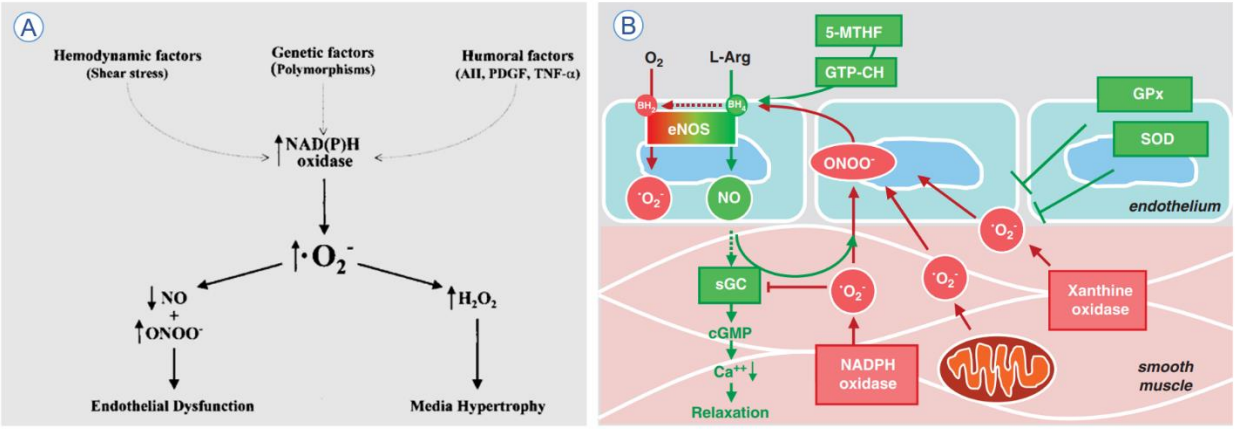

***Figure 13**: **(A)** NAD(P)H oxidase activation and functional consequences in arterial hypertension; **(B)** Schematic representation of the mechanisms by which reactive oxygen species lead to an impairment of nitric oxide (NO) bioavailability and vascular dysfunction (modified from - Schulz et al., 2011)*

Particularly due to its free radical nature, NO is scavenged both by reactive oxygen species (ROS) and by superoxide anions. For instance, as Figure 12 B represents, with an increase in superoxide anion levels, NO is scavenged more efficiently, resulting in the formation of peroxynitrite (ONOO), a powerful pro-oxidant that prevents eNOS bioactivity, and consequently reduces the bioavailability of NO [Deanfield et al., 2007]. More specifically, ROS cause BH_4 to uncouple from eNOS and to generate superoxide, which reduces NO bioavailability. Because it determines whether superoxide or NO is the predominant end-product of eNOS activity, the coupling of eNOS is of great importance (Figure 13 B). Although superoxide anion is broken down by the enzyme superoxide dismutase (SOD), the interaction between superoxide and NO happens three times more quickly than its degradation by SOD [Flammer and Lüscher, 2010].

In the blood vasculature, there are numerous enzymatic and non-enzymatic sources of ROS. Enzymatic sources of ROS in hypertension include xanthine oxidoreductase, uncoupled eNOS, mitochondrial respiratory enzymes, and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which is present in numerous vasculature cell types-the heart, kidney, and CNS [Montezano and Touyz, 2012]. In contrast to most ROS-producing enzymes that produce $\bullet O_2$ and/or H_2O_2 because of their catalytic activity or abnormally functioning in pathological situations, NADPH oxidase generates ROS solely, hence it is called a professional ROS generator [Lambeth, 2004].

The correlation between oxidative stress and high BP was originally proposed in individuals with essential hypertension and in spontaneously hypertensive rat (SHR) in the early 1990s [Nuyt,2008]. It is also believed that hypertension causes premature aging and increases the turnover of endothelial cells, which in turn are then replaced by regenerated endothelial cells. Endothelium-derived relaxing factors are less effectively released by the regenerated endothelium, which leads to an imbalance in favor of endothelium-derived constricting factors ultimately triggers vasoconstriction [Schiffrin, 2008]. In VSMCs, ROS activates profibrotic, proliferative, and apoptotic pathways, increasing extracellular matrix (ECM) buildup (fibrosis), hypertrophy, and fibrosis. Through adventitial fibroblasts and adipocytes, the adventitia plays a function in the modulation of cellular responses in vascular remodeling and is responsive to high levels of ROS.

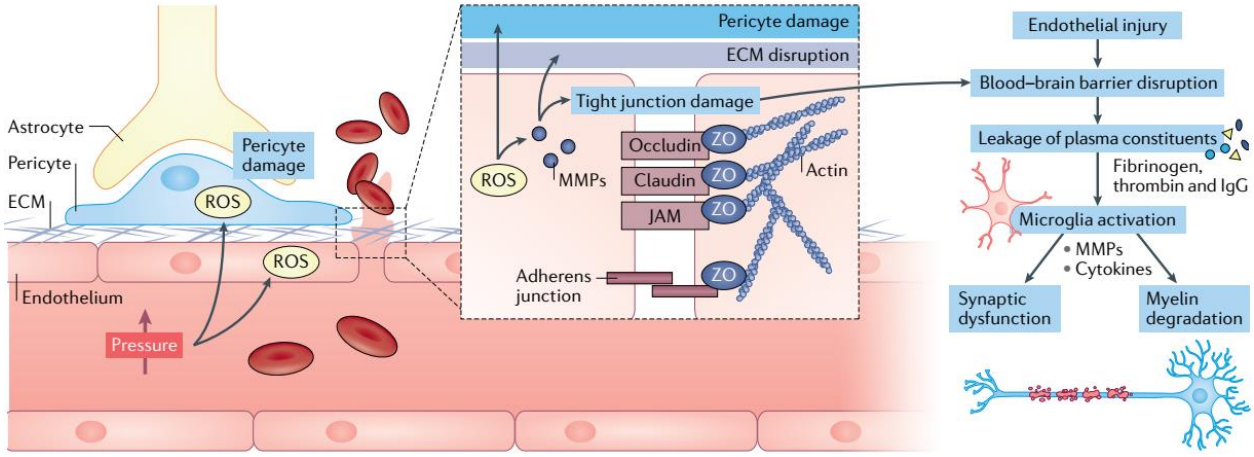

Figure 14: *Reactive Oxygen Species (ROS) mediated blood–brain barrier disruption in hypertension (modified from – Ungvari et al., 2021)*

Moreover, a substantial body of evidence indicates that oxidative stress in the brain causes neuronal damage following cerebral ischemia and reperfusion [Allen and Bayraktutan, 2009]. A ROS-induced change in microcirculation can alter blood flow resistance and, therefore, blood pressure regulation in the brain, having profound implications on vascular pathophysiology in the brain. Additionally, the inability of NVU cells to maintain a proper balance between ROS production and their neutralization results in a disruption of NUV and brain homeostasis, which leads to neurodegenerative disorders [Wevers and Vries, 2015]. According to studies, microglia and astrocytes produce high levels of ROS after an injury, which interferes with the expression of important molecules involved in BBB integrity such as ZO-1, claudin-5, occluding proteins (Figure 14). A critical role is played by pericytes in maintaining BBB integrity, which are located close to endothelial cells. Pericytes are highly susceptible to oxidative stress caused by overproduction of ROS under pathological conditions [Shah et al., 2013]. As endothelial cell signaling is very crucial to regulate the NVU's proper function, but due to excessive ROS formation, it disrupts the NVU's performance and coupling. There is substantial evidence that the NVU plays a major role in the initiation of neurodegeneration, according to recent studies [Nelson et al., 2016]. Consequently, ROS mediated impaired NUV increased susceptibility increases BBB permeability, which allows neurotoxic substances to enter the brain and ultimately affects brain homeostasis through subsequent neuroinflammation. This suggests that vascular alterations are

early events in cognitive deficits when vascular dysfunction is more marked in pathological conditions, including mild cognitive impairment (MCI). Indeed, it is reported that 60-90% of Alzheimer's disease (AD) patients exhibit cerebrovascular alterations such as cerebral amyloid angiopathy (CAA), microinfarcts and ischemic lesions, and microvascular degeneration [Bell and Zlokovic, 2009]. Moreover, it has also been found that oxidative stress-driven BBB permeability and vascular alterations exist in several diseases such as Parkinson's disease (PD), Huntington's disease, and cerebral small vessel disease [Carvalho and Moreira, 2018].

1.5 Causal link between hypertension and Neuroinflammation

Hypertension is a complex condition, with 90% of the cases classified as essential hypertension, also known as idiopathic hypertension, and considered as a multifactorial inflammatory disease [Agita and Alsagaff, 2017]. Studies indicate that inflammation could serve as the bridge between hypertension and target organ damage. The brain is an early target of hypertension-induced organ damage, which can manifest as stroke, subclinical cerebrovascular abnormalities, and cognitive impairment. However, interestingly, hypertension and inflammation have a somewhat controversial relationship. A recent study examined the effects of some important risk factors (such as obesity) on brain inflammation. According to their research, obesity, as well as other risk factors, may lead to inflammation of the brain, which in turn may lead to abnormal activation of the sympathetic nervous system, causing neurogenic hypertension. In contrast, several other authors affirm that hypertension-mediated vascular shear stress and subsequent vascular remodeling disrupt the vessel endothelium, which later upregulates endothelial oxidative stress and this leads to the activation of the inflammatory pathway ultimately [Tayebati et al., 2016*;* Harrison et al., 2011*;* Winklewski et al., 2015]. Furthermore, in terms of hypertension and neurodegenerative disease, both hypertensive patients and experimental models exhibit cerebral inflammation as well as microvascular injuries in the brain, including cerebral microhemorrhages and disruption of the BBB. The cerebral inflammatory state associated with hypertension is characterized by proinflammatory astrocytic and microglial phenotypes and an elevation in proinflammatory cytokines and cerebral oxidative stress [Youwakim et al., 2021].

Upon exposure to pathogenic stimuli, neuroinflammation is usually initiated by microglia, the brain's innate immune cells. As an immune cell, microglia act like a double-edged sword, whereas the M1 phenotype plays a role as pro-inflammatory and the M2 as anti-inflammatory. Surprisingly,

in the experimental model of hypertension, microglia switch from the immunoregulatory (M2) to the proinflammatory state (M1; microgliosis). Additionally, astrocytes are classified into A1 and A2 phenotypes, which possess neurotoxic and neuroprotective properties, respectively. As the primary responders to tissue injury, microglia have close interactions with other cells in the brain, including endothelial cells and astrocytes in the context of the NVU. Thus, inflammatory responses can be initiated quickly because microglia are constantly communicating with endothelial cells, which are able to detect blood-borne pathogens. In addition, triggering the inflammatory M1 state of microglia results in the following activation of nearby astrocytes into their pro-inflammatory or A1 fate. After activation, both cell types release inflammatory signals such as pro-inflammatory signals such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), which can then activate more astrocytes and microglia, initiating a self-feedback loop of microglial activation [Liu et al., 2020]. Because recent studies show that binding IL-1β to its IL-1 receptors on microglia, astrocytes, and endothelial cells, initiates producing a range of pro-inflammatory molecules, including ROS [Liu et al., 2019].

There has been a long-standing association between arterial hypertension and BBB disruption and altered BBB microanatomy has been suggested as an early marker of cerebrovascular disease. Microglia and astrocyte activation in ischemic brain tissue induces the production of inflammatory mediators and matrix metallopeptidases (MMPs). MMPs are peptidases that catalyze the breakdown of peptide bonds across several BBB components, such as type IV collagen, laminin, and fibronectin. Therefore, MMPs are known to degrade basement membranes and reduce tight junction connections between cerebral endothelial cells, thereby promoting BBB breakdown and increasing permeability [Yenari et al., 2006]. In addition, ROS production during gliosis causes pericytes to migrate from their usual microvascular location, culminating in thigh junction's proteins disruption and, as an outcome, BBB permeability to proinflammatory factors [Youwakim et al., 2021]. Hence, through this process, NVU impairment occurred and not only infiltrated blood-derived inflammatory cells in the brain parenchyma but also activated the endothelial cells in the microcirculation. Once cerebral endothelial cells are activated, they produce vast quantities of adhesion molecules including selectins, ICAM-1, and VCAM-1. Circulating leucocytes are attracted to these adhesion molecules and transmigrate into the brain parenchyma (Figure 15 A). Upon attachment, endothelial (E) and platelet (P) selectins facilitate leukocyte transmigration into the brain tissue, where they interact with perivascular cells such as astrocytes and microglia.

Cytokines derived from leukocytes, such as IL-1β, stimulate astrocyte adhesion molecule expression, which promotes leukocyte recruitment and thus propagates pro-inflammatory conditions [Evans et al., 2021].

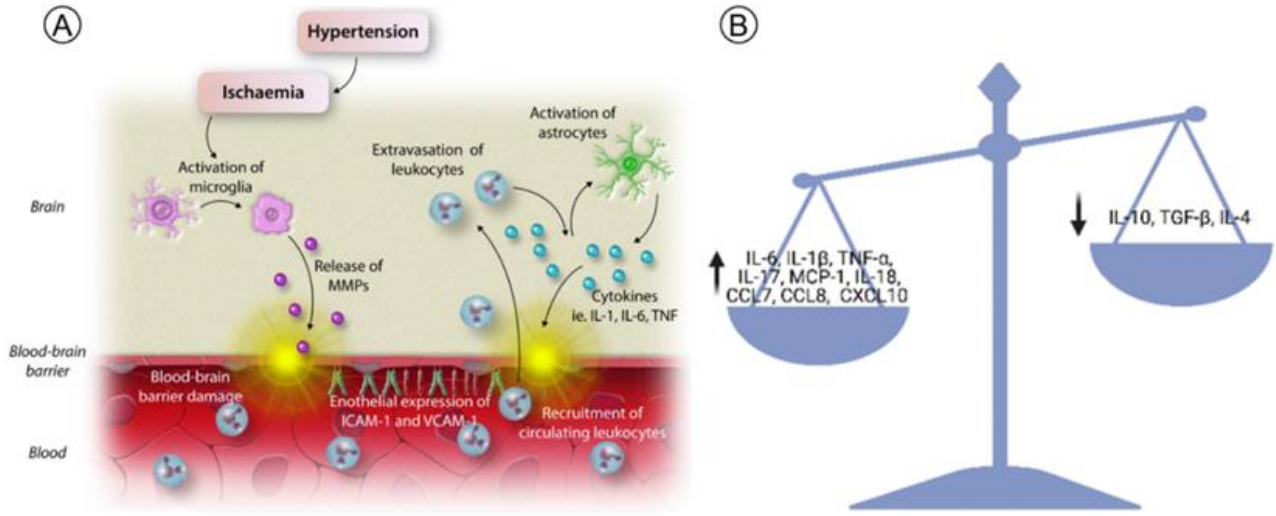

Figure 15: *(A)Hypertension mediated BBB damage and subsequent neuroinflammatory events; (B) Hypertension is characterized by pro- and anti- inflammatory cytokines (modified from – Evans et al., 2021 ; Youwakim et al., 2021).*

Moreover, at the tissue damage site, excessive release of ROS from inflammatory cells induces nuclear factor κB (NF-κB) activation, thus increasing expression of both cell adhesion molecules and the pro-inflammatory cytokines. These adhesion molecules, in turn, attract more immune cells to the area of oxidative stress. In a mouse model with a genetic NOX2 deletion, reducing ROS production significantly reduced microglial production of IL-1β, IL-6, and TNF-α in response to brain injury [Kumar et al., 2016]. Overall, it is believed that ROS production by brain-resident immune cells at the site of BBB damage promotes an inflammatory response, exacerbating local injury.

Moreover, cytokines and ROS have been shown to modulate the NVC in rodent models of hypertension, and mechanisms associated with NVC contribute to providing adequate brain energy. According to a study of wild-type mice consuming high salt diets, a higher systemic inflammatory state, characterized by Th17 cell differentiation in the distal small intestine and increased levels of circulating pro-inflammatory IL-17, was associated with NVC impairment and cognitive decline [Faraco et al., 2018]. Similarly, antioxidant treatments with $\bullet O_2^-$ scavengers such

as superoxide dismutase (SOD) and Tiron could normalize NVC, supporting the role of ROS in NVC impairments [Kazama et al., 2004]. Interestingly, evidence of astrogliosis was found in hypertension models, supporting a role for these cells in hypertension induced NVC impairment. At this point, it is apparent that Ang Il, through its ATIR, activates the JAK/STAT pathway, further enhancing proinflammatory cytokines produced by astrocytes and preventing neuroprotective anti-inflammatory cytokines from being released [Kandalam et al., 2012]. And as an overall scenario, this imbalance of pro- and anti-inflammatory cytokines may alter neurovascular functions and cause neurodegeneration **(**Figure 15 B**).**

1.5.1 Neuroinflammation in the Frontal cortex

Hypertension produces changes in the brain, such as vascular remodeling, impaired cerebral autoregulation, white-matter lesions, and cerebral microbleed. Furthermore, hypertension has been implicated in vascular dementia [Amenta et al 2002]. Hypertension causes major reductions in dendritic spine density with increases in the dendritic length in layer 3 and 5 pyramidal neurons of the medial part of the prefrontal cortex (PFC) and these may be linked in part with the cognitive impairment seen in. [Vega et al 2004, Joseph et al 2018].

The PFC is involved in cognitive processes, including learning and memory. The supra-limbic area of the medial PFC can alter memory and learning abilities [Fuster,2002, Wood,2003]. There are glutamatergic projections from the medial part of the prefrontal cortex to the ventral hippocampus as well as intracortical projections from the medial part of the PFC to a variety of limbic cortices. Medial PFC neurons are regulated by the activity of hippocampal neurons. In all cognitive processes, synapses between the hippocampus and the medial PFC may express different degrees of plasticity [Goto Y and O'Donnell P, 2001, Silva-Gómez et al 2003].

As a result of the loss of inputs into the medial part of the PFC, there may be a decrease in the spine dendrites in pyramidal neurons due to the loss of synapses on the spines [Konur et al 2003].. Chronic hypertension may also alter the integrity of the blood-CSF barrier in addition to altering the vascular structure of the brain, and several toxins may cross this barrier with a toxic effect on dendritic morphology. One might speculate that damage to the blood-CSF barrier might result in a decrease in the density of dendritic spines. In particular, the increase in total dendritic length

associated with a decrease in the dendritic spine may be explained by an inefficient synaptic connectivity mechanism in the PFC [Al-Sarraf and Philip et al 2003, Fiala et al 2002].

Acute neuroinflammation alters the regulation of cognitive integrity and neuronal activity in the PFC. Acute neuroinflammation altered the neuronal activity of fast-spiking interneurons, resulting in a hyperexcitable phenotype with increased input resistance and decreased rheobase current, as well as higher firing frequency, according to the electrophysiological properties of PFC neurons. Acute neuroinflammation within the PFC selectively increased the intrinsic excitability of fast-spiking interneurons [Feng et al 2021].

In general, pro-inflammatory cytokines adversely affect the development of dendritic spines [Bitzer-Quintero and González-Burgos, 2012]. IL-1β and IL-6, which are immediate early proteins secreted by microglia, have been shown to induce oxidative stress and reduce the length of dendrites. A high level of IL-6 within the brain results in abnormalities in dendritic spine shape, length, and distribution pattern, as well as alterations in excitatory/inhibitory synaptic transmission. TNF-α in conjunction with microglia regulates synaptic remodeling and affects long-term potentiation (LTP) as well as long-term depression (LTD) [Joseph et al 2018].

1.5.2 Neuroinflammation in the hippocampus

In the limbic system of the brain, the hippocampus plays a central role in regulating cognitive, affective, and behavioral functions. It has a high level of vulnerability to neuroinflammatory processes, with some of its subregions exhibiting specific neuroinflammatory pathology in ex-vivo studies. It will be possible to directly study the functional consequences of hippocampal neuroinflammatory pathology to optimize neuroimaging correlates of hippocampal neuroinflammation, as well as to define therapeutic end points for treatments targeting neuroinflammation, as well as their cognitive or affective consequences [Nwaubani P et al., 2022].

The role of neuroinflammatory mechanisms in hippocampal function and dysfunction can significantly contribute to the vulnerability of the hippocampus leading to cognitive impairment. Peripheral inflammatory responses in the hippocampus result in an interleukin-1 beta (IL-1β) activation with subsequent T and B lymphocyte proliferation and stimulation of natural killer cell

activity. IL-1β facilitates the production of other cytokines such as TNFα and interleukin-6 (IL-6), which acts on neural activity in the hippocampus [Chesnokova V et al 2016].

The increased levels of hippocampal IL-1β have been shown to completely block long-term potentiation (LTP) in the hippocampus in vitro and in vivo. [Kelly A et al.,2003]. Researchers found that increased levels of IL-1 stimulated reactive oxygen species (ROS) formation in the hippocampus, which activated members of the mitogen activated protein (MAP) kinase family, such as c-jun N-terminal kinase (JNK) and p38 [Vereker et al., 2000, Kelly et al., 2003, Minogue et al., 2003]. While activation of other members of the MAP kinase family, such as extracellular signal-regulated protein kinase (ERK), has been proven to result in neurite outgrowth, cell proliferation, or differentiation, [Seger and Krebs, 1995] activation of JNK and p38 has been proven to result in cell damage or cell death.

Neuroimmune signaling encompasses microglia and astrocyte activation, T-cell infiltration, and messengers such as cytokines, ROS and prostaglandins. The influence of the immune system is not restricted to states of an activated immune system. It has further been suggested that hippocampal-neuroimmunology interactions via neuroimmune signaling molecules including chemokines and cytokines contribute to the homeostatic balance of vulnerability, plasticity, and resilience mechanisms in the hippocampus [Williamson and Bilbo.,2013].

Alterations of the neuro-immune signature such as microglial sensitization seems to be a hallmark of anormal aging process resulting in facilitated and exaggerated neuroinflammatory response in the aging brain to an immune system. These mechanisms may lead to long lasting elevations in pro-inflammatory cytokines in the hippocampus resulting in impaired plasticity and cognitive deficits [Barrientos et al., 2015; Bartsch T, et al.,2015].

1.5.3 Neuroinflammation in *in-vitro* model: LPS activated BV-2 microglial cells.

Microglia are the resident phagocytes and innate immune cells of the brain. Over the past few decades, there has been an increased interest in microglia, as many investigators have recognized the importance of this cell in homeostasis, as well as various pathologies, of the CNS [Olson and Miller, 2004].

Microglia have activation states like that of macrophages and exhibit functional plasticity during activation states. The resting state, or 'ramified' state, is relatively inactive or 'quiescent' but seems to perform surveillance functions. Microglia cultures have been described as early as the 1930s; however, the use of cultures to study microglia function did not occur until after a method for obtaining and culturing large amounts of microglia was developed and improved upon. In contrast, when microglial cells are overactive, they release numerous pro-inflammatory mediators that contribute to neuroinflammation and neurotoxicity [Glezer et al., 2007].

Monitoring of microglial activation may represent an innovative proposal for the treatment of neurodegenerative diseases. This is because microglia play a significant role in several chronic neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease. [Kim SH et al 2018].

A microglia-like cell line would enable the acceleration of many research programs and the reduction of animal experimentation, provided that the cell line can reproduce the in vivo situation or primary microglia (PM) with high fidelity [Henn A et al 2009]. Two commonly used cell lines generated by infecting the cells with a retrovirus are the BV-2 and N9 microglia cell lines.

The BV-2 cells are an immortalized mouse microglial cell line exhibiting the morphological and functional characteristics of microglia [He BP et al 2002]. Cells of the BV-2 lineage are immortalized by v-raf/v-myc carrying J2 retrovirus. A BV-2 cell is characterized by the expression of nuclear v-myc and cytoplasmic v-raf oncogene products as well as surface expression of the environmental gp70 antigen. The BV-2 microglia cell line retains the morphological and functional characteristics of microglia. Due to the v-raf/v-myc expressed characteristics, in vitro BV-2 has a much higher metabolic and proliferation rate than other microglia [Luan W et al 2022].

Following lipopolysaccharide stimulation, transcriptome (480 genes) and proteome analyses indicated that BV-2 developed a reaction pattern that was similar to PM, although the average upregulation of genes was less prominent. These cells displayed normal regulation of NO production and functional response to IFN-gamma, two important parameters for appropriate interaction with T cells and neurons. BV-2 also stimulates other glial cells. NF-κB translocation was triggered in astrocytes, resulting in the production of IL-6. In this way, it appears that BV-2 cells can serve as a viable alternative to PM in many experimental settings, including studies involving complex cell-cell interactions (Figure 16) [Henn et al., 2009].

Microglia cell markers were tested on BV-2 cells and 90% were positive for nonspecific esterase activity. BV-2 cells were positive for MAC-1 and MAC-2, but negative for MAC-3. Additionally, they were negative for GFAP and GC antigens, markers for astrocytes and oligodendrocytes, respectively. Levels of IL-1 were assessed when cells were stimulated with LPS, and they increased dose-dependently. The presence of Aβ (1–42) fibrils stimulated phagocytosis by microglia in a dose- and time-dependent manner [Stansley, B 2012].

Activation of BV-2 cells by lipopolysaccharide (LPS) led to the release of proinflammatory mediators such as nitric oxide (NO), prostaglandin E2 (PGE2), and cytokines. LPS induces the production of pro-inflammatory mediators that are associated with nuclear factor-kappaB (NF-kB) nuclear translocation and mitogen-activated protein kinases (MAPKs) such as ERK1/2 and JNK [Lim JY et al 2013]. It has been demonstrated that LPS in gram-negative bacteria is a strong stimulator of microglial activation [Dansokho C et al 2018]. As a result of the activation of microglial cells by LPS, inflammatory responses are induced that promote disease progression in neurodegenerative models. [Subedi L et al 2017, Fellner A et al 2017]. In microglia, LPS interacts with Toll-like receptors (TLRs) such as TLR4 [Hines DJ et al 2013]. A signaling pathway downstream of TLR4 is activated by this interaction. It has been demonstrated that activated TLR4 signaling affects NFκB and/or other transcription factors in the nucleus and triggers the release of proinflammatory cytokines. [Lester SN et al 2014].

Upregulation of the cAMP/PKA signaling may inhibit the LPS-induced proinflammatory cytokine expression and oxidative stressin microglia and suppressing NF-κB p65 nuclear translocation [Park et al 2016]. In microglia cells, cAMP is an important intracellular regulator of homeostasis; its disruption via proinflammatory signaling results in microglial cell activation [Kim et al 2002, Woo et al 2003] . An activated PKA can act as a transcriptional regulator by interacting with NF-KB and CREB. There has been some evidence that PKA inhibits NF-κB activity by promoting its interaction with CBP and/or p300[Zhong et al 1998].

Therefore, modulating the interaction and/or activation of LPS and TLR is a potential therapeutic strategy in the prevention and treatment of neuroinflammation-related diseases. Moreover, resting microglial cells, activated with LPS, could represent an important in vitro model to evaluate the neuroinflammatory process in different CNS disease and the possible protective effects of drugs [Lopes,2016, Schmidt et al 2016].

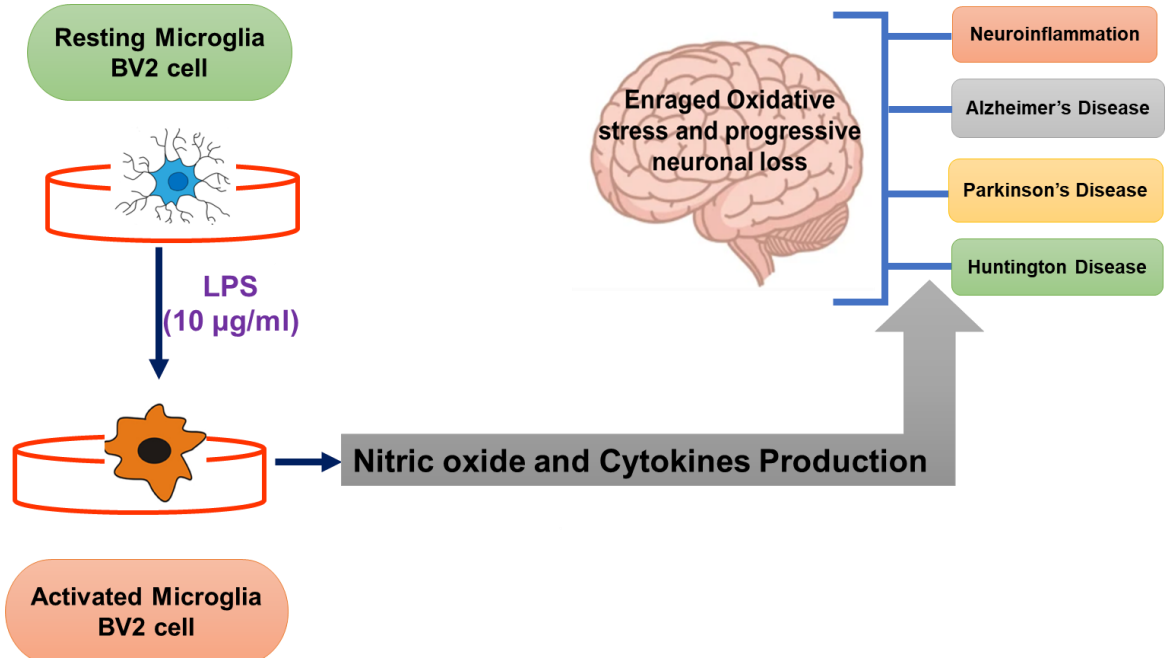

Figure 16. *LPS activated microglial cells as an in-vitro model for the study of neuroinflammation in neurodegenerative disease.*

1.6 Antioxidants and Cholinergic Precursors molecules

In experimental and human hypertension, there is an increase in the production of superoxide anions and hydrogen peroxide, a decrease in nitric oxide (NO) synthesis, and impairment in endogenous antioxidant bioavailability [Brito et al., 2015]. Hence, the most effective treatment for hypertension seems to be the use of antihypertensive drugs with antioxidant properties. Even if clinical trial data is contradictory, natural antioxidant supplementation may be a promising therapeutic tool for hypertension. In experimental evidence, early antioxidant treatment may be beneficial in preventing hypertension-mediated organ damage [Martinelli et al., 2021]. Additionally, hypertension contributes to both VaD and AD, the two most common causes of dementia, which account for about 85% of cases [Canavan and O'Donnell, 2022]. There is well-documented evidence that VaD and AD are associated with low cerebrocortical cholinergic input. It has also been reported that VaD can lead to cholinergic deficits, independently of concomitant

AD symptoms. Because cholinergic structures may be susceptible to ischemic damage, resulting in this impairment. As a result, intensive research on potential treatment methods towards improvement of cholinergic neurotransmission deficiencies is motivated by the hypothesis that impaired brain cholinergic transmission plays a significant role in the loss of cognitive functioning in AD and/or VaD [Tomassoni et al., 2006].

1.6.1 Thioctic Acid (alpha lipoic acid)

Alpha-lipoic acid (ALA), often referred to as thioctic acid (TIO) and 1,2-dithiolane-3-pentanoic acid, is a naturally occurring substance that is essential for the function of several enzymes involved in oxidative metabolism. TIO was first discovered by Esmond E. Snell in 1937 and then isolated by Lester J. Reed in 1951. The first clinical application of TIO was recorded in Germany in 1959 for the treatment of acute poisoning with *amanita phalloides*, also known as death cap. the same authors quickly afterward discussed its value in treating neuropathic complaints [Gomes and Negrato, 2014]. As well, TIO is synthesized in the organism, adsorbed intact from dietary sources, and accumulates transiently in many tissues after adsorption. As an antioxidant and detoxifying agent, it has been proposed to treat diabetic neuropathy, improve age-related cardiovascular, cognitive, neuromuscular deficits, and modulate inflammatory signaling. Thus, TIO has received increasing attention as a dietary supplement with potential therapeutic effects in the prevention and treatment of neurodegenerative diseases [Tomassoni et al., 2013]. Moreover, as a natural compound, TIO represents an important molecule to prevent fat accumulation. As previous reviewed oxidative stress plays an essential role in the development of co-morbidities in obese condition [Manna et al., 2015]. Obesity promotes oxidative stress through different biochemical mechanisms as shown in figure 17.

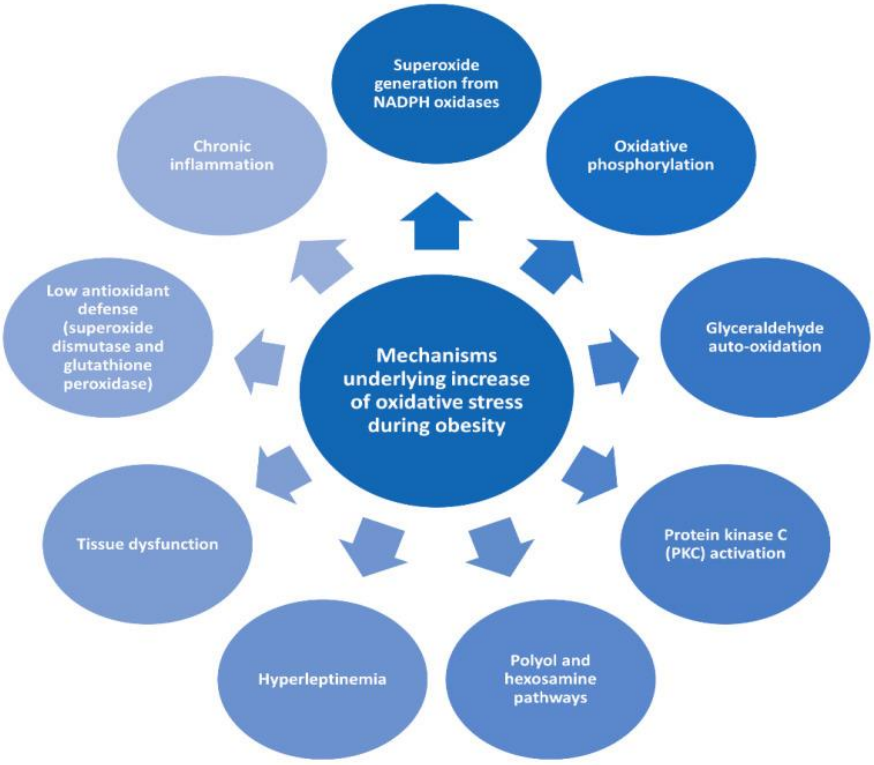

Figure 17. *Mechanisms underlying increase of oxidative stress during obesity. Modified from Roy et al., 2021.*

As a result of a chiral center at the C6 position, two lipoate enantiomers (S and R) of TIO exist (Figure 18A). However, only R-TIO is endogenously synthesized, suggesting that the R-enantiomer is preferred in biological systems. In fact, previous studies show that R-TIO (peak > 300 ng/ml) has a higher bioavailability than S-TIO (peak > 200 ng/ml) in patients with diabetic polyneuropathy, which indicates that R-TIO is more readily absorbed. Additionally, it is possible that S-TIO is metabolized more quickly. Therefore, R formation is considered the most active form. TIO can be both oxidized and reduced, the disulfide-containing ring is broken open to produce dihydrolipoic acid (DHLA) by the mitochondrial enzyme lipoamide dehydrogenase (Figure 18B). ALA/DHLA is a crucial cofactor for pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, and other mitochondrial enzymes, making LA an essential cofactor in catabolic and metabolic processes [Salinthone et al., 2008].

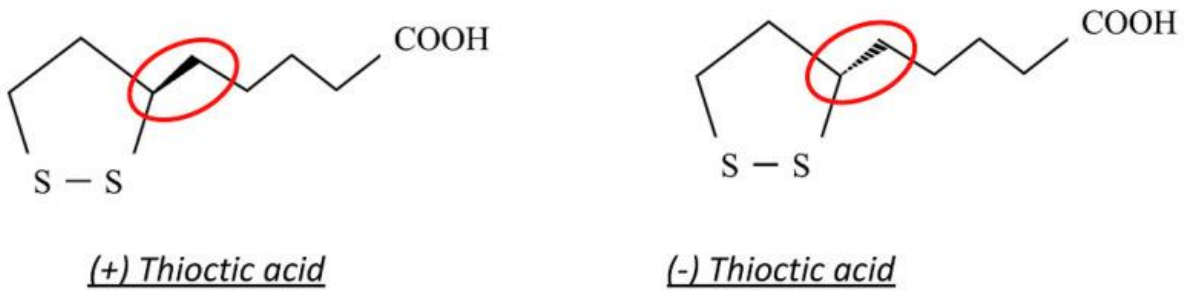

Figure 18. *Chemical structure of (+)-thioctic acid and (−)-thioctic acid. Red circles represent the chiral center of the molecule (Modified from Roy et al., 2021).*

In addition to their role as an enzyme cofactor, TIO and DHLA also participate in several other cellular and molecular functions, including their role as powerful antioxidants through several different mechanisms: removing reactive oxygen and nitrogen species, chelating metals, anti-inflammatory effects and repairing damaged proteins and lipids (Figure 14). They interact with reactive oxygen species (ROS) including hydroxyl, peroxyl, and superoxide radicals as well as reactive nitrogen species (RNS), hence their roles are a part of cellular defense systems against stress conditions, where oxidative stress is the key contributing factor to the underlying etiology. Even though TIO and its reduced form DHLA can scavenge free radicals directly, it is still unclear if TIO and DHLA can scavenge ROS directly. ALA indirectly inhibits oxidative processes through the transcription of genes involved in glutathione synthesis, and regeneration of reduced vitamin C, E, and glutathione from oxidized forms [Seifar et al., 2019]. As well as having direct antioxidant properties, TIO and DHLA chelate redox-active metals, such as copper, free iron, zinc, and manganese, albeit in different ways. According to Bush, chelating iron and copper with DHLA may explain the lower rate of free radical damage to the brain and improvements in AD pathobiology [Bush, 2002]. TIO treatment has also been shown to decrease iron absorption and iron pool size in lens epithelial cells and cerebral cortex. Additionally, several studies have shown that TIO modulates metal-induced free radical reactions where transitional metals accumulate. Furthermore, research also suggests that TIO alters the NF- κβ signal transduction. NF- κβ, a redox-sensitive transcription factor, plays a significant role in inflammation by regulating the expression of cytokines, inner tissue factors, and adhesion molecules in endothelial cells. Furthermore, the findings demonstrate that TIO therapy controls inflammatory cell infiltration into

the CNS and prevents NF-B-dependent production of MMP-9. Thus, TIO could play a pioneering role in the treatment of hypertension induced cerebrovascular alteration [Kim et al., 2007].

Also, TIO might increase glucose uptake in insulin-resistant neurons and lead to increased acetyl-CoA production during glycolytic metabolism, resulting in increased acetylcholine production. Sancheti et al. found that TIO feeding stimulated the PI3K/Akt insulin signaling pathway. A reversed synaptic deficit was seen in AD mice models in this context [Sancheti et al., 2013].
As reported in our previous paper:
"TIO has many clinically valuable properties [114]. It works as an enzymatic cofactor [93], and it is involved in glucose metabolism, lipid metabolism, and control gene transcription. TIO also restores the intrinsic antioxidant systems and supports their production or cell accessibility [94,95]. It efficiently removes heavy metals from the bloodstream. TIO is also responsible for oxidative stress [115]. The most identifiable characteristic of TIO over other antioxidant substances is that it reacts both as lipid and as water-soluble compounds [113]. TIO also has some other functions because it is involved in mitochondria-producing energy, acting as a cofactor for various enzymes that deal with metabolism [113]. ***[Roy et al., 2021 Appendix 2]****.*

Based on these findings, it is possible that TIO increases glucose absorption in insulin-resistant neurons, supplying more glycolytic metabolites, including acetyl-CoA, to these neurons. Since acetyl-CoA availability from glucose metabolism is essential for acetylcholine (ACh) synthesis, TIO may as well be able to directly elevate the concentration of the substrate acetyl-CoA for ACh synthesis (Figure 19). Thus, it could be concluded that TIO could be considered a promising therapeutic agent for the treatment of neurodegenerative diseases, for instance, cerebrovascular alteration mediated cognitive impairment and AD.

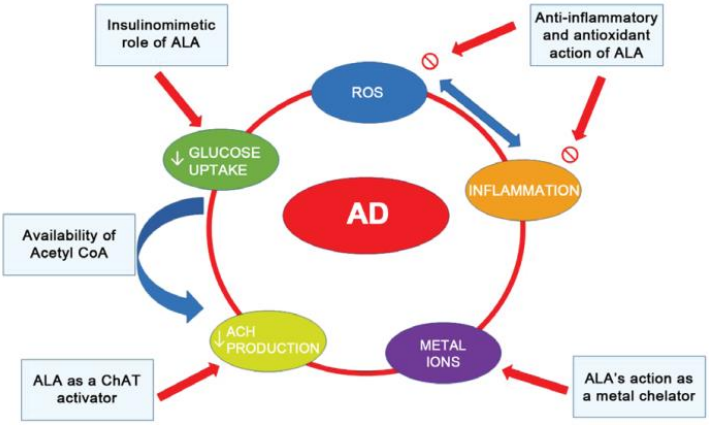

Figure 19: *Alpha Lipoic Acid (ALA) can improve cognitive performance and could be a potential treatment for the pathogenesis of neurodegenerative disease through several mechanisms (modified from - Seifar et al., 2019)*

Previous our evidences highlighted in the SHR highlights the greater effectiveness of (+)TIO compared to racemic form to reduce cardiac and renal damages related to the oxidative stress [Martinelli et al., 2021].

As reported in the published paper:

"According to other studies [25,81,82], we found greater effectiveness of (+)-ALA compared to (+/−)-ALA in reducing oxidative stress, demonstrated by the reduction of oxidized proteins levels, and by 8-oxo-dG expression, as well as cardiac and renal damages induced by hypertension. However, the limitation of our study is that we did not assess the effects of various forms of ALA on SHR renal and cardiac functions yet. Thus, further studies are necessary. The advantage in using (+)-ALA, as compared to the racemic form, may be linked to an amplified bioavailability and biological activity of this enantiomer that enhanced the antioxidant activity in SHR animals. The supplementation of lipoic acid as natural antioxidant has already been demonstrated to be safe in clinical trials [83], and to have multiple beneficial effects [57]. In particular, (+)-ALA showed the most pronounced activity, with extremely few acute and subchronic toxicities, compared to all the forms of ALA [84]". [***Martinelli et al., 2021 Appendix 3***].

To conclude, these findings propose (+) TIO as one of the more appropriate antioxidant molecules for slowing down organ alterations associated with hypertension.

1.6.2 Choline alphoscerate (L-alpha glycerylphosphorylcholine)

Cerebrovascular injury is typically accompanied by brain damage which consequences in cognitive dysfunction. Cerebrovascular pathology is acknowledged as a major cause of dementia, also known as VaD, and brain vascular injury exacerbates the course of Alzheimer's disease (AD). VaD is characterized by a steady decline in cognitive, functional ability impairment, and behavioral issues. The primary cause of VaD is ischemia injury or oligaemia to the brain regions responsible for cognition, memory, and behavior. Therefore, arterial hypertension is a major factor in brain vascular injury and may be linked to cognitive decline [Tayebati et al., 2009; Tomassoni et al., 2012].

Cholinergic neuronal processes regulate cerebral blood flow and cognitive functions. This could be linked to the pathogenesis of VaD, where cognitive impairment is caused by decreased brain blood flow, hypoperfusion, and complete or partial ischemia, but it may also be related to the course or complications of AD. Cholinergic structures and specific brain areas are vulnerable to ischemic damage which contributes to cognitive decline, functional impairments, and behavioral issues associated with VAD and AD. Similarly, study on the brains of AD individuals has revealed a notable reduction of the nicotinic cholinergic receptors and the choline acetyltransferase (ChAT), an enzyme that synthesizes Ach. Additionally, administering the muscarinic antagonist scopolamine to healthy young subjects causes a cognitive impairment resembling that seen in adult-onset dementia. It has been found that the decline in high affinity cholinergic receptors correlates with the loss of cortical cholinergic synapses. Considering these findings, the "**cholinergic hypothesis**" of geriatric memory dysfunction has been proposed [Tomassoni et al., 2012; Traini et al., 2013].

The hypothesis of impaired brain cholinergic transmission plays a significant role in the decline of cognitive function in AD and/or VaD sparked intense investigation into potential treatment strategies for addressing cholinergic neurotransmission deficiencies. Intervention with ACh precursors, stimulation of ACh release, use of muscarinic or nicotinic receptor agonists, and acetylcholinesterase (AChE) inhibition were among the various options proposed or attempted for treating impaired cholinergic neurotransmission in dementia. Interestingly, neurons cannot synthesize choline, which comes from food and is delivered to neurons through the bloodstream. ACh released from cholinergic synapses is hydrolyzed into choline and acetyl coenzyme A by

AChE. A high-affinity ChAT can recover almost 50% of choline-derived ACh hydrolysis (Figure 20A). Consequently, neurons must obtain additional choline to synthesize ACh. It appears that cholinergic neurons exhibit a particularly high affinity for choline. If choline is not provided in sufficient amounts, neurons can obtain it by hydrolyzing membrane phospholipids. It has been proposed that this auto-cannibalism mechanism may play a role in AD-associated cholinergic neuron degeneration [Amenta et al., 2001]. Consequently, decreased choline availability and increased phosphatidylcholine breakdown have been reported as relevant AD pathophysiologic conditions.

As a cholinergic precursor, choline alphoscerate (L-α-glycerylphosphorylcholine; GPC) plays a pivotal role in the preservation of the structural integrity of cell membranes (Figure 20 B). For instance, in oral administration, GPC cross the blood-brain barrier, affecting brain phospholipid metabolism and increasing levels and releases of choline and acetylcholine [Tayebati et al., 2017]. GPC was also subjected to pharmacodynamic studies during phases of development to examine its efficacy in potentiating cholinergic neurotransmission in the brain as well as interfering with its phospholipid metabolism. Researchers have shown that GPC increases acetylcholine release in the rat hippocampus, facilitates learning and memory, improves brain transduction mechanisms, and prevents frontal cortex and hippocampus structural changes associated with aging.

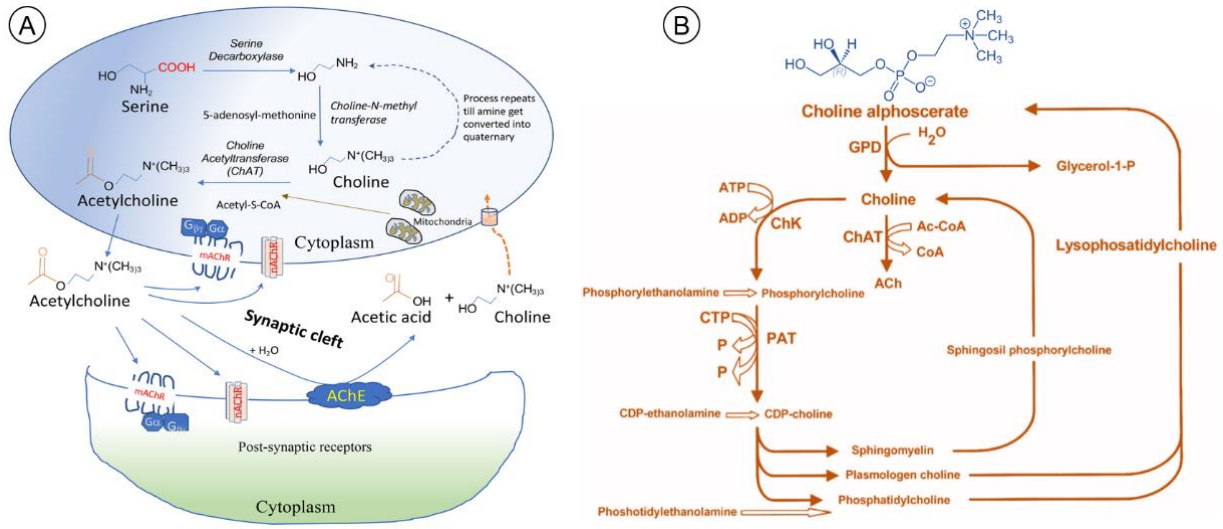

Figure 20: *(A) Synthesis and degradation of Acetylcholine; (B) Steps in which choline alphoscerate can influence neurotransmitter biosynthesis are shown (modified from - Traini et al., 2013).*

As well as other findings, GPC also showed neuroprotective effects when used alone or in combination with AChE/cholinesterase inhibitors in SHR as animal model of VaD. Although clinical efficacy study of these drugs showed an unsatisfactory result, to some extent they improve memory and other cognitive functions in AD and VaD [Tomassoni et al., 2012]. In this regard, it is important to note that although cholinergic therapy is believed to have different protective mechanisms in VaD and AD, increasing evidence suggests that anti-inflammation is at least partially responsible for the safeguarding effects. For instance, the cholinergic anti-inflammatory pathway, which comprises efferent vagus nerve, acetylcholine, and the α7 subunit of the nicotinic acetylcholine receptor (nAchRα7), has been shown to reduce excessive inflammation by inhibiting NF- κB nuclear translocation in essential conditions [Ren et al., 2017].

1.6.3 Mechanism of action of Choline alphoscerate

The anabolic pathways proceed with the production of choline molecule and paving another lead of glycerol-1-phostate which in turn utilized to transform glycerol-1-phosphorylcholine using glyceryl-phosphorylcholine diesterase enzyme which is hydrolysed to release acetyl choline from the cholinergic synapses.

The synthesis of acetyl choline by using choline, however phosphorylated glycerol-1-phosphate enters the pool of phospholipids. By product being acetyl choline serves to rearrange and organize nerve cell membrane components. Thus, choline administration can increase its availability for the synthesis of acetyl choline which functions as a neurotransmitter. In order to synthesize choline is uptake from the diet by the nervous tissue and couriered to neurons via blood stream.

Therapeutic strategies for countering MCI either of degenerative or vascular origin have not been established yet. There are currently no EMEA/FDA-approved therapies for MCI as no treatment trial to date has convincingly demonstrated a significant effect on cognition or symptom progression. Future trials will likely need to use strategies to define optimal treatment durations and develop sensitive assessments and reliable outcomes to detect treatment benefit in mildly impaired subjects. In meantime, in spite of the numeric differences of patients treated with (acetyl) cholinesterase inhibitors or with GPC, positive results obtained with this cholinergic precursor

probably would justify reconsideration of its effects as well as further investigations on it in larger carefully controlled studies [Enea Traini et al; 2013].

Supportive preclinical studies published in recent years have concentrated on the activity of choline alphoscerate in neurotransmitter transporter systems. Plasma membrane and vesicular transporters for acetylcholine and monoamines (dopamine, norepinephrine and serotonin) are specific proteins that play a role in the regulation of neurotransmission and could be a target for the action of therapeutic agents. Cholinergic transporters, including high-affinity choline uptake and vesicular acetylcholine transporters, control cellular mechanisms of acetylcholine synthesis and release into presynaptic terminals and load into synaptic vesicles.

Treatment of 4 weeks of spontaneous hypertensive rats with choline alphoscerate increased high-affinity choline uptake and vesicular acetylcholine transporters in the frontal cortex, striatum, and hippocampus as well as peripheral blood lymphocytes considered to be a marker for cerebral cholinergic transporters. This effect of GPC was considered consistent as it induces greater acetylcholine synthesis.

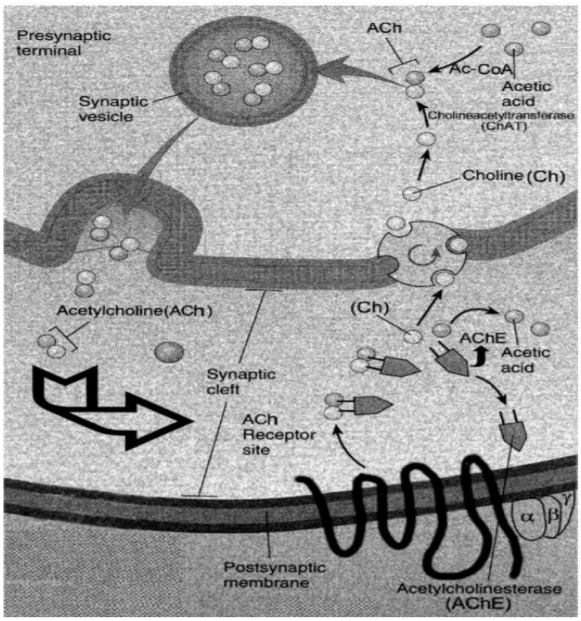

Figure 21: *Schematic representation of a cholinergic synapse with neurotransmitter anabolic and catabolic pathways [modified from: Amenta.F et al; 2001]*

The activity of choline-containing phospholipids in brain phospholipids biosynthesis may influence brain metabolism and various neurotransmitter systems. Based on the finding that

choline-containing phospholipids CDP choline has a monoaminergic profile, the activity of GPC on brain dopamine, serotonin levels and dopamine plasma membrane transporters, vesicular monoamine transporters 1 and 2, serotonin transporters and norepinephrine transporters have been investigated. Administration of the compound increased dopamine levels in frontal cortex and cerebellum and serotonin levels in frontal cortex and striatum. It also stimulated dopamine plasma membrane transporter in frontal cortex and cerebellum. This investigation concluded that GPC possesses also a monoaininergic profile and interferes to some extent with brain monoamine transporters.

Choline-containing phospholipids showed specific activities on all elements of NVU. As reported in our review:

Based on the evidence that administration of CCPLs, not only increases ACh levels and modulates the phospholipid breakdown, the administration of CCPLs has been studied as a possible therapeutic approach to treat neurodegenerative disorders (Tayebati et al., 2013). Several studies have clarified the properties of CCPLs, in particular of α-GPC, on the neuronal alterations in animal models of cerebrovascular disease (Tomassoni et al., 2006; Tayebati et al., 2009; Tomassoni et al., 2012; Tayebati et al., 2015). The beneficial effects of α-GPC may depend by an influence of the compound on brain phospholipids metabolism and/or by its documented activity of increasing free plasma choline levels (Gatti et al., 1992) and brain ACh bioavailability and release (Sigala et al., 1992). The α-GPC provides both free choline and phospholipids for synthesizing ACh and could reconstitute the components of the nerves cell membrane. Moreover α-GPC has been found to increase ACh levels and release in rat hippocampus (Sigala et al., 1992, Amenta et al., 2006). These preclinical findings are associated with results of clinical trials in which CCPLs were proposed as potential pharmacotherapies for adult-onset dementia disorders. In particular, α-GPC countered the degeneration occurring in the brain areas involved in learning and memory of AD patients (Amenta et al., 2020).

More recently in a rat model of dual stress, the administration of α-GPC (400 mg/kg) for 7 days, countered the increase of stress hormones, reduced hearing loss, and prevented neuronal injury. This effect has been considered due to the increase of choline acetyltransferase (ChAT) and reduction of brain neuro-infammation (Jeong Yu, H., et al., 2022). Moreover, in the hippocampus α-GPC enhances brain-derived neurotrophic factor (BDNF) expression and protects the activity of immature cells in the hippocampus (Jeong Yu, H., et al., 2022).

*The CCPLs show mainly a cholinergic profile and affect phospholipids biosynthesis, nervous tissue metabolism and neurotransmitter systems also may have a monoaminergic profile. Dopamine levels in brain areas increase after treatment for 7 days with α-GPC (150 mg/Kg/day) but not with CDP-choline (325 mg/Kg/day) whereas dopamine transporter expression was stimulated in frontal cortex and cerebellum by both CDP and α-GPC. α-GPC increased also serotonin levels in frontal cortex and striatum (Tayebati et al., 2013). CDP-choline in rats does not have effects in the forced swim test, but its primary metabolites showed opposing effects: cytidine has antidepressant-like actions, whereas choline has prodepressant-like actions (Calzezon et al., 2002). The results of this study are consistent with a clinical trial in which depression symptoms in mild to moderate stage AD patients, probably could benefit from stronger cholinergic stimulation induced by combined usage of donepezil and α-GPC (Carotenuto et al., 2022). [**Roy et al., 2022 Appendix 1**]*

A metanalysis of clinical study highlighted that GPC alone or in combination with donepezil improved cognition, behavior, and functional outcomes among patients with neurological conditions associated with cerebrovascular injury [Sagaro et al., 2023].

Moreover, as reported in our review:

"α–GPC has been on the pharmaceutical market since 1987, however its popularity has declined since the introduction of cholinesterase inhibitors. The last 10 years have seen a renewed attention on this compound, with preclinical studies, clinical investigations and review articles published in the literature (Traini et al. 2013).

The majority of clinical studies available on the effect of α–GPC on cognitive function in neurodegenerative and cerebrovascular disorders were detailed in two review articles as showed in (Parnetti et al. 2007; Traini et al. 2013). Administration of α–GPC improved congnitive functions, as well as affective and somatic symptoms (fatigue, vertigo). The effects of α–GPC were greater than those of placebo and of the same extent or superior to those of reference compounds (Amenta et al. 2001). Studied in rat models showed that the association of α–GPC with (acetyl)cholinesterase inhibitors enhance the effects of both drugs on cholinergic neurotransmission (Amenta et al., 2006). Based on this evidence, in Italy was performed an independent clinical trial: "Effect of association between a cholinesterase inhibitor and α–GPC on cognitive deficits in AD associated with cerebrovascular impairment" (ASCOMALVA). In accordance with literature data, in patients selected to the reference treatment group (donepezil + placebo), a slight time-dependent worsening of Mini Mental State Examination (MMSE) and The Alzheimer's Disease Assessment Scale–Cognitive Subscale (ADAS-cog) scores was found. In the active

treatment group, the administration of donepezil + α–GPC countered the decline of MMSE and ADAS-cog scores. The effect of the association on psychometric tests was statistically significant after 12 months of treatment (Amenta et al. 2014). The combination of donepezil plus α–GPC was more effective than donepezil alone in countering symptoms of apathy in AD. This suggests that the availability in the brain of a higher amount of acetylcholine may affect apathy in AD subjects with spared executive functions (Rea et al., 2015).

A supplementary contribution of the ASCOMALVA trial was the study of the influence of the combine treatment of α–GPC and donepezil on brain atrophy in AD. Cerebral atrophy is a common feature of neurodegenerative disorders. This pathology includes a loss of gyri and sulci in the temporal lobe and parietal lobe, and in parts of the frontal cortex and of the cingulate gyrus. Patients enrolled in the ASCOMALVA trial underwent yearly Magnetic Resonance Imaging analysis for diagnostic purposes. In 56 patients who achieved three years of therapy, brain MRI were examined by voxel morphometry techniques. At the end of three years of treatment, in patients enrolled in the active group of donepezil plus α–GPC, a reduction of the volume loss of the grey matter (with a concomitant increase of the volume of the ventriculi and cerebrospinal fluid space) was observed, compared to the reference group (just donepezil). Frontal and temporal lobes, hippocampus, amygdala and basal ganglia represented the areas in which brain atrophy was more sensitive to combination treatment. No significant differences were noticeable in other areas, between the two groups. Morphological data were confirmed by neuropsychological assessment performed alongside the trial (Traini et al., 2020).

*In conclusion, a cholinergic precursor-loading strategy with α–GPC in combination with donepezil counters to some extent the atrophy occurring in some brain areas of AD patients. In these patients, the parallel observation of an improvement in cognitive and functional tests suggests that morphological changes observed could have functional relevance (Traini et al., 2020). [**Roy et al., 2022 Appendix 1**]*

1.7 Animal model of hypertension

Animals are valuable research tools for understanding the pathophysiology of diseases and developing therapeutic interventions. Various animals have been reported as useful models for understanding human and animal diseases. Based on the feasibility and similarities to human and drug safety, animals are deployed in biomedical research. For instance, rats and mice share about 98% of their DNA with humans, and because of their short life span, they can be studied over their whole life cycle or even across several generations [Leong et al., 2015]. Small animal models are

the most widely utilized in the research of hypertension, and they offer valuable insights. These models, for instance, may focus on characteristics that have been associated to the pathophysiology of human hypertension, such as salt sensitivity, renin-angiotensin-aldosterone system (RAAS) activation, and genetic variables. Rats and mice are more cost-effective, have shorter gestation times, and are easier to genetically modify than larger animal models. However, accurate blood pressure measurement in small animals is difficult, surgical operations are technically complex, and the quantity of available samples, notably plasma and urine, may be limited. Nevertheless, some of these problems have been resolved by recent developments in imaging and surgical procedures, which have substantially expedited the evaluation of target organ damage [Lerman et al., 2019].

Basically, primary and secondary hypertension is defined in animal models based on etiology. Figure 22 illustrates a schematic classification of animal models of hypertension and the corresponding human hypertension [Risler et al., 2005].

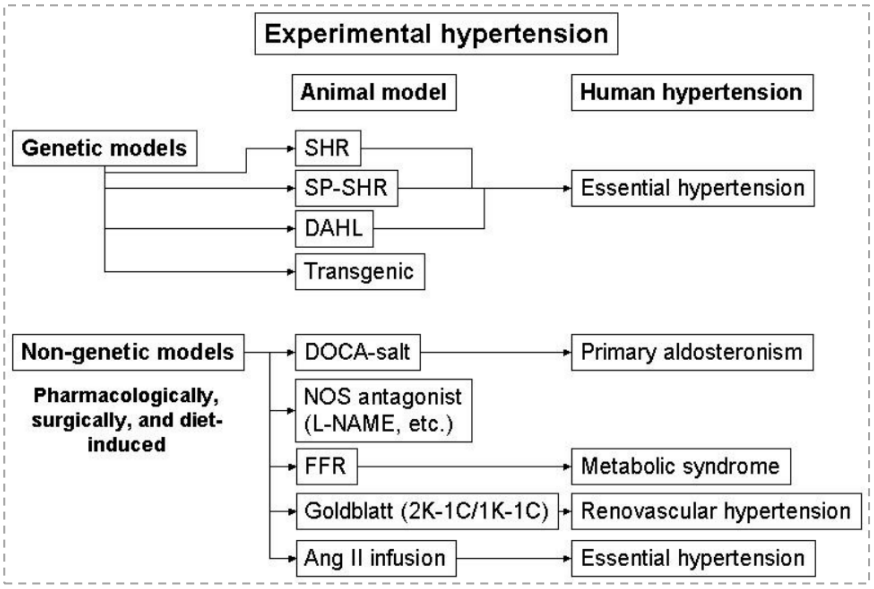

Figure 22: *Animal models of hypertension and the corresponding human hypertension (modified from - Risler et al., 2005).*

There are several strains of rats that exhibit genetic hypertension, including Spontaneously Hypertensive Rat (SHR), Dahl Salt-Sensitive (DSS), Fawn-Hooded Hypertensive Rats (FHH),

Milan Hypertensive Rats, Lyon Hypertensive Rats, Sabra hypertensive rats, genetically hypertensive rats, and inherited stress-induced arterial hypertension rats. Among these, the SHR is the most studied. The term spontaneously hypertensive rats have been indexed in PubMed [https://pubmed.ncbi.nlm.nih.gov/] for more than 4500 articles over the past 10 years. In contrast, the DSS rat, the second most cited model over the same period, was indexed 585 times, while other genetic rat strains were indexed less frequently. In addition to the inbred strains mentioned above, several congenic and transgenic animals also express hypertensive phenotypes [Lerman et al., 2019].

1.7.1 Spontaneously Hypertensive Rat (SHR)

SHR is an excellent animal model of hypertension which shares several characteristics with human essential hypertension. It has a distinct and uniform genetic predisposition, allowing researchers to investigate the causes, mechanisms, and pathophysiology of hypertension, as well as its behavioral effects. SHRs are normotensive at birth and develop sustained hypertension within the first 6 months of life, offering them the best-known model of essential hypertension available today [Amenta et al., 2010; Tayebati et al., 2012]. The SHR strain was first developed in the 1950s by Okamoto and colleagues. Hypertensive rats were selected by mating male Wistar Kyoto rats (WKY) with mild hypertension (systolic pressure 145–175 mmHg) with females with relatively high BP (systolic pressure 130–140 mmHg). Through their efforts to select hypertensive offspring by continuously measuring blood pressure, they were eventually able to generate a colony of rats developing hypertension without exception and described them as SHR in 1963. Both neuronal and vascular changes have been linked to the genetic causes of hypertension in SHR [Amenta and Tomassoni, 2011].

Furthermore, SHR is a model that has been widely researched for measuring hypertensive brain damage and treatment options. Hypertensive brain injury in humans shares several characteristics with this condition, including the time-dependent rise in arterial blood pressure, the incidence of brain atrophy, the loss of nerve cells, and glial response. As a result, SHR may be considered as a plausible model of brain damage brought on by hypertension and perhaps prevented by effective antihypertensive therapy. Cerebrovascular changes associated with hypertension are hypertrophy

or remodeling and are closely linked to the degree of blood pressure elevation. Previous research found that cerebral arteries of SHR aged 24 weeks show significant lumen diameter reduction when compared to normotensive WKY rats. The wall-to-lumen ratio values are an important parameter in the analysis of vascular changes. Thus, vascular alterations are evident in the animal model of SHR. Brain injury, swelling, and other experimental procedures affect the expression of brain aquaporins. In rodents, AQP4 is regulated in astrocytes following cerebral oedema caused by focal brain ischemia and hyponatremia. In rat models of brain injury, AQP4 is overexpressed in astrocytes at the region where the BBB is impaired. Tayebati and colleagues conducted research on the expression of AQP4 in the SHR brain, and the immunohistochemistry results showed a remarkable increase in immune reaction in the cerebral vessels of SHR. This data indicates that increased expression of AQP4 in the brain of SHR may be implicated in impaired autoregulation of CBF and water content in this animal model [Tayebati et al., 2012; Taybeti et al., 2015]. The occurrence of cytotoxic oedema in SHR cannot, however, be excluded based on these data. It is possible that this contributes to the development of the brain lesions documented in SHR. And damage in the BBB could accelerate the neuroinflammation process in the brain. Thus, cerebrovascular inflammation cannot be excluded and interestingly, signs of inflammation have been observed in this rat's brain (table1). The hippocampus is a cerebral area particularly sensitive to ischemia and thus is a site of predilection for brain damage after hypoxic or ischemic injury and is also involved in memory and learning. It has also been found that in previous studies, Sabbatini and coworkers found significant microanatomical changes in the hippocampus of 4- and 6-month-old SHR rats in comparison with age-matched WKY rats, which were characterized by decreased hippocampus volume, neuronal loss, and astroglial response [Sabbatini et al., 2000]. Vascular dementia is described clinically as this situation. SHR also exhibits impairments in the brain cholinergic system which can lead to changes in memory, conditional avoidance, and spatial learning abilities. There are also behavioral abnormalities in SHR, such as hyperactivity, low attention, inhibition deficit, and hyper-reactivity to stress. These resemble the behavioral abnormalities of attention deficit hyperactivity disorder (ADHD) [Amenta et al., 2010].

Neuroinflammation has been observed in several experimental models of hypertension. SHR is a model of neurogenic hypertension, and brain inflammation can precede BBB disruption and cerebrovascular inflammation [Tomassoni et al., 2004; Sun et al., 2006; Tayebati et al., 2016].

Interestingly, signs of inflammation have been found in different brain areas of this rat as summarized in Table3.

Table 3: Cerebral markers of inflammation in spontaneously hypertensive rat (SHR);

Cerebral inflammatory markers		**Brain Regions**
Astrogliosis		Frontal Cortex,
		Striatum,
		Hippocampus
		Occipital Cortex
		Deep cortical region
Microgliosis		Corpus callosum
		Fimbria-fornix
		Paraventricular nucleus
Increased IL-1β expression		Amygdala
		Hypothalamus
		Hippocampus
		Brainstem
		Whole brain homogenate
Increased TNF-α expression		Hippocampus
		Whole brain homogenate
Decreased TGF-β expression		Cerebral Endothelial Cells (CECs)
		Hippocampus
Decreased IL-10 levels		Cerebrospinal fluid (CSF)
Increased NLRP3 expression		Amygdala
		Hypothalamus
		Hippocampus
		Brainstem
Increased COX-2 expression		Hippocampus
Increased ICAM-1 expression		CECs of brain micro vessels
		Whole brain homogenate
		Myeloid leukocytes
Increased VCAM-1 expression		CECs
Increased NF-κB levels		Amygdala
		Hypothalamus
		Hippocampus
		Brainstem
Increased iNOS expression		Amygdala
		Hypothalamus

		Hippocampus
		Brainstem
		Whole brain homogenate
Reduced nNOS expression		Brainstem
Increased nNOS expression		Whole brain homogenate
NOS down regulation		CECs
		Brainstem
Reduced eNOS expression		Cerebral vessel
Increased Nox2 subunit ($gp47^{phox}$) expression		Hippocampus
Increased NK cells count		Brain microvasculature
Increased number of adherent and infiltrating macrophages		Cerebral vessels

modified from Youwakim et al., 2021

Furthermore, cholinergic system is altered in brain areas of SHR rats. The activity of choline acetyltrasferase (ChAT), a marker for cholinergic activity, was reported to be enhanced in the brain, expecially in the brain stem of SHR. Other studies underlined in SHR-SP alterations of the cholinergic cerebral system. In fact, in the SHR-SP, in comparison to WKY rats a decrease of the levels of choline and acetylcholine in the cerebral cortex, hippocampus and cerebral spinal fluids The alterations of ACh levels in the cerebral cortex and hippocampus, has been correlated with a decrement of the functions cognitive, of learning and memory. Pre-clinical studies in SHR have shown an alteration in the abilities of learning and memory explored with behavioral test The reduced in central nicotinic acetylcholine receptors in prefrontal cortex, caudate-putamen, and enthorinal cortex but not in the hippocampus of 15-month-old SHR could in part explain the modest performance decrements of the water maze tasks by SHR compared to WKY

Previous study showed a higher expression of VAChT in the hippocampus of six-month-old SHR compared age-matched WKY rat, whereas no differences was evident in the younger rat strain.

Speculatively the increased expression of VAChT observed in SHR represents a tentative to compensate cholinergic impairment in the first stages of hypertensive brain damage. With worsening of hypertensive brain damage cholinergic dysfunction probably becomes no longer compensable through an up/regulation of VAChT [Amenta et al., 2011].

2. AIM

Hypertension is a highly prevalent condition that affects one-third of the world's adults and two-thirds of adults over the age of 65 [Mills et al., 2016]. The relationship between hypertension, brain structure alterations and VCI has been extensively studied [Rodrigue and Gérard, 2016]. There is increasing evidence that arterial hypertension is the most important modifiable cerebrovascular risk factor, with a continuous, consistent, and independent correlation with brain diseases. High BP increases the risk of cerebral small and large vessel disease, resulting in brain damage and VAD. A decline in cerebrovascular reserve capacity and developing degenerative arterial wall alterations are responsible for brain infarcts, hemorrhages, and white matter hyperintensities [Lanari et al., 2007; Gąsecki et al., 2013].

Several studies have shown that cerebrovascular alteration may initiate vascular disease in the brain of hypertensive individuals by either reducing NO bioavailability or by participating in vascular remodeling through ROS production. Moreover cerebrovascular alteration is often linked to the initiation of an inflammatory process that frequently affects vascular endothelium, BBB permeability, that may lead to neuroinflammation by activating proinflammatory neuroglial cells [Carvalho and Moreira, 2018; Takata et al., 2021]. In addition, vascular changes also impaired the cholinergic neurotransmission that plays a key role in the autoregulation of cerebral blood flow [Tayebati et al., 2012; Hamner et al., 2012].

The cholinergic precursor drug, GPC therapies have demonstrated promising effects on cognitive improvement which may act through the improvement of cholinergic pathway by stimulating the production of ACh. Dextrorotatory form of Thioctic acid [(+)TIO], is a naturally occurring antioxidant eutomer that has been shown to have antioxidant and anti-inflammatory effects [Tayebati et al., 2015; Martinelli et al., 2021].

The aim of this study, that represented the main field of my PhD activities, is to investigate the effects of GPC and (+) TIO on brain alterations in SHR, as animal model of cerebrovascular disease, to determine their efficacy in terms of neuroprotection and reduction of neuroinflammation. SHR, which is normotensive at birth and develops sustained hypertension

between three and six months of age, is the model most extensively investigated for evaluating hypertensive brain damage and its treatment. The time-dependent increase in arterial blood pressure, as well as the development of brain atrophy, nerve cell death, and glial response, are somewhat similar to what happens in the hypertensive human brain. For these reasons SHR may provide a plausible model of essential hypertension-related brain damage [Amenta et al., 2010].

24-weeks old SHR were treated for 4 weeks with GPC (150 mg/kg/day) and (+)TIO (125 µmol/kg/day) alone or in combination[Tomassoni et al ., 2012, Tomassoni et al., 2013]. We used age matched normotensive Wistar Kyoto (WKY) rats as control. The frontal cortex and the hippocampus, as areas involved in motor and cognitive functions, were collected for western blot and immunohistochemistry investigations of oxidative stress, neuronal, glial, BBB and neuroinflammatory markers. Moreover, to evaluate the possible anti-inflammatory effects of the two molecules, cells culture experiments were performed on lipopolysaccharide activated microglial BV-2 cells, treated at different concentrations with (+) TIO and GPC.

3. MATERIALS AND METHODS

3.1 Animal Handling and drugs treatment

Male SHR aged 24 weeks (n=32) and age-matched male normotensive Wistar-Kyoto (WKY) rats (n=8) were used. SHR were treated or not (controls) for 4 weeks with a dose (125 µmol/kg/day) of (+) TIO and 150 mg/kg/day of GPC dissolved in physiological solutions and administered intraperitoneally. Control SHR and WKY rats received the same amounts of vehicle. Rats were housed one per cage under constant temperature (22-24°C) with 12 h light/dark cycle (light on at 07:00), food and water available *at libitum* (Figure 23).

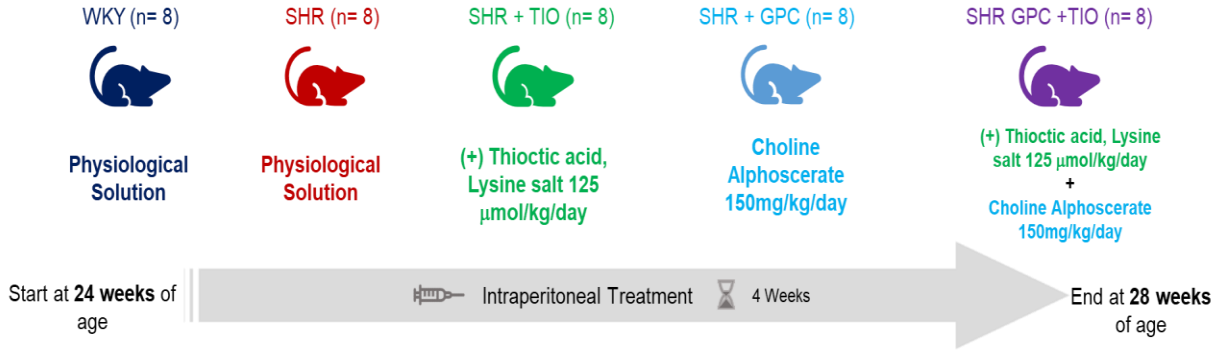

Figure 23. *Animal models with different treatment groups.*

Rats were handled according to internationally accepted principles for care of laboratory animals (European Community Council Directive 86/609, O.J. n° L358, Dec. 18, 1986) and approved by the Ministry of Health based on the D.lgs 26/2014 (Authorization n°163/2019-PR February 25, 2019) after the acceptance of the Committee "Organismo Preposto al Benessere degli Animali" of University of Camerino.

Body weight and food intake were controlled daily. Blood pressure values were measured once a week by an indirect tail-cuff method in conscious rats. Rats were gently handled to immobilize them, tail was warmed. The tail was inserted through a cuff equipped with a photoelectric pulse detector, which enabled recording of systolic and diastolic BP, by detection of the first oscillation following the gradual reduction of the cuff pressure. One week before sacrifice, different behavioral and memory tests were performed to detect potential behavioral abnormalities that might influence performance on rats: open field test as a means of assessing locomotor activity and as an index of anxiety. Memory deficits were measured using

Passive Avoidance task. Before the sacrifice, after BP measurement, blood withdrawals were performed from the tail vein. 800µl of blood was collected in tubes with L-heparin. The blood samples were then centrifuged for 10 min at 3000 RPM.

For paraffin embedding, right cerebral hemispheres were immediately immersed for 72 h in 4% paraformaldehyde in 0.1M phosphate buffer saline (PBS) solution pH 7.4. After fixation, the samples were gradually dehydrated in ethanol using solution of crescent concentration from 70% to 100%. Once successfully dehydrated, xylene was infiltrated through the tissues. Finally, the samples were embedded in paraffin through successive steps from 42 to 60°C (1 hour for each step). Once the paraffin samples are ready, we began to cut the tissues using the Microtome into sections of various thicknesses (10 and 6µm thick) that were placed on the slides and processed for morphological staining technique or for immunohistochemistry analysis.

The left hemisphere samples were dissected in the different brain areas (frontal cortex and hippocampus) and they were frozen at -80°C for western blot analysis and other biochemical analysis.

Based on previously obtained results in SHR, treated with racemic and eutomer form of TIO in heart and renal parenchima ***[Martinelli et al., 2021, Appendix 3***], and the basis of evidence of anti-inflammatory activity of natural antioxidant compound on heart of obese rats ***[Martinelli et al., 2022, Appendix 4]*** heart samples were dissected and processed for morphological, immunochemical and immunohistochemical techniques, to evaluate possible anti-inflammatory activities of (+) TIO. The results of these different experiments were presented at 74° Congress of Societa Italiana di Anatomia ed Istologia (SIAI) [***Appendix 5***] and submitted for publication on BMC Journal of Complementary Medicine and Therapies [***Roy et al., 2023, Appendix 6***].

3.2 Behavioral Tests

3.2.1 Open Field

The Open Field test assays activity, locomotors, and anxiety-related effects of neurobiological manipulations and to evaluate the exploration using photo beams connected to a computer to monitor the rat's activity and record its placement within the arena (Figure 24). The Open Field consists of exposing an animal to an open arena, a new environment without any clearly aversive or appetitive stimuli and let explore it freely for a fixed amount of time. In this sense, it is the classical non-aversive and non-associative task. Session duration may range from 5 to 10 min, especially during daytime experimentation; 2-3 min may be the minimum time to assure it habituate to the context, and more than 10 minutes seems to be useless, because the animal will start grooming and/or resting, even sleeping, since there seems to be no

novelties /risks around. The test was performed without having access to palatable food. Locomotion in the entire open field and in the central zone and entries into the central zone were computed based on interruptions of infrared light beams. Increased locomotors activity in the entire field is considered a sign of behavioral arousal, and reduced locomotors activity in the central zone and numbers of entries into the central zone are considered signs of increased emotionality, anxiety or fear in mice and rats.

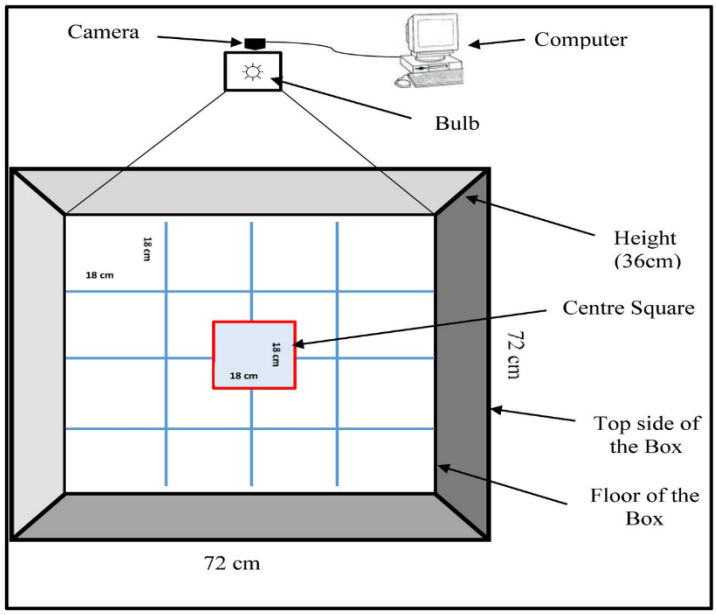

Figure 24. *The Open Field Arena (modified from – Arika et al., 2019)*

3.2.2 Passive Avoidance Task

Passive avoidance is fear-motivated tests classically used to assess short-term or long-term memory on small laboratory animals. Passive avoidance paradigm requires the subjects to behave contrary to their innate tendencies for preference of dark areas and avoidance of bright ones. The apparatus chamber used in this test is composed of a black poorly illuminated compartment and a white illuminated compartment. The chamber is divided into a lit compartment and a dark compartment, with a gate between the two (Figure. 25). Animals are allowed to explore both compartments on the first day. On the following day, they are given a mild foot shock in the dark compartment. Animals will learn to associate certain properties of the chamber with foot shock. To test their learning and memory, the rats are then placed back in the compartment where no shock was delivered. Rats with normal learning and memory will avoid entering the chamber where they had previously been exposed to the shock. This is measured by recording the latency to cross through the gate between the compartments (http://med.stanford.edu). In the passive avoidance protocols were identified three different steps: Habituation, Training, and testing.

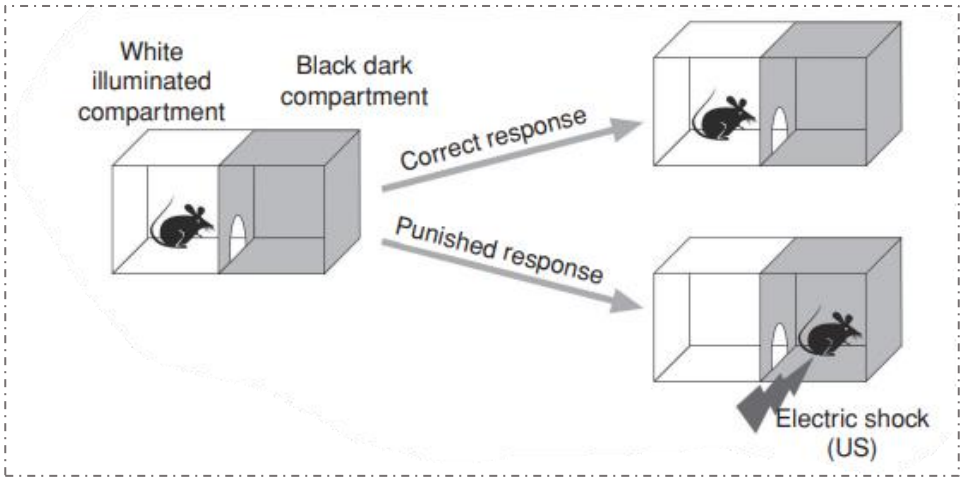

Figure 25. *Passive avoidance learning: step-through passive avoidance test. One compartment is white, brightly illuminated and the other is black and dark. The animal is placed in the white compartment and learns to withhold the spontaneous response of entering the dark compartment to avoid a mild electric foot-shock (modified from* Branchi and Ricceri, 2013*)*

Habituation: on habituation day, rats are placed in the lighted compartment, facing away from the dark compartment, and allowed to explore for 30 seconds. After 30 seconds the door is raised, and the rat is allowed to explore freely. When the rat enters the dark compartment with all four paws, the guillotine door is closed, and the latency to enter is recorded (from the time the door is lifted). Once the rat crosses the darkened chamber the door closes, and the rat is immediately removed and returned to the home cage.

Training: on training day, rat is placed in the lighted compartment, facing away from the dark compartment, and allowed to explore for 30 seconds. After 30 seconds the guillotine door is lifted. When the rat enters the dark compartment with all four paws, the guillotine door is closed, and the latency to enter is recorded (from the time the door is lifted). 3 seconds after the door is closed a foot shock (0.5 mA, 2 seconds duration) is delivered. 30 seconds after the foot shock the rat is removed to the home cage.

Testing: on test day (48 hours after training), the rat is returned to the lighted compartment, facing away from the dark compartment. After 10 seconds, the guillotine door is lifted. When the rat enters the dark compartment with all four paws, the guillotine door is closed, and the latency to enter the dark compartment is recorded (from the time the door is lifted).

3.3. Biochemical Analysis

3.3.1. OxyBlot

The OxyBlot methodology evaluates general oxidative stress. Carbonyl group formation on protein side chains is a common biochemical marker of oxidative stress that is seen in a wide range of acute and chronic neurological diseases, including stroke, AD, and PD. Given that proteins are frequently the immediate targets of cellular oxidative stress, it is critical to understand how reactive electrophile adductions and other oxidative reactions can irreversibly alter protein structure and function. Protein adduction was previously thought to be a random process, but it has recently become clear that these protein modifications are specific and selective [*Stankowski et al., 2011*].

Protein carbonyl groups (aldehydes and ketones) on protein side chains, particularly prolines, arginine, lysine, and threonine, are a common biochemical marker of oxidative stress. Carbonyl groups are formed primarily from lipid electrophiles generated under oxidative stress and are composed of a carbon atom double bonded to an oxygen atom. Protein structure and function can be irreversibly altered by electrophile adduction and other oxidative reactions. The accumulation of oxidatively modified and damaged proteins is a desirable way to assess cell stress, and novel techniques have been developed to help researchers determine the identity of adducted proteins [*Dalle-Donne et al., 2003*].

As carbonylated proteins are relatively stable, they can be chemically modified with 2,4-dinitrophenylhydrazine (DNPH), resulting in the formation of a stable dinitrophenyl (DNP) hydrazone product (Figure 26). Following the separation of oxidatively modified proteins by electrophoresis, Western

blot technology and anti-DNP antibodies enable the determination of total protein carbonyl formation through rapid and highly sensitive separation of the protein's DNP moiety.

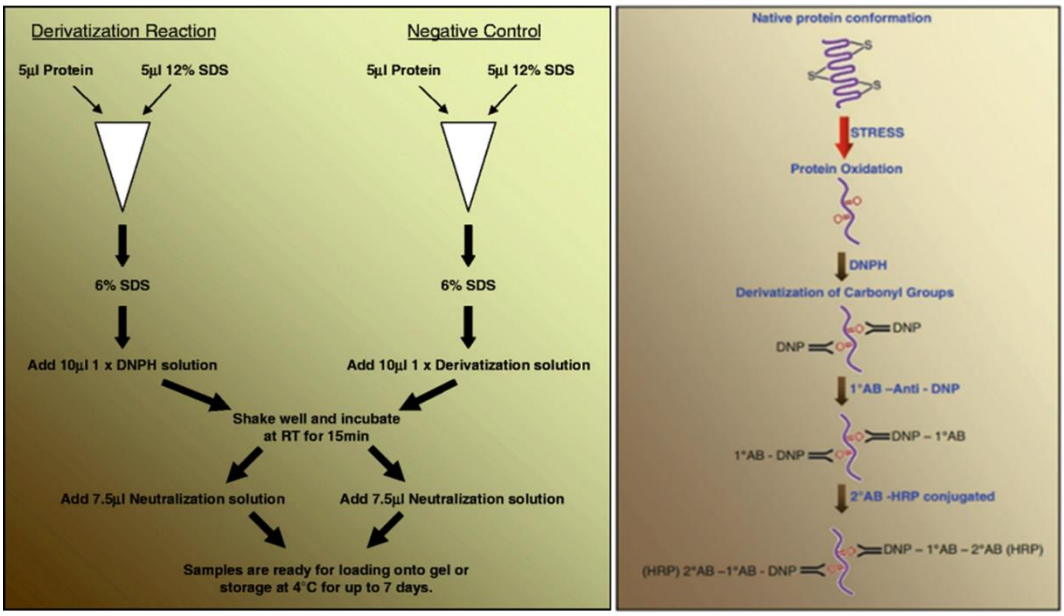

Figure 26. *Schematic of the derivatization of protein carbonyl groups. Cellular exposure to stress results in protein damage characterized by the formation of protein carbonyl groups (side chains). The OxyBlot methodology uses 2,4-dinitrophenylhydrazine (DNPH) to derivatize protein carbonyl groups leading to the formation of a stable dinitrophenyl (DNP) hydrazone product. The primary antibody is directed against the DNP moiety of the protein. The secondary, HRP-conjugated antibody allows for the use of chemiluminescent reagent to visualize bands of oxidized proteins upon exposure to film (modified from - Stankowski et al., 2011).*

OxyBlot Protein detection kit (Millipore, USA, Cat. No. S7150):

Protocol:

Two aliquots of each sample to be analyzed: one aliquot is subjected to the derivatization reaction; the other aliquot is used as a negative control, by substituting the 1X Derivatization-Control Solution instead of the 1X DNPH Solution.

1. Transfer 5µL of a protein sample, containing 20 micrograms of protein, into each of 2 Eppendorf tubes (0.5mL). The volume of protein samples can vary depending on the well size of the gel and the concentration of the sample. When a sample volume other than 5µL is used, adjust the volume of other reagents accordingly.

2. Denature each 5µL aliquot of protein by adding 5µL of 12% SDS for a final concentration of 6% SDS.

3. Derivatize the sample by adding 10µL of 1X DNPH solution to one of the tubes. To the aliquot designated as the negative control, add 10µL of 1X Derivatization-Control Solution instead of the DNPH solution.

4. Incubate both tubes at room temperature for 15 minutes. Do not allow the reaction to proceed more than 30 minutes, as side reactions other than hydrazone linkage may occur.

5. Add 7.5µL of Neutralization Solution to both tubes. If reduction of the protein sample is desired and a reducing reagent was not present during lysis, add 2-mercaptoethanol to the sample mixture to achieve a final concentration of 0.74 M solution (1-1.5µL; 5%v/v).

3.3.2 Western Blot

Blotting is the transfer of proteins to microporous membranes, and it includes both "spotting" (manual sample deposition) and transfer from planar gels. Proteins resolved on Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE) are typically transferred to adsorbent membrane supports under the influence of an electric current in a procedure known as western blotting (WB) or protein blotting. Through gel electrophoresis, a mixture of proteins is separated by molecular weight, which determines their type. In the next step, the results are transferred to a membrane where a band is produced for each protein. Following this, the membrane is incubated with antibodies specifically directed towards the protein of interest (Figure 27). The unbound antibody is washed away, leaving only the antibody that is bound to the protein of interest. The developed film then detects the bound antibodies. Only one band should be visible because the antibodies only bind to the protein of interest. The thickness of the band corresponds to the amount of protein present; thus, performing a standard can indicate the amount of protein present [*Mahmood and Yang., 2012*; *Kurien and Scofield., 2015*].

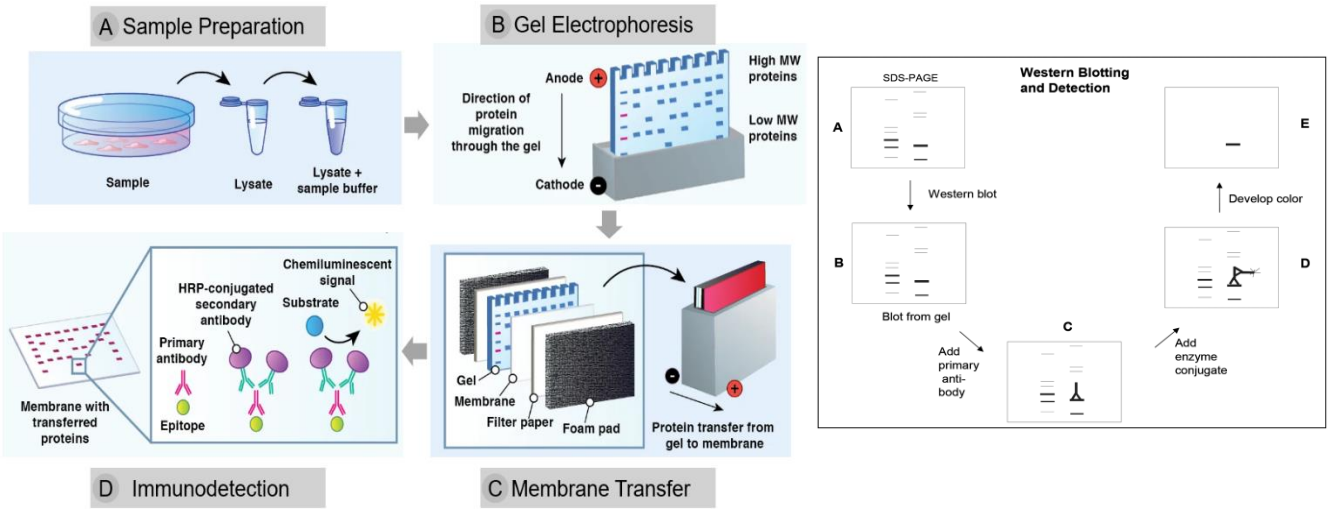

Figure 27: *Schematic representation of Western blotting and detection procedure. (A) Sample preparation (B) Sample loading and separation according to molecular weight (C) Sample transfer from gel to nitrocellulose membrane (D) Immunodetection by using primary antibody and subsequent horseradish (HRP) peroxidase conjugated secondary antibody (modified from – antibodies.com, Kurien and Scofield., 2015).*

Preparation of Protein Lysate

Protein lysate was obtained mixing the tissue in a Mixer Mill for 10 minutes, using a specific lysis buffer containing: Tris-buffer 1M pH 7.4, NaCl 1M, EGTA 10mM, NaF 100mM, Na_3VO_4 100 mM, PMSF 100mM, Deoxycholate 2%, EDTA 100mM, Triton X100 10%, Glycerol, SDS 10%, $Na_4P_2O_7$ 0.1M, Inhibitor Cocktail, Distilled H_2O. Protein concentration was calculated spectrophotometrically by Bio-Rad protein assay, reading the absorbance a 595 nm.

BIO-RAD protein assay

BIO-RAD protein assay (Cat.No.500-0001, BIO-RAD, Munich, Germany) is a colorimetric assay, in which the colour change occurs in response to the concentration of proteins. This method is based on the Bradford method using the Coomassie Brilliant Blue dye. The resulting values are plotted on a standard curve to measure the protein concentration. Equal amount of protein (40μg) were obtained and mix with an appropriate volume of Laemmli sample buffer (SB).

Western Blot protocol

Electrophoresis:

1. Preparation of 8%, 10%, 14% and SDS-PAGE gels.

2. Treatment of samples: add equal volumes (40 μg of proteins) of 2X reducing laemmli buffer to protein sample solutions, vortex gently to mix, and heat in boiling water bath for 5 minutes.

3. Prepare the running and the stacking gel for loading the samples.

4. Start the electrophoresis process at 100V and then, when the proteins have completely migrated into the stacking gel, increase at 200V. Run gel at constant current.

5. Electrophoresis is complete then transfer the gel into the transblot turbo apparatus (Bio-Rad Laboratories, Inc. Germany) at 1.3 A and 25 V for 7 minutes for the transfer of the protein in the nitrocellulose membrane.

Antibodies incubation:

1. Rinse the blot in water and stain in Ponceau-red solution to check the transfer quality.

2. Incubation in BSA 5% in PBS-T, for 1 hours

3. Incubation with primary antibody, for Overnight at 4 °C, as detailed in Table 4.

4. Wash in 0.05% PBS-T, 3 times 5 minutes each.

5. Incubation with secondary antibody for 1.30 hours at room temperature, as detailed in Table 4.

6. Wash in 0,05% PBS-T, 3 times 5 minutes each

7. Incubation with chemiluminescent substrate and acquisition by image Lab software (ChemiDoc XRS+ Bio Rad, Bio-Rad Laboratories, Inc. Germany).

Table 4: Primary and Secondary Antibodies used for Western Blotting Analysis

Primary Antibody	Company	Dilution	Secondary Antibody	Dilution	Company
4 Hydroxynonenal (4-HNE)	Merck Millipore Cat. MAB393207	1:2000	Anti-Rabbit HRP conjugate	1:5000	Jackson Immuno Research C.N:711-035-152
Neuronal Nuclear Antigen (NeuN)	Merck Millipore Cat. MAB377	1:1000	Anti-mouse HRP conjugate	1:5000	Jackson Immuno Research C.N:711-035-151
Microtubule associated protein (MAP 2)	Gene Tex Cat. GTX133109	1:1000	Anti-mouse HRP conjugate	1:5000	Jackson Immuno Research C.N:711-035-151
Neurofilament (NF)	Merck Millipore Cat. MAB5262	1:1000	Anti-mouse HRP conjugate	1:5000	Jackson Immuno Research C.N:711-035-151
Myelin basic protein (MBP)	Merck Millipore (SMI-99) NE1019	1:1000	Anti-mouse HRP conjugate	1:5000	Jackson Immuno Research C.N:711-035-151
Postsynaptic density protein 95 (PSD-95)	Abcam Cat.ab2723	1:500	Anti-mouse HRP conjugate	1:5000	Jackson Immuno Research C.N:711-035-151
Synaptophysin (SYN)	Abcam Cat.ab8049	1:1000	Anti-mouse HRP conjugate	1:5000	Jackson Immuno Research C.N:711-035-151

Glial Fibrillary acid protein (GFAP)	Merck Millipore Cat. MAB3402	1:1000	Anti-mouse HRP conjugate	1:5000	Jackson Immuno Research C.N:711-035-151
Ionized calcium-binding adaptor molecule 1 (IBA -1)	Thermo Fisher Scientific Cat. PA5-27436	1:5000	Anti-Rabbit HRP conjugate	1:5000	Jackson Immuno Research C.N:711-035-152
Interleukin 1 beta (IL-1 β)	BIO-RAD Datasheet: AAR15G	1:500	Anti-Rabbit HRP conjugate	1:5000	Jackson Immuno Research C.N:711-035-152
Interleukin 6 (IL-6)	Gene Tex Cat. GTX110527	1:200	Anti-Rabbit HRP conjugate	1:5000	Jackson Immuno Research C.N:711-035-152
Tumor necrosis factor alpha (TNF-α).	BIO-RAD Datasheet: AAR33B	1:500	Anti-Rabbit HRP conjugate	1:5000	Jackson Immuno Research C.N:711-035-152
Vascular cell adhesion molecule-1 (VCAM-1)	Abcam Cat.ab134047	1:2000	Anti-Rabbit HRP conjugate	1:5000	Jackson Immuno Research C.N:711-035-152
Aquaporin-4 (AQP4)	Abcam Cat.ab259318	1:1000	Anti-Rabbit HRP conjugate	1:5000	Jackson Immuno Research C.N:711-035-152
Glucose Transporter 1 (GLUT 1)	Merck Millipore Cat. MABS132	1:1000	Anti-mouse HRP conjugate	1:5000	Jackson Immuno Research C.N:711-035-151
Zonula occludens-1 (ZO-1)	Gene Tex Cat. GTX108592	1:1000	Anti-Rabbit HRP conjugate	1:5000	Jackson Immuno Research C.N:711-035-152
Vesicular Acetylcholine transporter (VAChT)	Santa Cruz Biotechnology Cat. sc7717	1: 250	Anti-goat HRP conjugate	1:5000	Jackson Immuno Research C.N:711-035-147
Alpha-7 nicotinic Acetylcholine receptor (nAChR α7)	Proteintech Cat. 21379-1-AP	1:500	Anti-Rabbit HRP conjugate	1:5000	Jackson Immuno Research C.N:711-035-152
Anti-Choline Acetyltransferase (ChAT)	Chemicon/ Millipore Cat. AB144P	1: 150	Anti-goat HRP conjugate	1:5000	Jackson Immuno Research C.N:711-035-147
Acetylcholinesterase (AChE)	Santa Cruz Biotechnology Cat. sc6430	1:500	Anti-goat HRP conjugate	1:5000	Jackson Immuno Research C.N:711-035-147
Myelin basic protein (MBP)	Merck Millipore Cat. mAB(SMI-99)	1:1000	Anti-mouse HRP conjugate	1:5000	Jackson Immuno Research C.N:711-035-151

β – Actin	Merck-Millipore A2228	1:3000	Anti-mouse HRP conjugate	1:5000	Jackson Immuno Research C.N:711-035-151

3.4 Morphological and Immunohistochemical Analysis

3.4.1 Nissl's Staining

Cresyl violet acetate is used for staining tissue and nerve cells and fresh tumor tissue for biopsy. It's a high purity biological stain to use for Nissl staining of spinal cord and brain paraffin sections and to stain mast cell and cartilage granules. Nissl's staining solution is composed of: Cresyl Violet 1,5g, distilled water 98ml, acetic acid 1M 1ml.

Nissl's Staining protocol:

1. Bring the sections to water by xylene and ethanol
2. Wash in tap water 5 minutes and distilled Water 1 Minute
3. Incubate the sections with the staining solutions for 8 minutes
4. Countered in 95% ethanol for different minutes
5. Ethanol 100 I 30 seconds
6. Ethanol 100 II 30 seconds
7. Ethanol-Xylene 5 Minutes
8. Xylene I 5 Minutes
9. Xylene II 5 Minutes
10. Coverslip with Mounting Medium

3.4.2. Immunohistochemistry

Immunohistochemistry is a procedure that employs the use of antibodies (immuno-) in tissues (histo-) to highlight a protein of interest. Immunohistochemistry consists of two major steps:

I) Sample preparation: fixing the sample to preserve the architecture of the collected tissue and prepare it for antibody labelling.

II) Sample labeling: using antibodies to label the protein(s) of interest.

Before labeling the protein of interest with an antibody, a blocking step is required to minimize non-specific antibody binding (false positives). This is accomplished by incubating the tissue in solutions containing proteins or other molecules that will bind to readily reactive nonspecific sites, preventing the antibody from binding to these nonspecific sites. This is known as blocking, and it is done prior to antibody treatment. Normal serum and protein solutions, such as bovine serum albumin (BSA), are common blocking solutions.

In addition to blocking non-specific antibody binding, it may be necessary to inhibit endogenous expression of peroxidase and alkaline phosphatase depending on the detecting system. For example, if a secondary antibody is conjugated to horseradish peroxidase, we want this antibody to detect peroxidase specific to the primary antibody, so endogenous peroxidase could be blocked by (3-10%) hydrogen peroxidase (H_2O_2) before using the antibody. Moreover, the need for this blocking step is determined by tissue type because of endogenous expression of these enzymes and molecules varies between tissue types. Blocking endogenous peroxidase activity, for example, is important in kidney tissue, which has high peroxidase activity, but not in adipose tissue [Ramos-Vara JA., 2005].

After blocking the non-specific binding, primary antibodies are applied to spot the antigen of interest. They can be conjugated to a fluorophore or a chromogenic enzyme, allowing the antibody-antigen interaction to be visualized. Conjugated secondary antibodies can also be used to bind to unconjugated primary antibodies (that detect the antigen) to visualize or amplify the signal (Figure 28).

The type of conjugate bound to an antibody determines how the signal is processed. Antibodies can be conjugated to fluorophores or enzymes that process chromogenic substrates to generate signal (for example, horseradish peroxidase degrades 3,3'-diaminobenzidine (DAB) and alkaline phosphatase degrades 3-amino-9-ethylcarbazole (ABC).

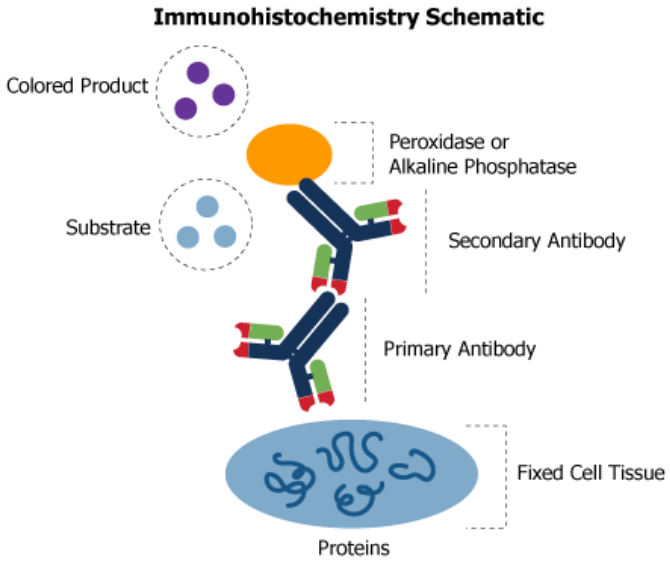

Figure 28. *Schematic presentation of immunohistochemistry techniques (Modified from - Rockland Immunochemical)*

Immunohistochemistry Protocol

Paraffin-embedded brains were cut into sagittal sections of 10 µm and mounted on microscope slides to perform the immunohistochemical (IHC) analysis, as previously described. After deparaffinization with xylene and ethanol, two cycles in microwave, 5 min each, with Tris-HCl, EDTA, pH 9/ Citrate buffer, pH 6 antigen retrieval were used to unmasking binding site of antibodies.

Sections were incubated with peroxidase blocking (3% H_2O_2) and after incubation with BSA 3% for 60 minutes and sections were incubated overnight at 4 °C solution of primary antibodies (Table 5). Through preliminary experiments, the specificity and the concentration of the antibodies were established. The sections were incubated with the specific biotinylated secondary antibodies (Table 5) and after washing in PBS were incubated with avidin-biotin-peroxidase complex (Vectastain ABC Elite kit; Vector Laboratories, Inc., Burlingame, CA, USA). Finally, the immunoreaction was revealed using a 3,3′-diaminobenzidine tetrahydrochloride (DAB) substrate kit (Vector Laboratories, Inc., Burlingame, CA, USA) according to the manufacturer's protocol. Sections were counterstained with hematoxylin solution and coverslip with Mounting Medium.

Table 5: Primary and Secondary Antibodies used for Immunohistochemical analysis.

Primary Antibody	Company	Dilution	Secondary Antibody	Dilution	Company
Neuronal Nuclear Antigen (NeuN)	Merck Millipore Cat. MAB377	1:500	Anti- mouse biotinylated	1:200	Vector REF:BA-2000
Microtubule associated protein (MAP 2)	Gene Tex Cat. GTX133109	1:200	Anti- mouse biotinylated	1:200	Vector REF:BA-2000
Neurofilament (NF)	Merck Millipore Cat. MAB5262	1:200	Anti- mouse biotinylated	1:200	Vector REF:BA-2000
Myelin basic protein (MBP)	Merck Millipore (SMI-99) NE1019	1:1000	Anti- mouse biotinylated	1:200	Vector REF:BA-2000
Synaptophysin (SYN)	Abcam Cat.ab8049	1:50	Alexa fluor 594 dye (Red) Anti-rabbit	1:100	Invitrogen REF: A11032
Glial Fibrillary acid protein (GFAP)	Merck Millipore Cat. MAB3402	1:200	Alexa fluor 488 dye (Green) Anti-mouse	1:100	Invitrogen REF: A11001
Ionized calcium-binding adaptor molecule 1 (IBA -1)	Thermo Fisher Scientific Cat. PA5-27436	1:200	Alexa fluor 594 dye (Red) Anti-rabbit	1:100	Invitrogen REF: A11032
Interleukin 1 beta (IL-1 β)	BIO-RAD Datasheet: AAR15G	1:750	Alexa fluor 594 dye (Red) Anti-rabbit	1:100	Invitrogen REF: A11032
Interleukin 6 (IL-6)	Gene Tex Cat. GTX110527	1:200	Anti- rabbit biotinylated	1:200	Vector REF:BA-1000
Tumor necrosis factor alpha (TNF-α).	BIO-RAD Datasheet: AAR33B	1:750	Anti- rabbit biotinylated	1:200	Vector REF:BA-1000
Aquaporin-4 (AQP4)	Abcam Cat.ab259318	1:1000	Anti- rabbit biotinylated	1:200	Vector REF:BA-1000
Glucose Transporter 1 (GLUT 1)	Merck Millipore Cat. MABS132	1:500	Anti- mouse biotinylated	1:200	Vector REF:BA-2000

Vesicular Acetylcholine transporter (VAChT)	Santa Cruz Biotechnology Cat. sc7717	1: 50	Anti- Goat biotinylated	1:200	Jackson Immuno Research C.N:711-035-003
Anti-Choline Acetyltransferase (ChAT)	Chemicon/ Millipore Cat. AB144P	1: 150	Anti- Goat biotinylated	1:200	Jackson Immuno Research C.N:711-035-003
Acetylcholinesterase (AChE)	Santa Cruz Biotechnology Cat. sc6430	1:100	Anti- Goat biotinylated	1:200	Jackson Immuno Research C.N:711-035-003
Alpha-7 nicotinic Acetylcholine receptor (nAChR α7)	Proteintech Cat. 21379-1-AP	1:200	Anti-Rabbit biotinylated	1:200	Vector REF:BA-1000
Myelin basic protein (MBP)	Merck Millipore Cat. mAB(SMI-99)	1:1000	Anti- mouse biotinylated	1:200	Vector REF:BA-2000

3.5 Morphological Analysis

After mounting, sections were analyzed with the computer-assisted semi-quantitative method: images were reported from a light microscope (Leica DMR, Germany) by using a camera (Nikon DS-Ri2, Japan) to an image analysis software (Nikon Nis Element, Japan).

The area of frontal cortex and hippocampus (Plate 82 lateral 1.40 mm) were analyzed (Figure 29). Particularly for the frontal cortex the morphological analysis was performed at the levels of fifth (V) and sixth (VI) layers. In hippocampus campus the morphological analysis was performed at the levels of the CA1 and CA3 subfields and at the level of dentate gyrus. Densitometric analysis of the area of immunoreaction was performed for AQP4 and GLUT-1 and the values of mean intensity of immunoreaction was express as arbitrary optical density unit (ODU) ranged between 0 (no immunoreaction) and 256 the maximum intensity.

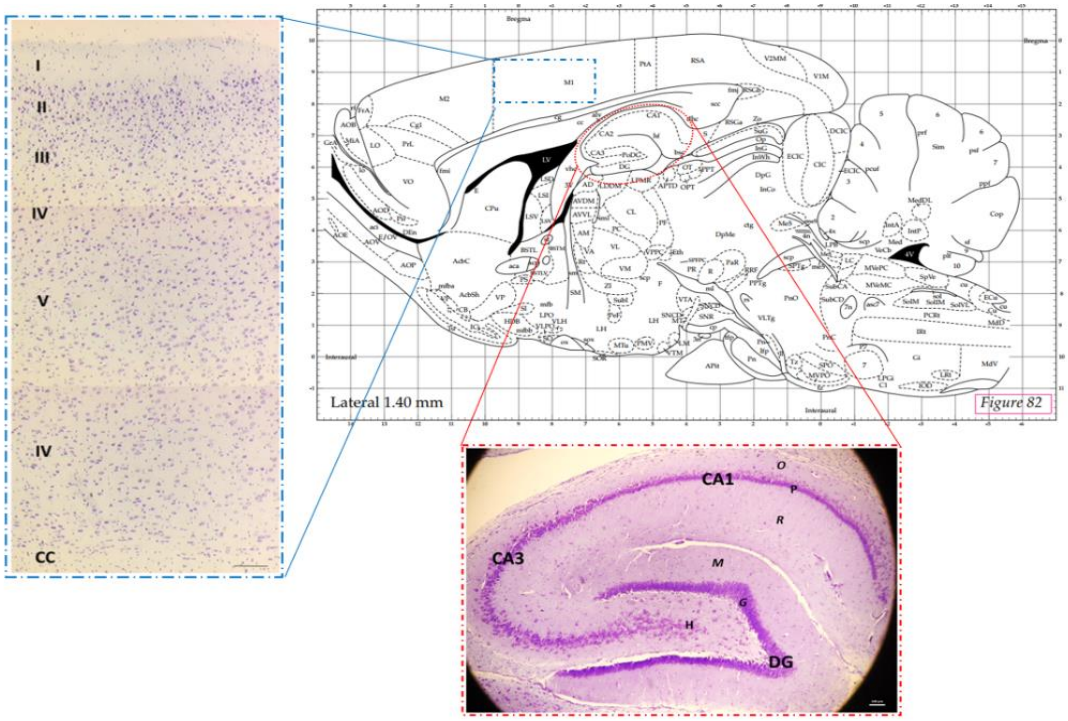

Figure 29: *Sagittal diagram of brain rat (Plate 82). The rat brain in stereotaxic coordinates. Paxinos& Watson Fourth edition. 1998. Section of hippocampus (lower panel in red box) and frontal cortex stained with Cresyl Violet. CA1: "Corno di Ammone" subfield 1; CA3: "Corno di Ammone" subfield 3; DG: dentate Gyrus; H: Hilum of dentate Gyrus; P: Pyramidal Neurons; O: stratum oriens; r: stratum radiatum: m: molecular layer; G: granule neurons. Calibration bar: 100 µm. Section of frontal cortex of WKY rats staining with the Nissl's procedure. The Romanic number indicates the different sixth layers of cortex (left panel in blue box). Calibration bar: 50 µm*

The area of GFAP immunoreactive astrocytes (Figure 30) and the area of IBA1 immunoreactive microglial cells was defined by a specific option of the program, and the values expressed as μm^2.

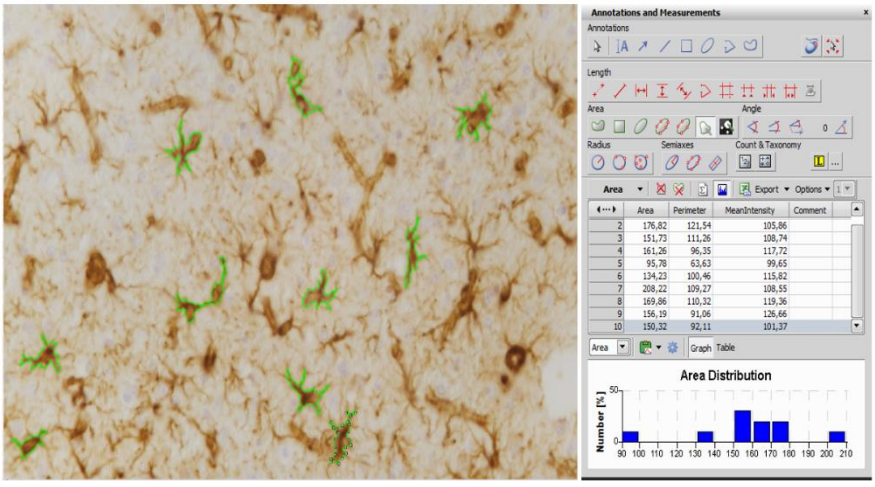

Figure 30. *Image acquisition and astrocyte area selection; Right: astrocyte measurement (Magnification: 40x)*

3.6 BV-2 Cell Culture

BV-2 cells, a type of microglial cell derived from C57/BL6 murine microglia were cultured in Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS) 10% and penicillin/streptomycin 1%. (5% CO2 incubator at 37°C). Cells ($5x10^5$/well) were pretreated or not with lipopolysaccharide (LPS, Cell Signaling code 14011S).

BV-2 microglial cells were treated with LPS (10 µg/ml) for 30 min and then, for other 24 hours, with different concentrations of GPC and (+) TIO (Figure 30)

Optimal concentrations of LPS (10, 20 or 30 or 60 µg/ml), α-GPC (10, 20, 40 mM) and (+) TIO (50,100,200 µm) were assessed at first based on MTT assay and IL-β expression (Figure.31).

MTT assay, protein extraction, western blot ad immunocytochemistry for IL-1β, NF-κb and IBA-1 were performed applied the same protocols for brain, using specific antibodies (Tables 6 and 7). All data were expressed as mean ± SD.

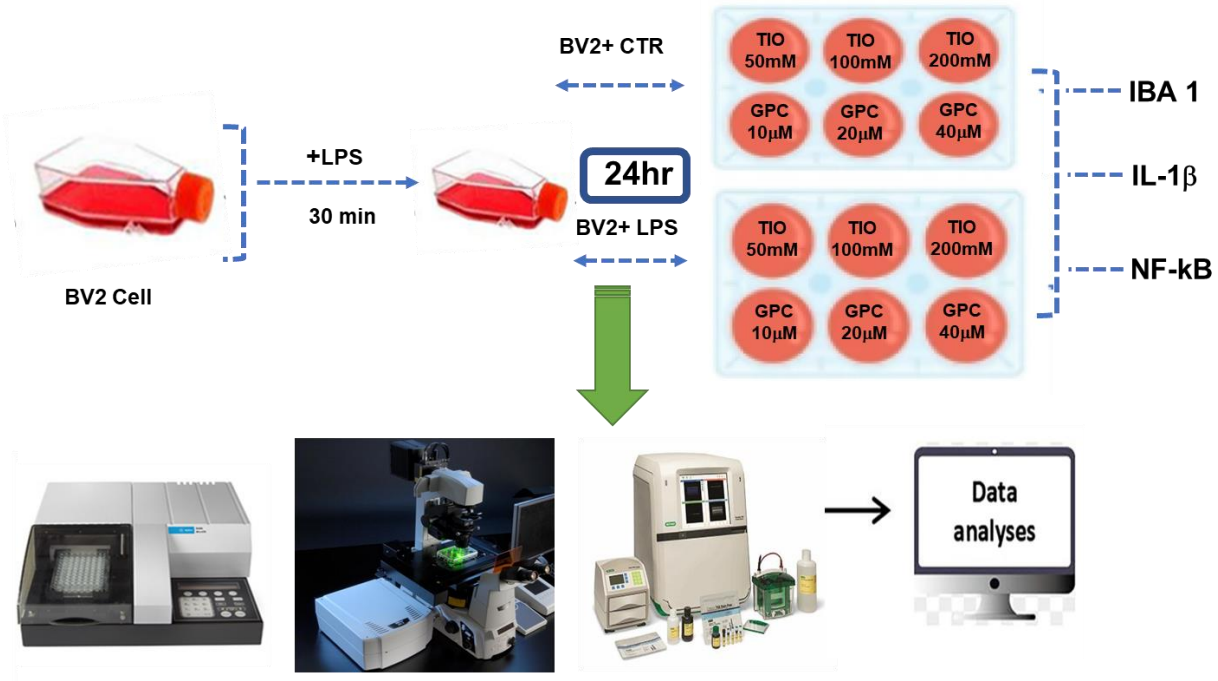

Figure 31. *Experimental approach for cell-culture analysis.*

***Table 6**: Primary and Secondary Antibodies used for Western blot analysis on BV2 Microglial cell.*

Primary Antibody	Company	Dilution	Secondary Antibody	Dilution	Company
Interleukin 1 beta (IL-1 β)	Novus Datasheet: NB600-633	1:1000	Anti rabbit HRP conjugated	1:5000	Jackson Immuno Research C.N:711-035-152
Ionized calcium-binding adaptor molecule 1 (IBA -1)	Thermo Fisher Scientific Cat. PA5-27436	1:5000	Anti rabbit HRP conjugated	1:5000	Jackson Immuno Research C.N:711-035-152
Nuclear factor kappa B (NF-κB)	Santa Cruz Biotechnology Cat. Sc114	1:100	Anti rabbit HRP conjugated	1:5000	Jackson Immuno Research C.N:711-035-152
GAPDH	Cell signaling: 2118	1:5000	Anti rabbit HRP conjugated	1:5000	Jackson Immuno Research C.N:711-035-152

***Table 7**: Primary and Secondary Antibodies used for Immunohistochemical analysis on BV2 Microglial cell.*

Primary Antibody	Company	Dilution	Secondary Antibody	Dilution	Company
Interleukin 1 beta (IL-1 β)	Novus Datasheet: NB600-633	1:300	Alexa fluor 594 dye (Red) Anti-rabbit	1:100	Invitrogen REF: A11032
Ionized calcium-binding adaptor molecule 1 (IBA -1)	Thermo Fisher Scientific Cat. PA5-27436	1:200	Alexa fluor 594 dye (Red) Anti-rabbit	1:100	Invitrogen REF: A11032
Nuclear factor kappa B (NF-κB)	Santa Cruz Biotechnology Cat. Sc114	1:100	Alexa fluor 488 dye (Green) Anti-rabbit	1:100	Invitrogen REF: A11001

3.7 Statistical Analysis

Means of different parameters investigated were calculated from single animal data, and values of standard error of mean (S.D.) were then obtained from single animal values. The data are expressed as mean ± S.D. The significance of differences between means was analysed by analysis of variance (ANOVA) followed by the Tukey's multiple comparisons test, considering as level of significance a p value < 0.05.

4. RESULTS

4.1 General and blood parameters

Data of body weight (Figure 32A) were similar among the different experimental groups without significant difference. The brain weight was significantly lesser in all SHR groups, compared to the WKY control (Figure 32 B). The two drugs treatment do not show any effects on brain weight (Figure 32 B).

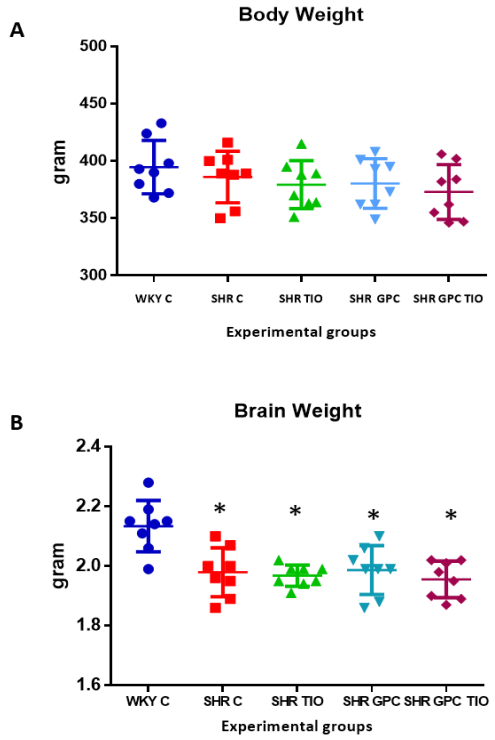

Figure 32. *Value of body weight (A) and brain weight (B) in the different experimental groups at the end of treatment. WKY C: Wistar Kyoto rats; SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Data are the mean ± S.D.* *******= p<0.05 vs WKY; #=p<0.05 vs SHR*

As shown in Figure 33 (A) and (B) both systolic and diastolic blood pressure were significantly increased in the SHR respect to normotensive WKY rats. The treatments with (+) TIO, GPC and the combination decrease the systolic blood pressure compared to the SHR control rats (Figure 33

A). While only the treatment with (+) TIO plus GPC reduced remarkably the diastolic blood pressure (Figure 33 B).

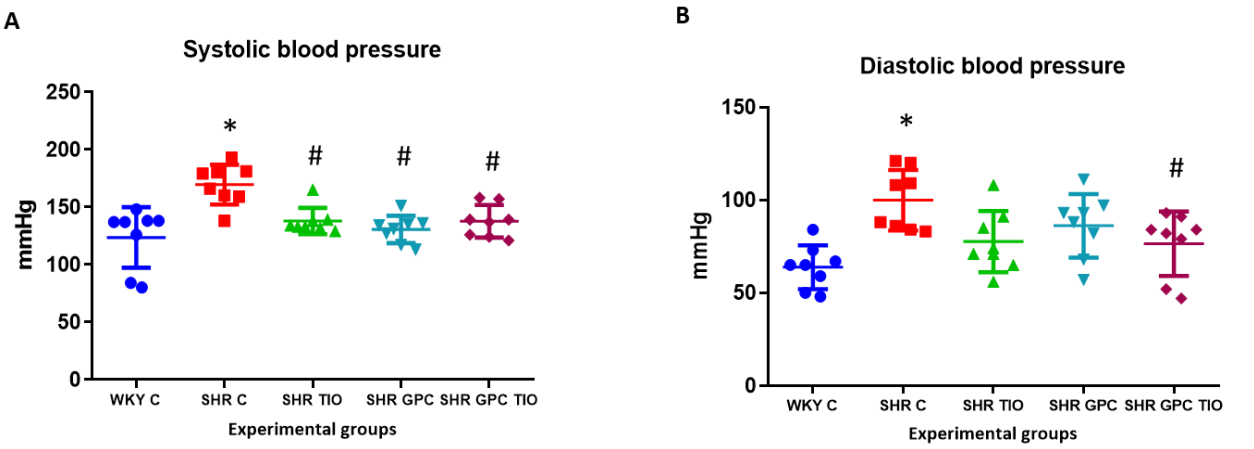

Figure 33. *Value of systolic (A) and diastolic (B) blood pressure in the different experimental groups at the end of treatment. WKY C: Wistar Kyoto rats; SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate.; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Data are the mean ± S.D.* *******= p<0.05 vs WKY; #=p<0.05 vs SHR*

4.2 Behavioral Tests

4.2.1 Open Field

The behavioral test reported a significant increase of distance travelled in SHR rats compared to WKY, demonstrating an anxiety status. However, no changes are reported after the injections of the different compounds compared to SHR control (Figure 34 A). In addition, the events of zone entries were similar in all the groups with a great variability between animals (Figure 34 B).

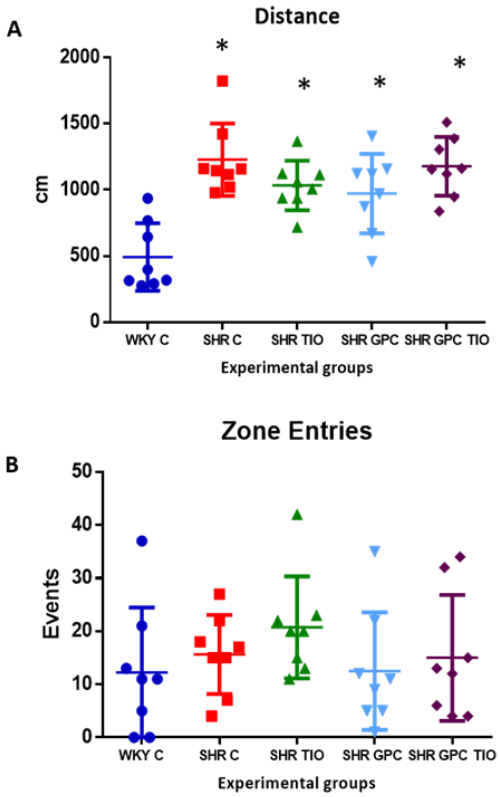

Figure 34. *Open field test. Values of total distance run from animals after 5 minutes in the box (A) and number of entries in central zone (B) after 5 minutes in the box. WKY C: Wistar Kyoto rats; SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Data are the mean ± S.D.* *******= $p<0.05$ vs WKY; #=$p<0.05$ vs SHR*

In the open-field test, the SHR rats increased their cumulative distance travelled, their number of ambulatory counts, jumps, vertical counts, and total entries in the central zone (Figures 35 A and B). Possibly, these alterations are related to the hyper-reactivity of the rats, as evidenced by the increase in systolic blood pressure, which is not mediated by anxiety. It is also found that, both (+) TIO and GPC treatment, alone or in combination, did not modify spontaneous hypertension-induced behavioral alterations (Figures 35 A and B)

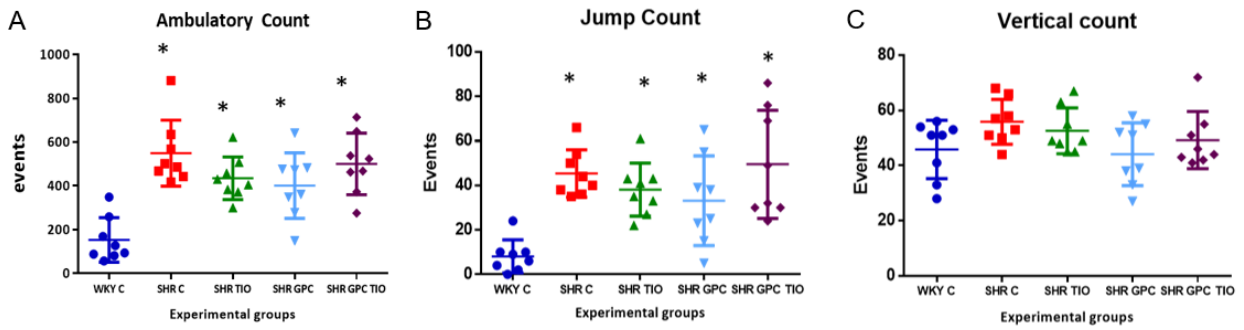

Figure 35. *Open field test. Values of ambulatory count (A), jump count (B), and vertical count (C) after 5 minutes in the box. WKY C: Wistar Kyoto rats; SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate.; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Data are the mean ± S.D.* *******= p<0.05 vs WKY; #=p<0.05 vs SHR*

4.2.2 Passive Avoidance Task

In the passive avoidance test, SHR rats showed no difference in retention latency time compared to WKY rats (Figure 36). Additionally, treatment with GPC and (+) TIO alone or in association did not affect latency time (Figure 36).

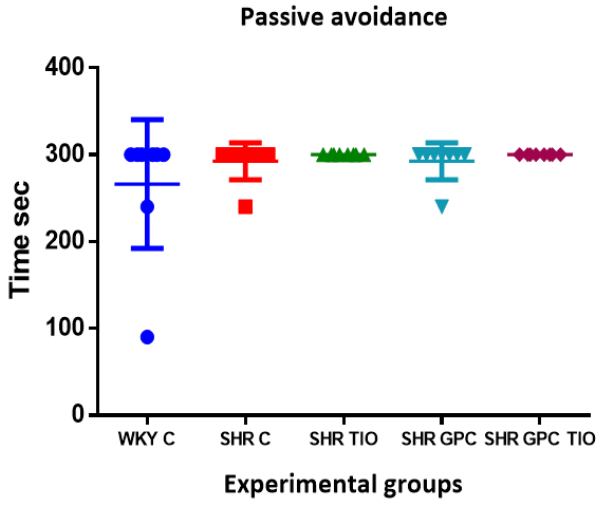

Figure 36. *Passive avoidance test. Values of latency time. WKY C: Wistar Kyoto rats; SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate.; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Data are the mean ± S.D.* *******= p<0.05 vs WKY; #=p<0.05 vs SHR*

4.3 Western blot and Immunohistochemistry results

4.3.1 Oxidative Stress and Lipid peroxidation

OxyBlot Kit provides reagents for the detection of carbonyl groups, which indicate the oxidation status of proteins, in a simple and sensitive manner. 4-Hydroxynonenal (HNE) is a quantitatively significant product of lipid peroxidation. When oxidative stress or redox disbalance occurs, the formation of HNE-protein adducts is one of the accompanying processes. The 4-HNE and the oxyblot analysis showed an evident increase of the oxidative status of the proteins in the hippocampus of SHR compared WKY animals (Figure 37A and B). All the treatments with (+) TIO and GPC slightly decreased the levels of oxidized proteins compared to SHR in hippocampus. On the other hand, all the treatments are showing some tendency to recover the protein oxidation status.

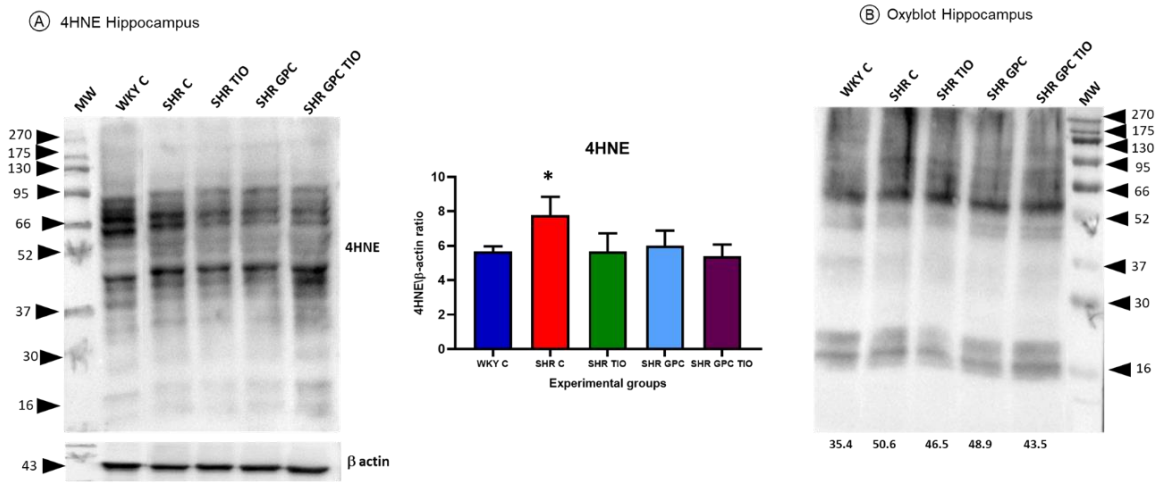

Figure 37. *4HNE (A) and Oxyblot(B)western blot analysis in the lysate of hippocampus of different experimental groups. WKY C: Wistar Kyoto rats: SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate.; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Data are the mean ± S.D.* *******= p<0.05 vs WKY; #=p<0.05 vs SHR*

In the frontal cortex this analysis showed the same increasing event were like the hippocampus. In this case GPC significantly decreased the levels of oxidized proteins compared to SHR in the frontal cortex area (Figure 38A). Moreover (+) TIO and the two compounds in association were

able to decrease the oxidized protein in the frontal cortex of SHR rats, as showed by the densitometric analysis of all line of the western blot (Figure 38B).

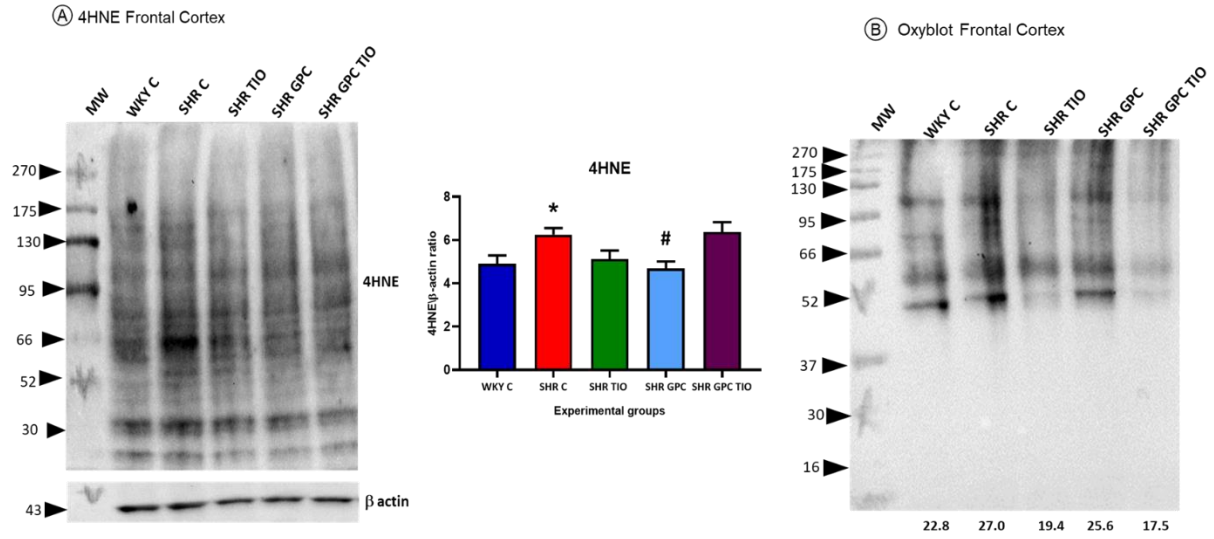

Figure 38. *4HNE (A) and Oxyblot (B) analysis in the lysate of frontal cortex of different experimental groups. WKY C: Wistar Kyoto rats; SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate.; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Data are the mean ± S.D.* *******= p<0.05 vs WKY; #=p<0.05 vs SHR*

4.3.2 Neuronal Markers

Neuronal Nuclei (NeuN) protein.

In 1992, Mullen and colleagues developed monoclonal antibodies by immunizing mice with brain cell nuclei. One of these antibodies detected a nuclear protein called NeuN (Neuronal Nuclei Protein) that is expressed by most of the neurons in the central and peripheral nervous systems. NeuN antibodies are still used by neuroscientists today as a reliable marker for identifying mature neurons [Mullen et al., 1992]. Additionally, NeuN is highly specific to nuclear proteins expressed by nearly all neurons but not the glial cells. Thus, unlike Nissl staining, this allows neurons to be visualized separately from glia.

In WB techniques, the hippocampus and the frontal cortex membrane were exposed to NeuN antibody, and they developed a band at approximately 48 KDa. Densitometric analysis of bands of different experimental groups was normalized by the intensity of reference protein band (β-

actin) (Figure 39 A and 40A). The result indicates that at the level of the hippocampus NeuN expression was significantly lower in the control hypertensive animals as compared to the normotensive control. The treatment with GPC alone and combination with (+) TIO was efficient enough to restore it to its normal level (Figure 39A). The immunohistochemistry (IHC) result for NeuN indicates that, in the hippocampal region, the protein was expressed at the level of nuclei of pyramidal neurons in the different Ammon Horn subfields (CA1 and CA3) and in the molecular layer of the dentate gyrus (Figure 39B). In the SHRs, the appearance of NeuN protein was evidently decreased compared to the age matched WKY. Only sections of SHR treated with GPC showed slightly increase in the NeuN expression compared to the control (Figure 39B).

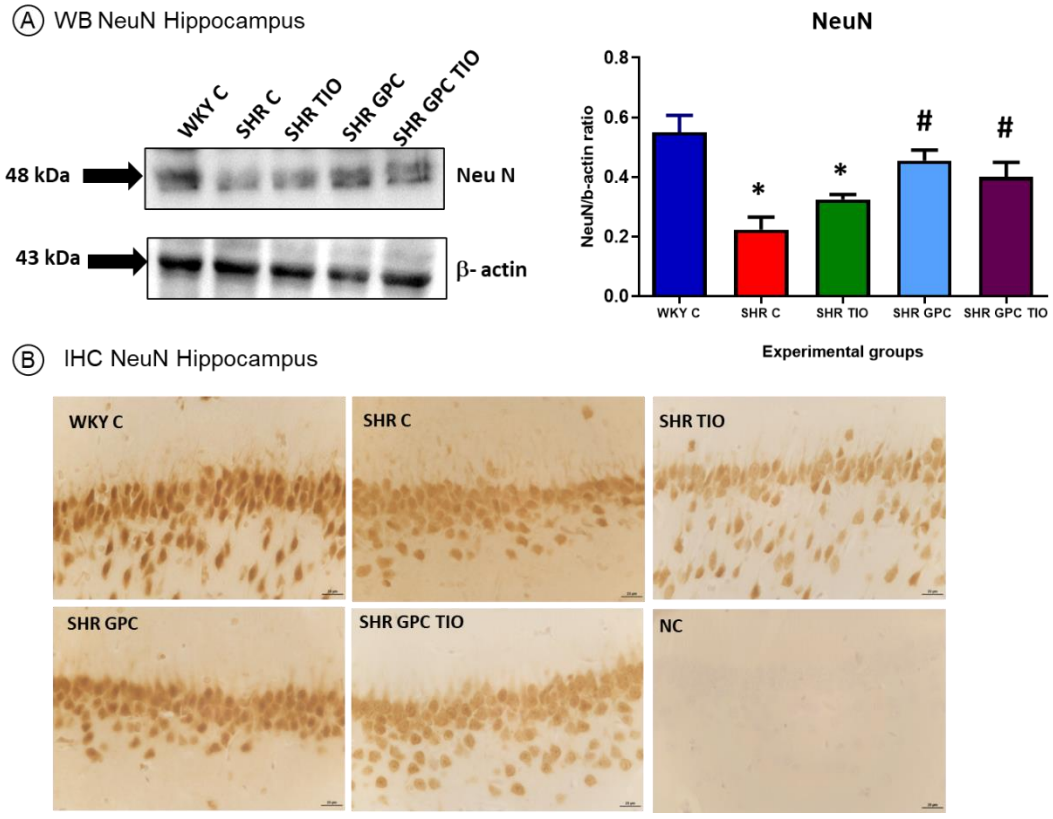

Figure 39. *Western blot analysis of neuronal nuclear (Neu N) proteins at the level of the hippocampus (A) and the relative densitometric analysis express as ratio between protein and β actin as reference. Immunohistochemical analysis for Neu N in the CA1 subfiled of hippocampus (B) in different experimental groups. WKY C: Wistar Kyoto rats; SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate.; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Calibration bar: 25 μm. Data are the mean ± S.D.* = $p<0.05$ vs WKY; # = $p<0.05$ vs SHR*

On the other hand, in the frontal cortex, NeuN expression also decreased in SHR as compared to WKY, and the treatment group remained almost unchanged (Figure 40A). Moreover, the pyramidal neurons at the V layer in the frontal cortex showed a significantly lower expression in hypertensive rats compared to normotensive ones. Treatment with (+) TIO alone and in combination with GPC showed a higher response (Figure 40B).

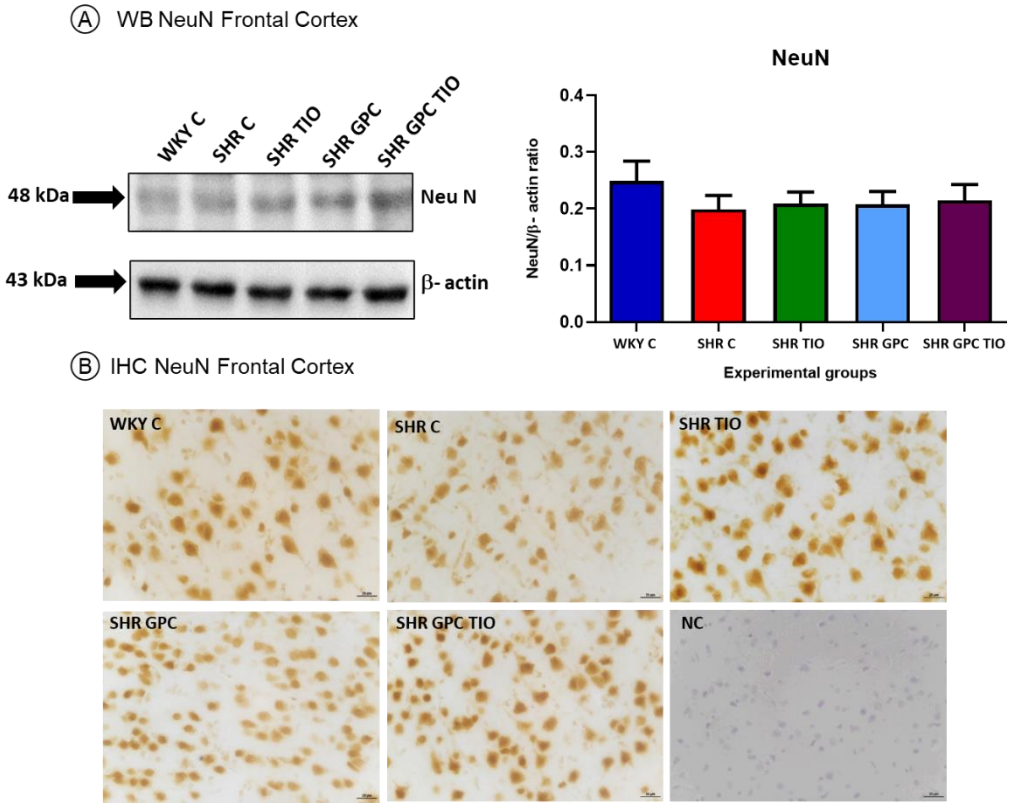

Figure 40. *Western blot analysis of neuronal nuclear proteins (Neu N) at the level of the Frontal Cortex (A) and the relative densitometric analysis express as ratio between protein and β actin as reference. Immunohistochemical analysis for Neu N in the V layer of the frontal cortex (B) in different experimental groups. WKY C: Wistar Kyoto rats: SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate.; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Calibration bar: 25 μm. Data are the mean ± S.D.* *******= p<0.05 vs WKY; #=p<0.05 vs SHR*

Microtubule-Associated Protein 2 (MAP-2)

Microtubule-associated protein 2 (MAP-2) is a structural protein and specifically expressed in the neuronal cell bodies and dendrites. Thus, MAP2 also acts as a marker of synaptic plasticity.

Moreover, MAP-2 is one of the family of proteins which functions to stabilize neuronal shape by promoting microtubule synthesis and cross-linking with other components of the cytoskeleton.

MAP-2 and the reference protein β-actin developed bands at 280 KDa and 43 KDa, respectively (Figure 41A and 42A). In hippocampus the ratio of MAP-2/β-actin densitometric values of bands revealed a significant reduction in all experiment groups as compared to the normotensive control rats. In fact, no difference was reported between the treatments (Figure 41A). The IHC results demonstrate immunoreaction at the level of the peripheral part of the soma and in the dendritic arborizations in the pyramidal neurons of the hippocampus (Figure 41B). MAP2 expression in SHR was significantly lower than WKY in the hippocampus. Downregulation of MAP-2 was not prevented by treatments (Figure 41B).

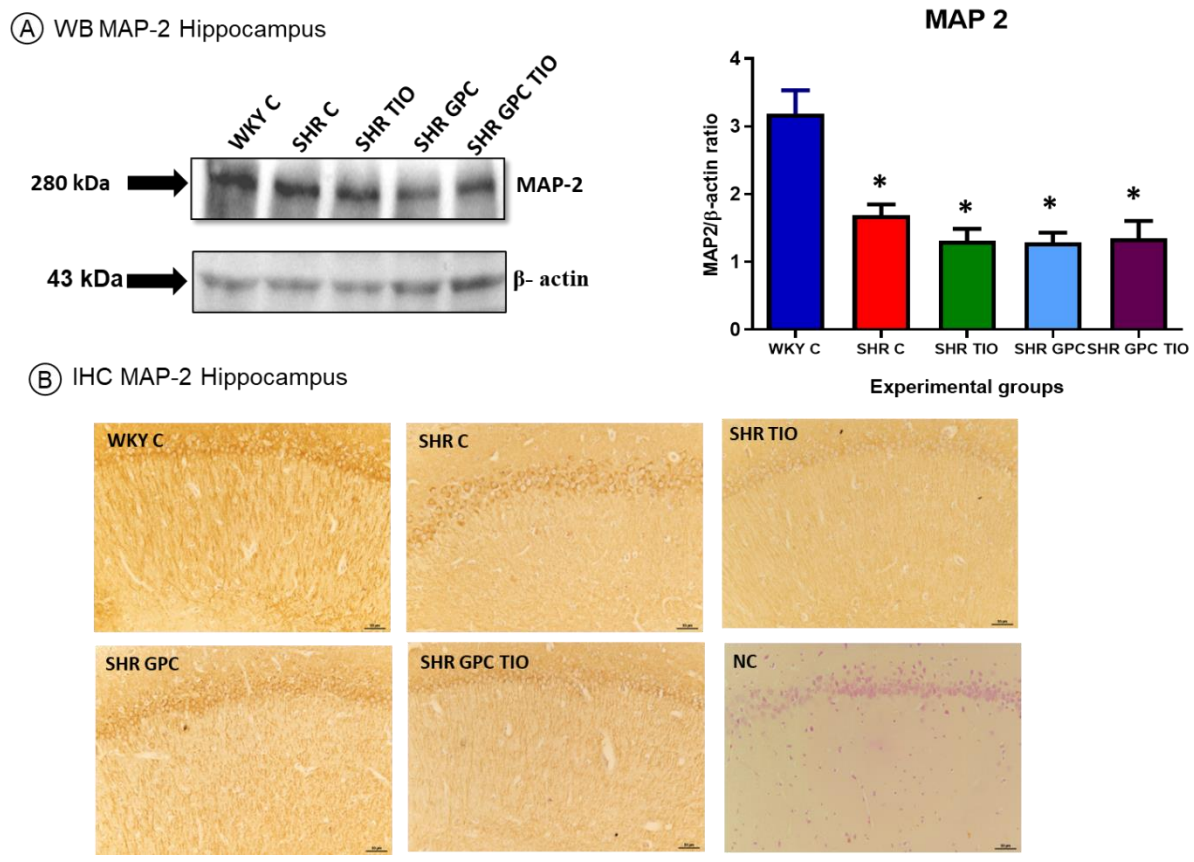

Figure 41. *Western blot analysis of the microtubular associated protein (MAP 2) at the level of the hippocampus (A) and the relative densitometric analysis express as ratio between protein and β-actin as reference. Immunohistochemical analysis for MAP-2 in the CA1 subfield of hippocampus in the different experimental groups. WKY C: Wistar Kyoto rats; SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats*

treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate.; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Calibration bar: 50 μm. Data are the mean ± S.D. ***= p<0.05 vs WKY; #=p<0.05 vs SHR***

However, in the region of the frontal cortex, the MAP-2 expression was significantly lower in both SHR and (+) TIO group compared to the WKY. Treatment with GPC alone and in combination with TIO showed a slight, but significant upregulation of MAP-2 protein. (Figure 42A). The IHC results showed dendritic arborizations in the pyramidal neurons of the V and VI layers of frontal cortex (Figure 42B). Treatment with GPC alone has shown slight increases in frontal cortex (Figure 42B).

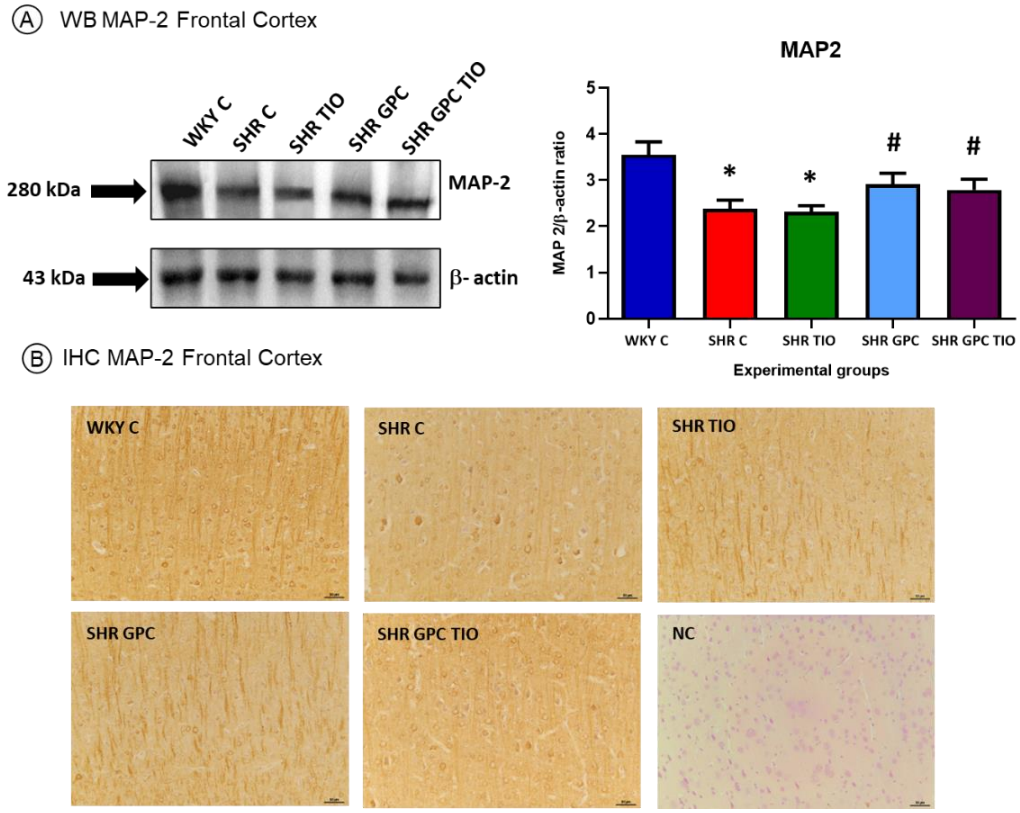

Figure 42. *Western blot analysis of the microtubular associated protein (MAP/2) at the level of the Frontal Cortex (A) and the relative densitometric analysis express as ratio between protein and β-actin as reference. Immunohistochemical analysis for MAP/2 in the V layer in the frontal cortex region (B) in the different experimental groups. WKY C: Wistar Kyoto rats: SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate.; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Calibration bar: 50 μm. Data are the mean ± S.D.* ***= p<0.05 vs WKY; #=p<0.05 vs SHR***

High molecular weight Neurofilament (NF)

High molecular weight Neurofilaments (NF) are classed as type IV intermediate filaments found in the cytoplasm of neurons. Neurofilament light chain, lysosomal enzymes, and markers of neuroinflammation provide promising preliminary results as candidate biomarkers. A change or disruption of the neurofilament network can cause pathological signs of neurodegeneration and even lead to the death of neurons.

NF and the reference protein β-actin developed bands at 200 KDa and 43 KDa, respectively (Figure 43A and 44A). In hippocampus the ratio of NF/β-actin densitometric values of bands, showed a reduction in SHR group compared to the WKY rats (Figure 43A). Treatment with the combination of GPC and (+) TIO countered to a greater extent phosphorylated neurofilament breakdown (Figure 43A). Evaluation of phosphorylated neurofilament immunoreactivity revealed a decreased immune reaction area in the CA1 subfield in SHR compared to age-matched WKY rats (Figure 43B). The condition of the phosphorylated neurofilament were improved with the combination of GPC and (+) TIO treatment.

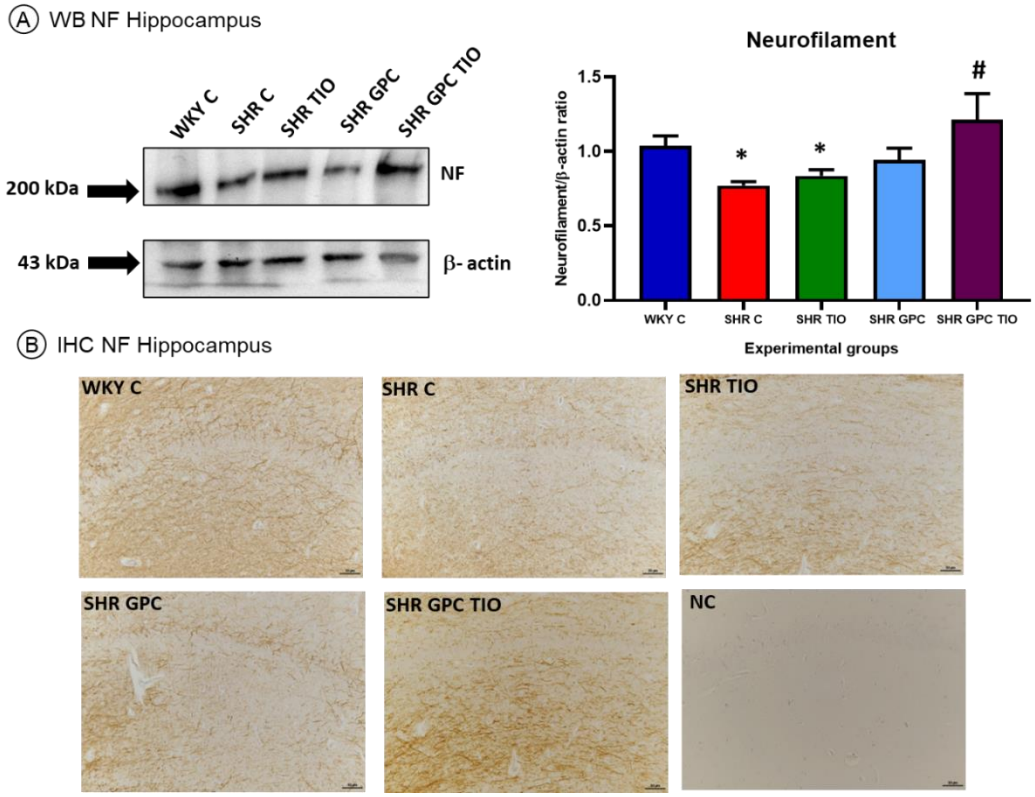

Figure 43. *Western blot analysis of the Neurofilament (NF) at the level of the Hippocampus (A) and the relative densitometric analysis express as ratio between protein and β actin as reference. Immunohistochemical analysis for NF in the CA1 subfield of hippocampus (B) in the different experimental groups. WKY C: Wistar Kyoto rats: SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate.; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Calibration bar: 50 μm. Data are the mean ± S.D.* *******= p<0.05 vs WKY; #=p<0.05 vs SHR*

In case of frontal cortex the phosphorylated neurofilament showed a reduction in SHR group compared to the WKY rats. The breakdown of the phosphorylated neurofilament was countered by the combination of GPC and (+) TIO as similar as hippocampus (Figure 44A). There were no significant differences in immunoreaction reported among all the control and treated groups (Figure 44B).

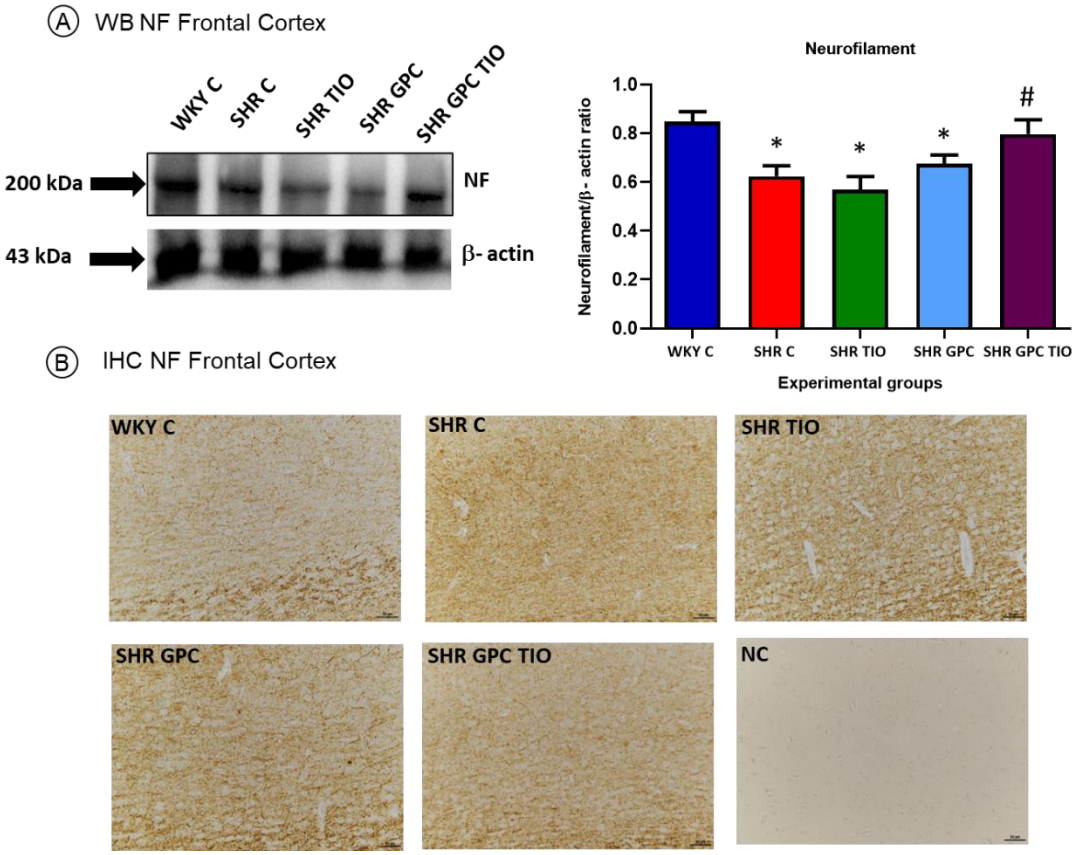

Figure 44. *Western blot analysis of the Neurofilament (NF) at the level of the Frontal Cortex (A) and the relative densitometric analysis express as ratio between protein and β actin as reference. Immunohistochemical analysis for NF in the in the V layer of the frontal cortex (B) in the different experimental groups. WKY C: Wistar Kyoto rats: SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate.; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Calibration bar: 50 μm. Data are the mean ± S.D.* *******= p<0.05 vs WKY; #=p<0.05 vs SHR*

Myeline Basic Protein (MBP)

Throughout the CNS, myelin plays an important role in the functioning of neuronal axons. Myelin protects neuronal axons and enables rapid propagation of electrical impulses to support CNS functions, such as cognition. The processes of learning and memory require good structural integrity of myelin, which is formed by the formation of myelin. Hypermyelination occurs with aging and neurodegenerative diseases due to disruption of these myelin properties. As myelin growth is enlarged, uncompacted myelin becomes thicker, unravels, and forms protrusions (referred to as out folding), and myelin integrity is lost through degeneration.

In WB, upon exposing the membrane to MBP and β-Actin, revealed bands at 17 KDa and 43 KDa respectively in both hippocampus and frontal cortex. (Figure 45 and 46). The GFAP/β-actin ratio of densitometric values of bands in the hippocampus revealed that the SHR group had a considerable decrease in MBP expression compared to the normotensive control, different treatment group were unable to improve the protein level (Figure 45A). The same results were evident with immunohistochemistry (Figure 45B).

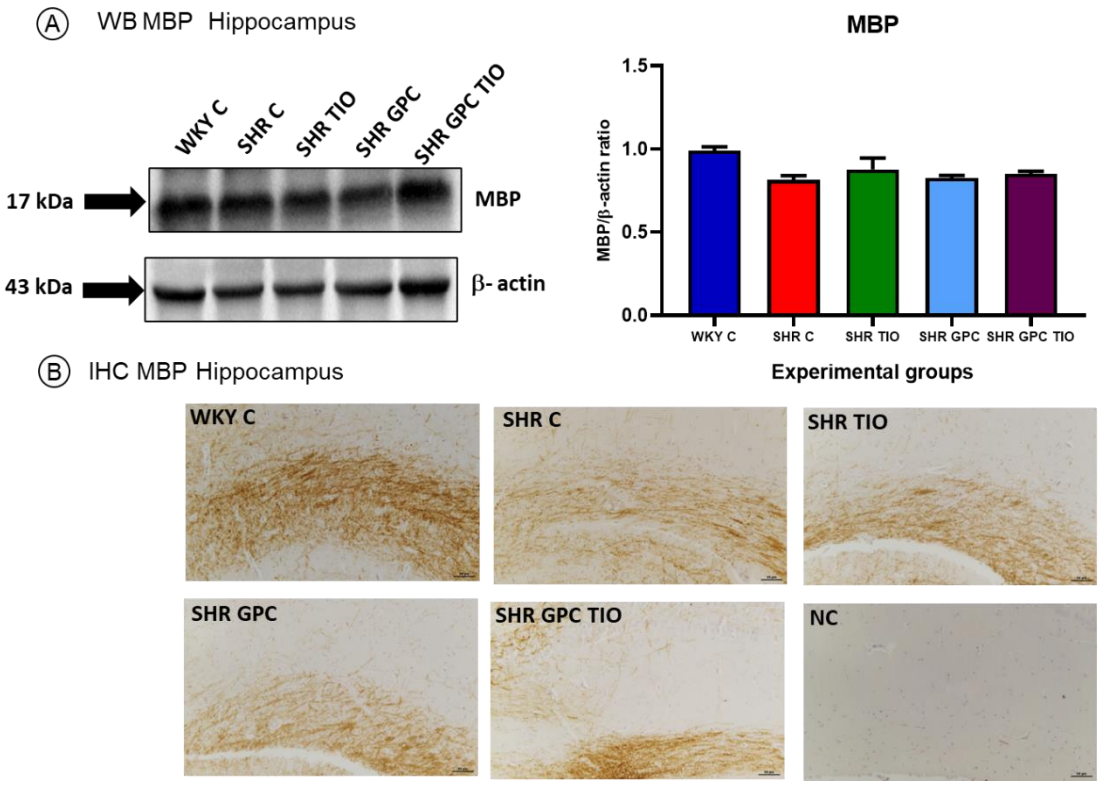

Figure 45*. Western blot analysis of the Myeline Basic Protein (MBP)at the level of the Hippocampus (A) and the relative densitometric analysis express as ratio between protein and β actin as reference. Immunohistochemical analysis for Myeline Basic Protein (MBP) in the CA1 subfield of hippocampus (B) in the different experimental groups. WKY C: Wistar Kyoto rats; SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate.; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Calibration bar: 50 μm. Data are the mean ± S.D. *= p<0.05 vs WKY; #=p<0.05 vs SHR.*

On the other hand, in frontal cortex the western blot analysis revealed that the MBP expression were decrease on the hypertensive model compared to the normotensive control, whereas our treatment with GPC and association with (+) TIO were able to improve the MBP protein level (Figure 46). IHC results showed a decrease expression of the MBP in SHR rats compared to the WKY rats whereas our treatment with GPC alone was able to improve its conditions.

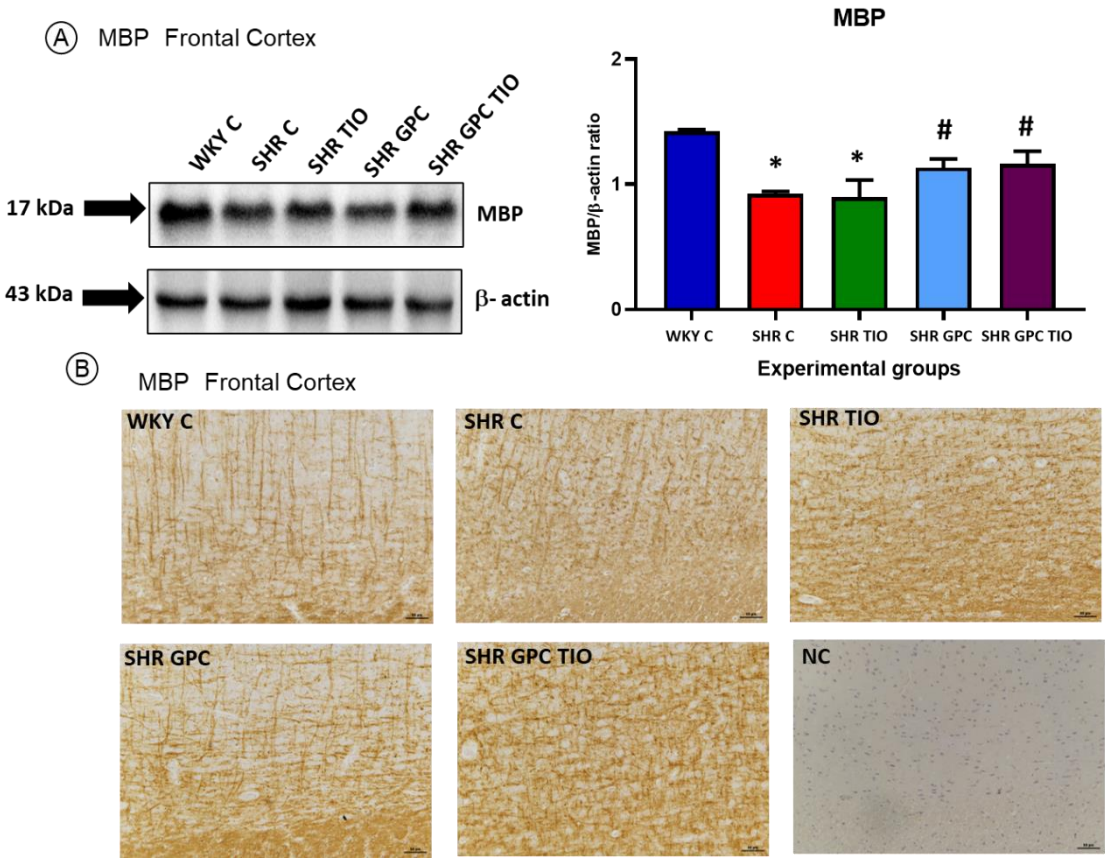

Figure 46. *Western blot analysis of the Myeline Basic Protein (MBP) at the level of the Frontal Cortex (A) and the relative densitometric analysis express as ratio between protein and β actin as reference. Immunohistochemical analysis for Myeline Basic Protein (MBP)in the VI layer of frontal cortex (B) in the different experimental groups. WKY C: Wistar Kyoto rats: SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate.; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Calibration bar: 50 μm. Data are the mean ± S.D.* ******* *= p<0.05 vs WKY; #=p<0.05 vs SHR*

Synaptophysin (SYN)

Synaptophysin (SYN) is a calcium-binding glycoprotein, which is the most abundant integral membrane protein constituent of synaptic vesicles of neurons. It is also present in a wide spectrum of Neuroendocrine cells and in many of their corresponding tumours. The exposure of the membranes at Synaptophysin antibody developed at 38KDa compared to β-actin developed at 43 KDa. The Western blot results did not show statistical differences in SYN levels in the hypertensive condition compared to the control in the hippocampus (Figure 47A). The quantification of SYN immunoreaction decreased in the hippocampus of SHR compared to the control. Only the GPC treatment showed improvement on the expression of SYN (Figure 47B).

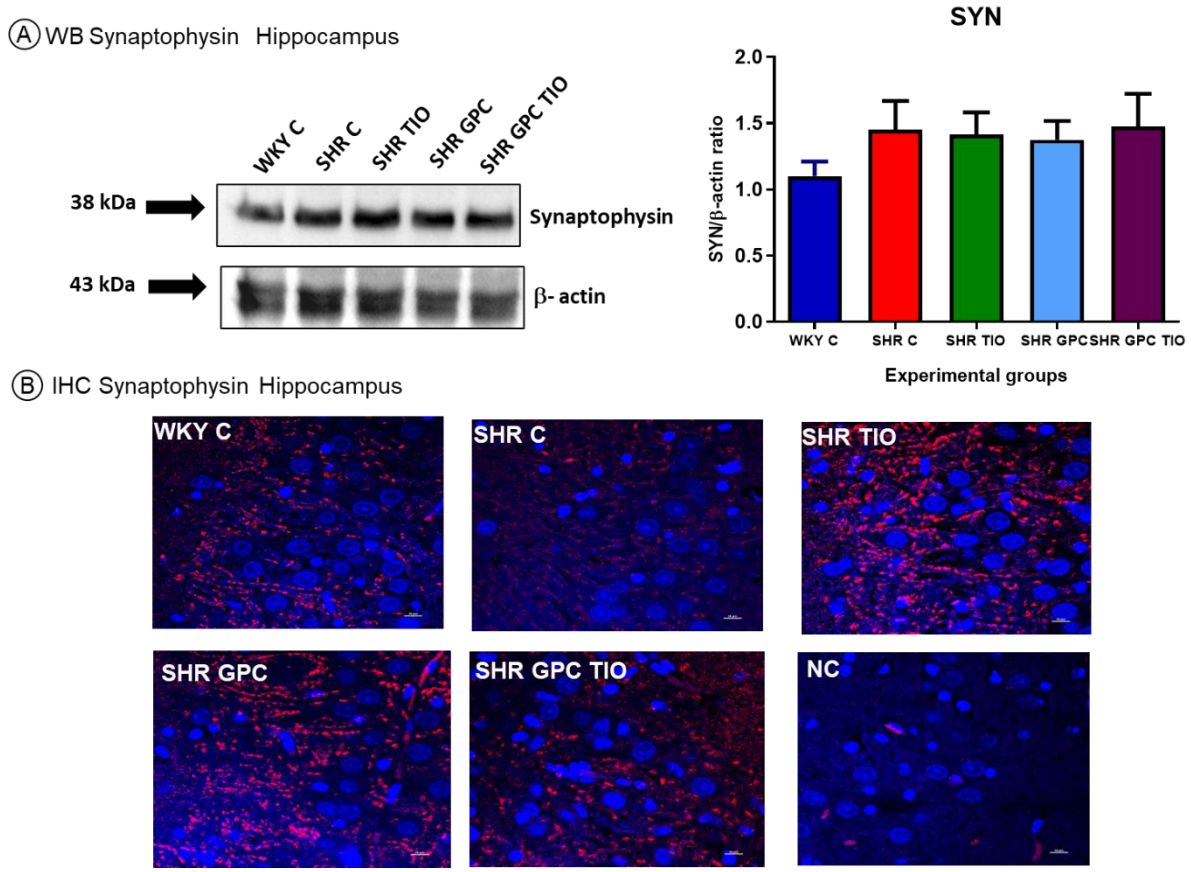

Figure 47. *Western blot analysis of the Synaptophysin (SYN) at the level of the Hippocampus (A) and the relative densitometric analysis express as ratio between protein and β-actin as reference. Immunohistochemical analysis for NF in the CA1 subfield of hippocampus (B). in the different experimental groups. Confocal image of SYN (Alexa Fluor® 594 dye in Red). WKY C: Wistar Kyoto rats: SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate.; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Calibration bar: 10 μm. Data are the mean ± S.D.* *******= p<0.05 vs WKY; #=p<0.05 vs SHR*

Similar results were obtained in frontal cortex, in which no changes of synaptophysin expression were revealed in the different experimental groups both in the western blot and immunohistochemistry (Figure 48A and B).

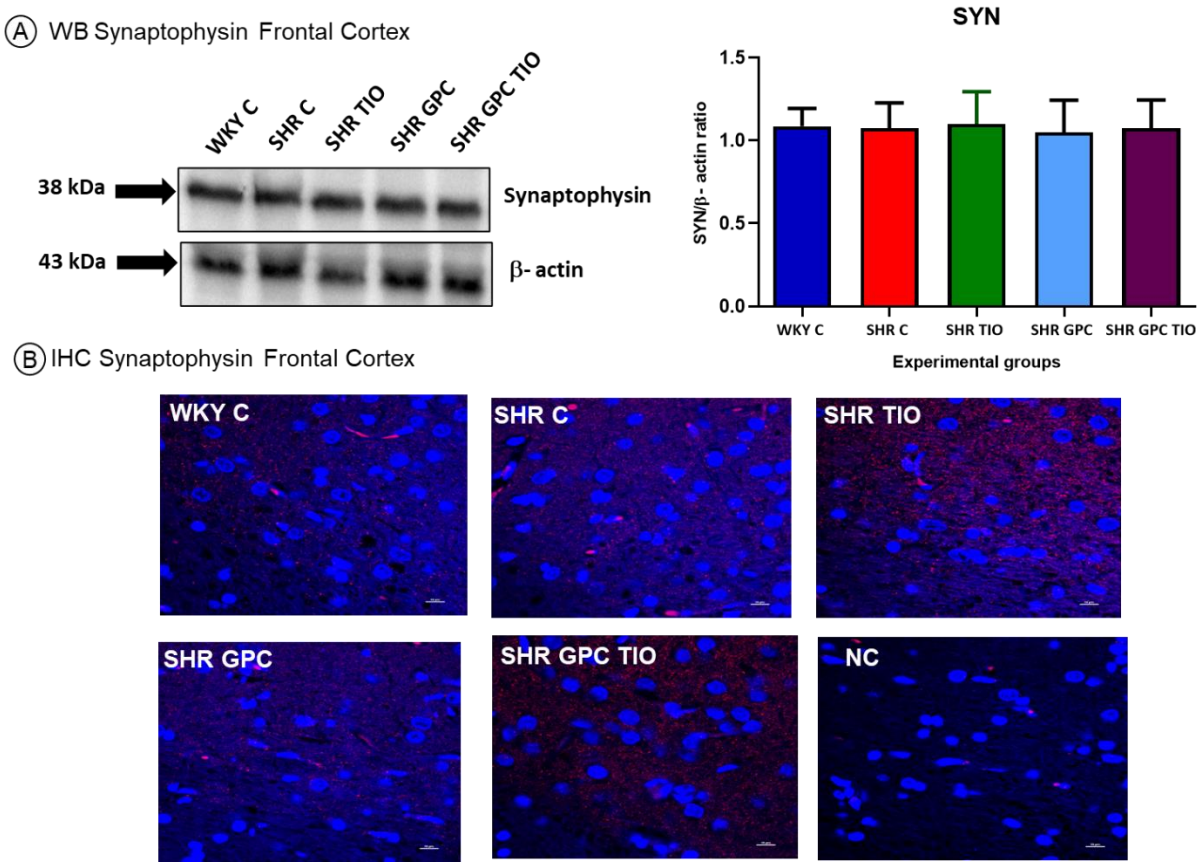

Figure 48. *Western blot analysis of the Synaptophysin (SYN) at the level of the Frontal Cortex (A) and the relative densitometric analysis express as ratio between protein and β-actin as reference. Immunohistochemical analysis for NF in the in the V layer of the frontal cortex (B) in the different experimental groups. Confocal image of SYN (Alexa Fluor® 594 dye in Red). WKY C: Wistar Kyoto rats; SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate.; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Calibration bar: 10 μm. Data are the mean ± S.D.* ******* *= $p<0.05$ vs WKY; #=$p<0.05$ vs SHR*

Postsynaptic density protein 95 (PSD-95)

Postsynaptic density protein-95 (PSD-95) is an important scaffolding protein that regulates a wide variety of receptors, channels, and signaling molecules at excitatory synapses. The PSD-95 protein binds neuroligins, which play an important role in cell adhesion. There is a strong expression of PSD-95 in the cortex, hippocampus, and striatum, but only a moderate or weak expression in other brain regions. A pivotal role is played by this postsynaptic protein in synaptic plasticity and function.

Data of western blots analysis showed that the ratio Postsynaptic density protein 95 (PSD-95) on β-actin (95KDa band and 43 KDa band, respectively) was significantly lower in the hippocampus and in the frontal cortex of SHR rats compared to WKY (Figure 49A and B). Treatment with the GPC alone or in association, showed an increase of PSD-95 both in hippocampus and frontal cortex (Figure 49A and B).

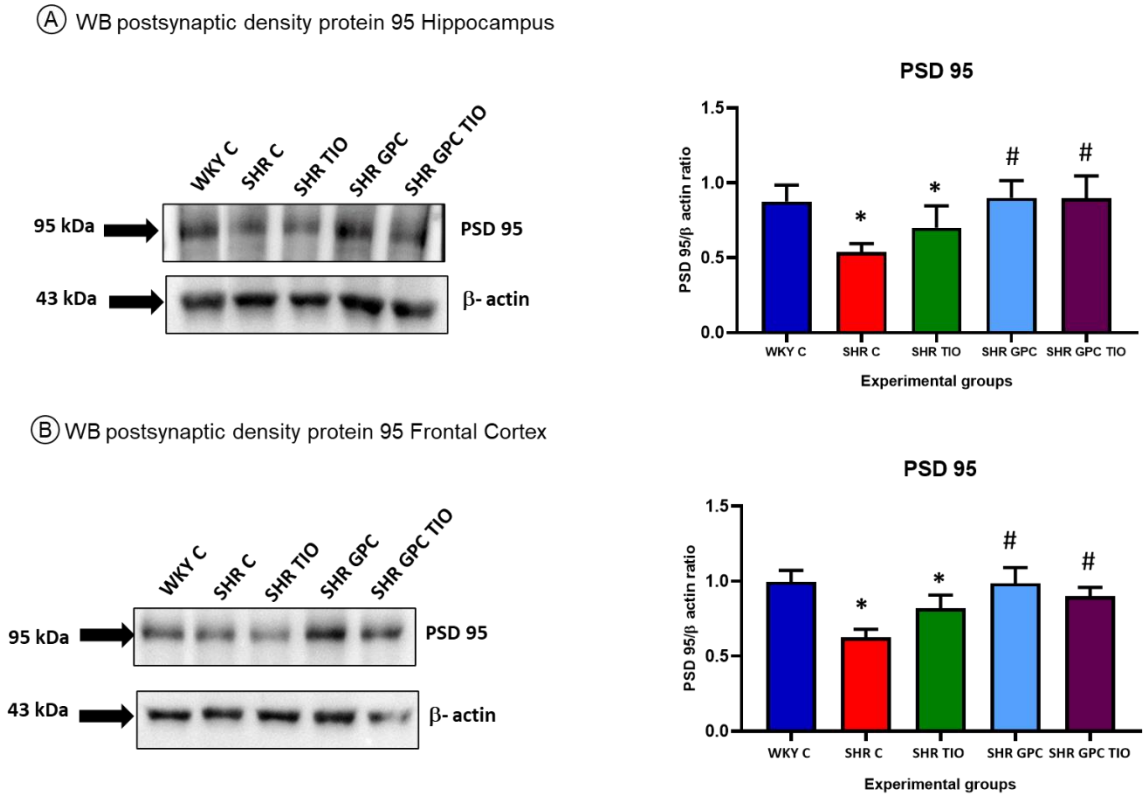

Figure 49. *Western blot analysis of the Postsynaptic density protein-95 (PSD-95) at the level of the Hippocampus(A) and Frontal cortex (B) in the different experimental groups and the relative densitometric analysis express as ratio between protein and β-actin as reference. WKY C: Wistar Kyoto rats: SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate.; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Data are the mean ± S.D.* *******= p<0.05 vs WKY; #=p<0.05 vs SHR*

4.3.3 Inflammatory Marker

Glial Fibrillary Acidic Protein (GFAP)

Astrocytes (astroglia) are distinguished by the presence of a distinct structural protein, glial fibrillary acidic protein (GFAP), which was isolated and identified by Eng in 1969. GFAP is a key intermediate filament III protein that is responsible for astrocyte cell cytoskeleton structure and mechanical strength, as well as supporting neighboring neurons and the BBB (Eng et al.,1969). GFAP is one of the most efficient markers for astrocyte activation in the CNS following injury or stress. The elevated expression of GFAP is one of the most important markers of astrocyte activation.

In WB, upon exposing the membrane to GFAP and β-Actin, revealed bands at 50 KDa and 43 KDa respectively. The GFAP/β-actin ratio of densitometric values of bands in the hippocampus revealed that the SHR group had a considerable increase in GFAP expression compared to the normotensive control (Figure 50A). Only the synergic effects of (+) TIO and GPC remarkably reduced the express of GFAP, whereas either treatment alone did not have any significant effects on normalizing the astrocyte activation (Figure 50A). Furthermore, in confocal laser microscopy sections of the hippocampal and frontal cortex processed for immunohistochemistry against GFAP as markers of reactive astrocytes, GFAP antibody exposure revealed the presence of astrocyte cells (Figure 50B and 51B). A high number of hyper-reactive astrocytes (H/R) as well as hypertrophic/hyper-immunoreactive astrocytes (H/H) were observed in the white matter of the frontal cortex and at the level of CA1, CA3, and DG of the hippocampus. According to morphological analysis, hypertensive control rats showed a significant increase in astrocyte immunoreactivity in the hippocampus in comparison to normotensive animal. In the hippocampus TIO, GPC, and the two drugs in combination were able to reduce the activation of astrocytes (Figure 50 B)

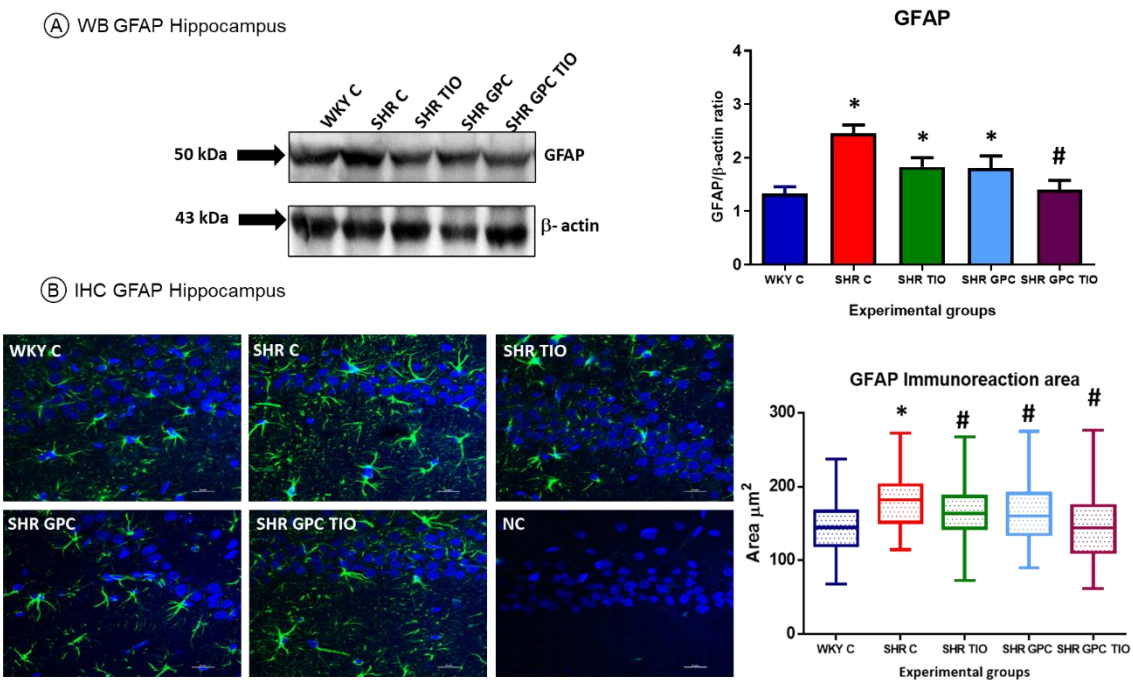

Figure 50. *Western blot analysis of the Glial Fibrillary acid protein (GFAP) in Hippocampus (A) and the relative densitometric analysis express as ratio between protein and β-actin as reference. Immunohistochemical analysis for GFAP in the CA1 subfield of hippocampus (B). Confocal image of GFAP (Alexa Fluor® 488 dye in green) and subsequent counterstained with DAPI (blue color) as nuclear dye (left panel). The mean immune reaction area of reactive astrocytes in each experimental group (right panel) was expressed as mean ± SEM. *= p<0.05 vs WKY; #=p<0.05 vs SHR. The bands are representative of the different animals. WKY C: Wistar Kyoto rats: SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate.; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Calibration bar: 20 µm. Data are the mean ± S.D.* ******* *= p<0.05 vs WKY; #=p<0.05 vs SHR*

However, in a similar way of hippocampus, the GFAP expression was upregulated in the frontal cortex of SHR in contrast with WKY and the effects were counteracted by the treatment with (+) TIO and GPC (Figure 51A). Furthermore, the immunohistochemistry results showed at the level of the frontal cortex, only (+) TIO and combination of (+) TIO and GPC significantly diminished the hyperactivity of astrocytes (Figure 51 B).

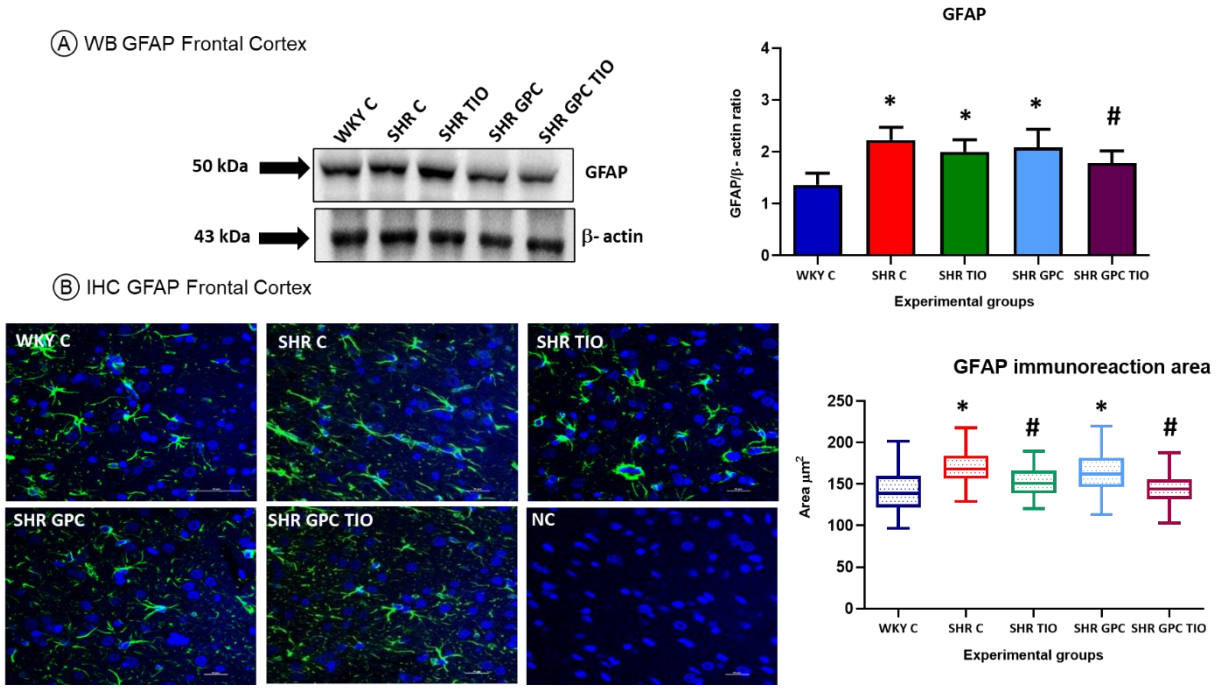

Figure 51. *Western blot analysis of the Glial Fibrillary acid protein (GFAP) in the Frontal Cortex and the relative densitometric analysis express as ratio between protein and β-actin as reference. Immunohistochemical analysis for GFAP in the in the v layer of frontal cortex (B). Confocal image of GFAP (Alexa Fluor® 488 dye in green) and subsequent counterstained with DAPI (blue color) as nuclear dye (left panel). The mean immune reaction area of reactive astrocytes in each experimental group (right panel) was expressed as mean ± SEM. *= p<0.05 vs WKY; #=p<0.05 vs SHR. The bands are representative of the different animals. WKY C: Wistar Kyoto rats: SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate.; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Calibration bar: 20 µm. Data are the mean ± S.D.* ******* *= p<0.05 vs WKY; #=p<0.05 vs SHR*

Ionized calcium binding adaptor molecule 1 (IBA1)

Ionized calcium binding adaptor molecule 1 (IBA1) is a calcium-binding protein which is specific to microglia and macrophages. In activated microglia, IBA1 has actin-bundling activity and participates in membrane ruffling and phagocytosis. Calcium ions are key signal mediators that function by interacting with numerous calcium binding proteins. IBA1 is a 17 KDa protein which corresponds to the large EF-hand-family of proteins with the EF-hand-motif. The immunohistochemical analysis highlights the resting (ramified form) or activated (phagocytic state).

The WB result showed a band around 17 KDa. Both at the level of hippocampus and frontal cortex, the IBA1/ β-actin ratio did not show any significant alterations in SHR groups compared to the control WKY (Figure 52A and 53A). Neither of the treated groups exhibit any significant variation in IBA1 expression (Figure 52A and 53A).

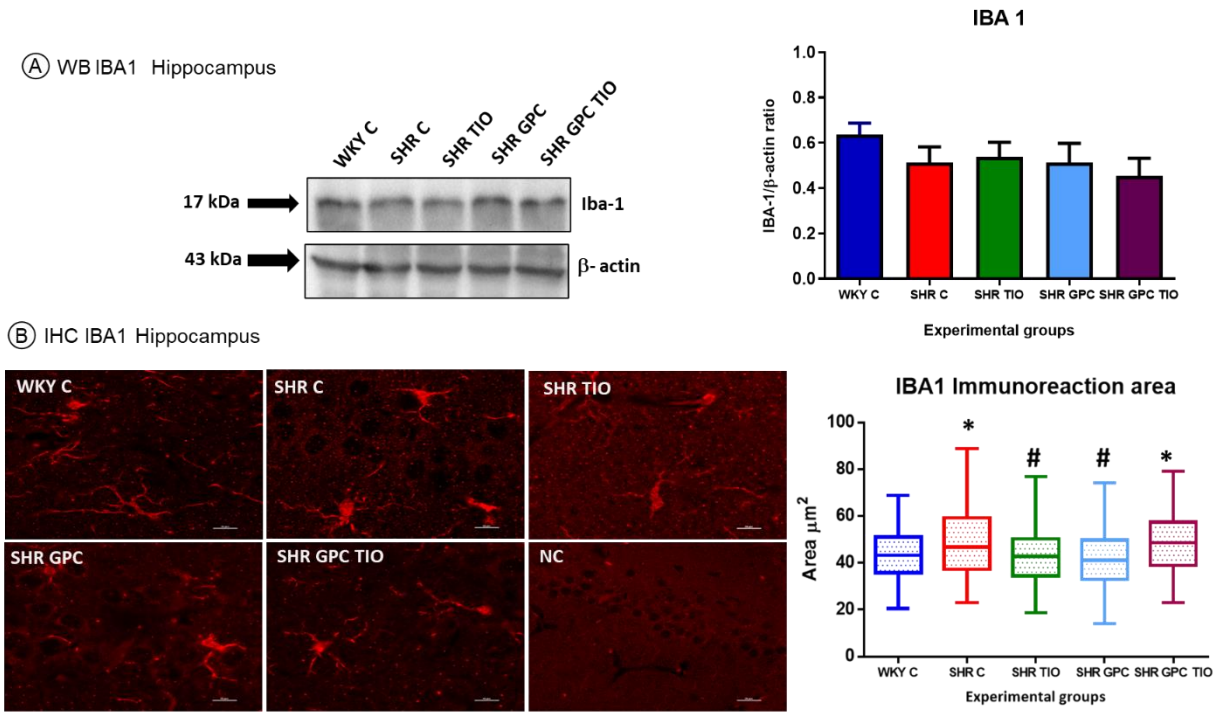

Figure 52. *Western blot analysis of the Ionized Calcium Binding Adaptor Molecule 1 (IBA1) in Hippocampus (A) and the relative densitometric analysis express as ratio between protein and β actin as reference. Immunohistochemical analysis for IBA1 in the hippocampus. Confocal image of IBA 1 (Alexa Fluor® 594 dye in red) (left panel). The mean immune reaction area of reactive microglia in each group (right panel) was expressed as mean ± S.E.M. *= p<0.05 vs WKY; #=p<0.05 vs SHR. WKY C: Wistar Kyoto rats: SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate.; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in assocation. Calibration bar: 10 µm. Data are the mean ± S.D.* ******* *= p<0.05 vs WKY; #=p<0.05 vs SHR*

Moreover, hippocampal, and frontal cortex sections were analyzed morphologically for activation of microglia using specific IBA1 antibody in confocal laser microscopy (Figure 52B and 53B). Upon activation, microglia undergo a rapid morphological change, characterized by retraction of their fine processes and gradual acquisition of the shape of amoebas. Activated microglia were

visible in red color. In addition, several groups' immunological responses to activated microglia were compared. Both in the hippocampus and frontal cortex, the area of the central part of the cells, feature of activated microglia, significantly increase in the spontaneously hypertensive rats compared to WKY. Microgliosis was counteracted by the treatment of two compounds alone in hippocampus (Figure 52 B). Contrary to this, none of the treatments showed any noticeable effects at the level of the frontal cortex (Figure 53 B).

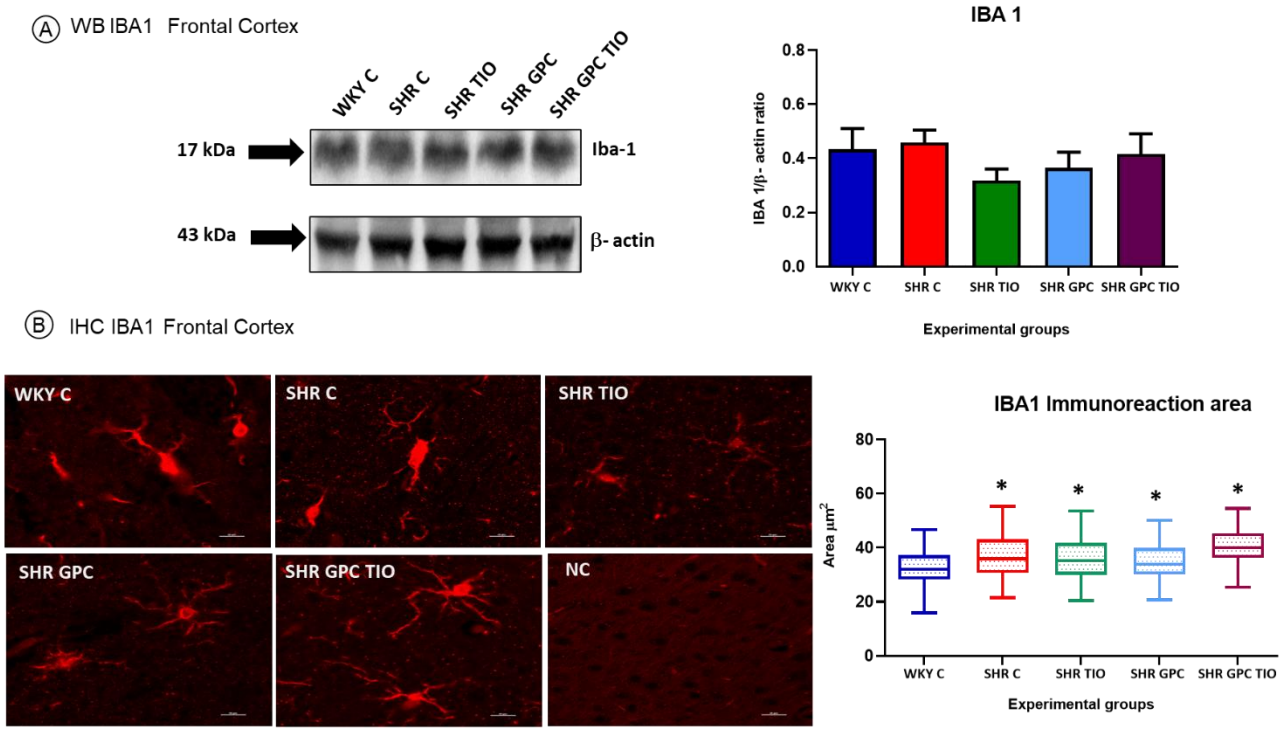

Figure 53. *Western blot analysis of the Calcium Binding Adaptor Molecule 1 (IBA1) at the region of the Frontal Cortex (A) and the relative densitometric analysis express as ratio between protein and β actin as reference. Immunohistochemical analysis for IBA1 in the frontal cortex (B). Confocal image of IBA 1 (Alexa Fluor® 594 dye in red) (left panel). The mean immune reaction area of reactive microglia in each group (right panel) was expressed as mean ± S.E.M. *= p<0.05 vs WKY; #=p<0.05 vs SHR. WKY C: Wistar Kyoto rats; SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate.; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Calibration bar: 10 µm. Data are the mean ± S.D.* ***=** *p<0.05 vs WKY; #=p<0.05 vs SHR*

Pro-inflammatory biomarkers are defined as regulatory proteins that are capable of detecting inflammation. Upon entry of a foreign pathogen, the glial cells (astrocytes and microglia) become activated, triggering the release of numerous neuroinflammatory markers (NMs) such as tumor necrosis factor (TNF), interleukin-1 (IL-1) and interleukin 6 (IL-6).

Tumor necrosis factor-alpha (TNF-α)

Data of western blots showed that the ratio tumor necrosis factor alpha (TNF-α) on β-actin (17KDa) band and 43 KDa band, respectively) was significantly higher in the hippocampus of SHR rats compared to WKY. The co-treated group GPC and the combination GPC (+) TIO showed significantly reduced inflammation in SHR animals (Figure.54A). Sections processed for TNF alpha immunohistochemistry developed dark-brown staining along the hippocampus. The density values of immunoreactivity were significantly increased in the SHR compared to the control. whereas the GPC alone and the synergic effects of (+) TIO and GPC significantly reduced the immunoreactivity (Figure.54B).

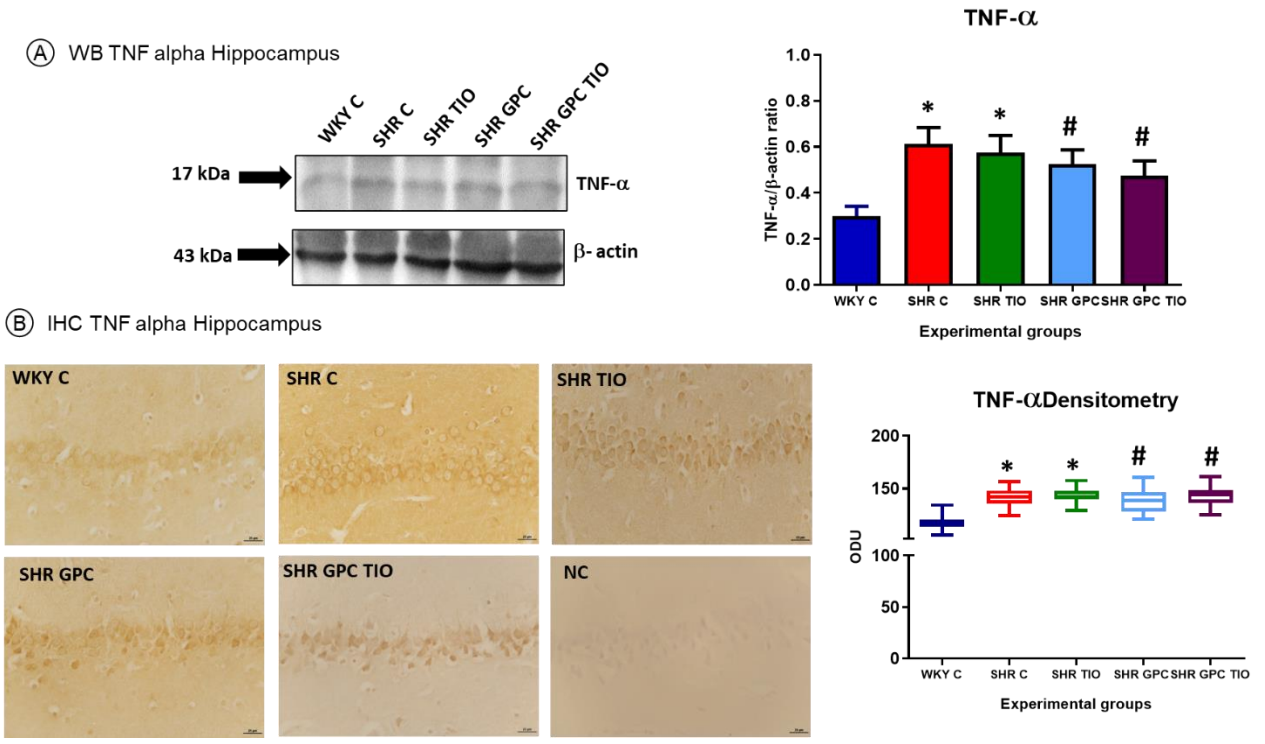

Figure 54. *Western blot analysis of the tumor necrosis factor alpha (TNF α) at the level of the Hippocampus (A) and the relative densitometric analysis express as ratio between protein and β actin as reference. Immunohistochemical analysis for TNF α in the CA1 subfield of hippocampus (B) in the different experimental groups. WKY C: Wistar Kyoto rats: SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate.; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Calibration bar: 25 μm. Data are the mean ± S.D.* ******* *= p<0.05 vs WKY; #=p<0.05 vs SHR*

Moreover, in the frontal cortex western blot and the immunohistochemistry both results showed and increase expression of the TNF-α in SHR compared to the WKY rats. Treatment with the GPC alone and the combination reduced the immunoreaction in the level of frontal cortex (55A and B).

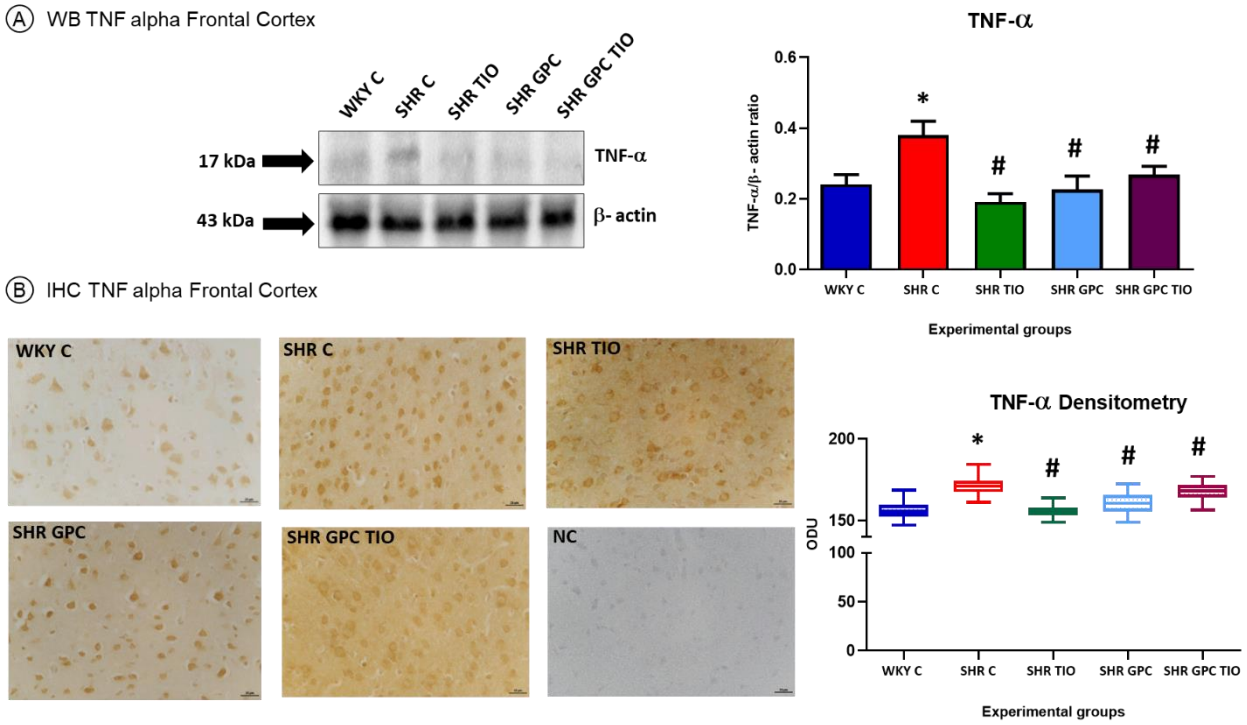

Figure 55. *Western blot analysis of the tumor necrosis factor alpha (TNF α) at the level of the Frontal Cortex (A) and the relative densitometric analysis express as ratio between protein and β actin as reference. Immunohistochemical analysis for TNF α in the V layer of frontal cortex (B) in the different experimental groups. WKY C: Wistar Kyoto rats: SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate.; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Calibration bar: 25 μm. Data are the mean ± S.D.* ******* *= p<0.05 vs WKY; #=p<0.05 vs SHR*

Interleukin-1 beta (IL-1β)

The exposure of the membranes with IL-1β antibody showed a band at approximately developed at 31KDa in both hippocampus and the frontal cortex. (Figure 56A and 57A). A slight, but not significant increase in the expression of the interleukin was evident in all SHR groups, without differences between the different drug treatments (Figure 56A). Section processed for immunohistochemistry against IL-1β, as inflammatory markers were observed in all subfields of

hippocampus around the cell body neurons. Morphological analysis highlighted a clear increase of immune reaction around the cell bodies of SHR control group and showed a trend of decreasing in all experimental treated SHR groups (Figure 56B).

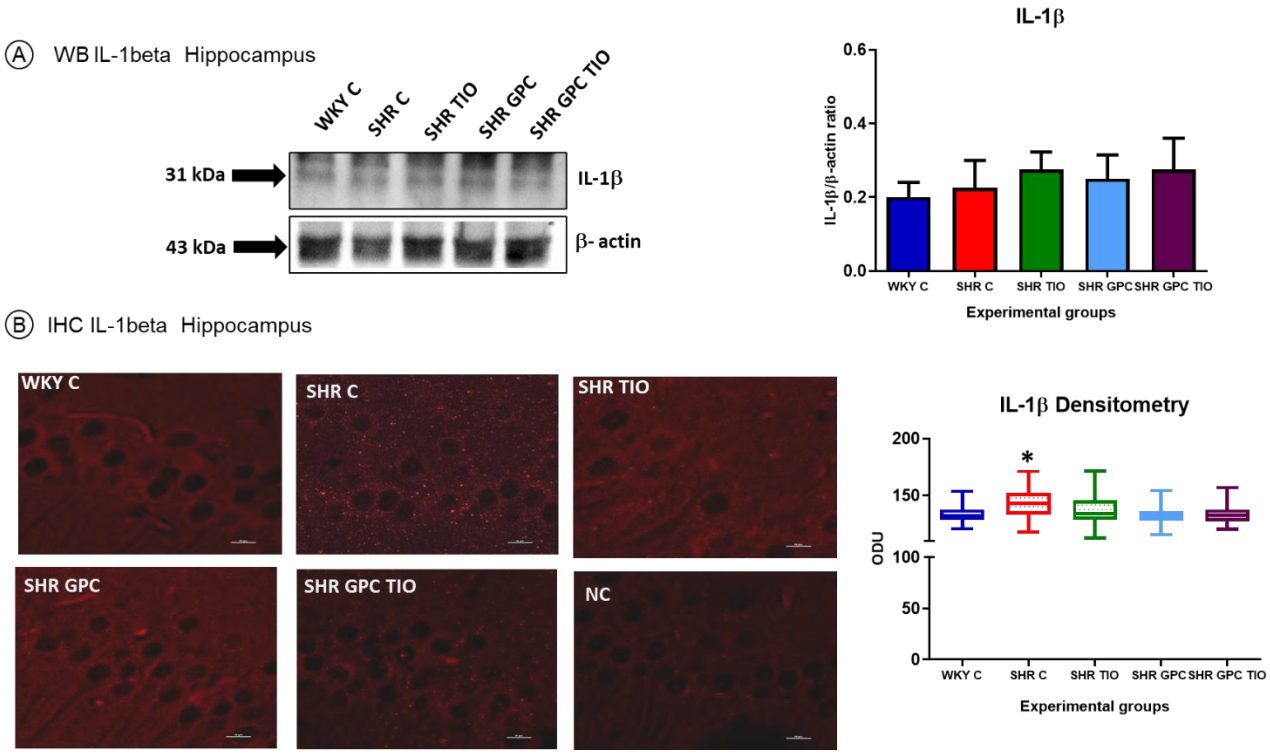

Figure 56. *Western blot analysis of the Interleukin-1 beta (IL-1β) at the region of hippocampus (A) and the relative densitometric analysis express as ratio between protein and β actin as reference. Immunohistochemical analysis for IL-1β in the hippocampus. Confocal image of (IL-1β) (Alexa Fluor® 594 dye in red) (left panel). The mean immune reaction area of reactive microglia in each group (right panel) was expressed as mean ± S.E.M. *= p<0.05 vs WKY; #=p<0.05 vs SHR. WKY C: Wistar Kyoto rats; SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate.; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Calibration bar: 10 μm. Data are the mean ± S.D.* ******* *= p<0.05 vs WKY; #=p<0.05 vs SHR*

In the frontal cortex western blot analysis showed an elevated immunoreaction in the SHR group compared to the normotensive rat. This condition is only counteracted by the synergic combination. Eventually the other treatment also showed a tendency to decline immunoreactivity (Figure 57A). Moreover, the pyramidal neurons at the V layer in the frontal cortex showed a significantly higher expression in hypertensive rats compared to normotensive ones. Treatment with thioctic acid alone and GPC showed a tendency to improve the condition (Figure 57B).

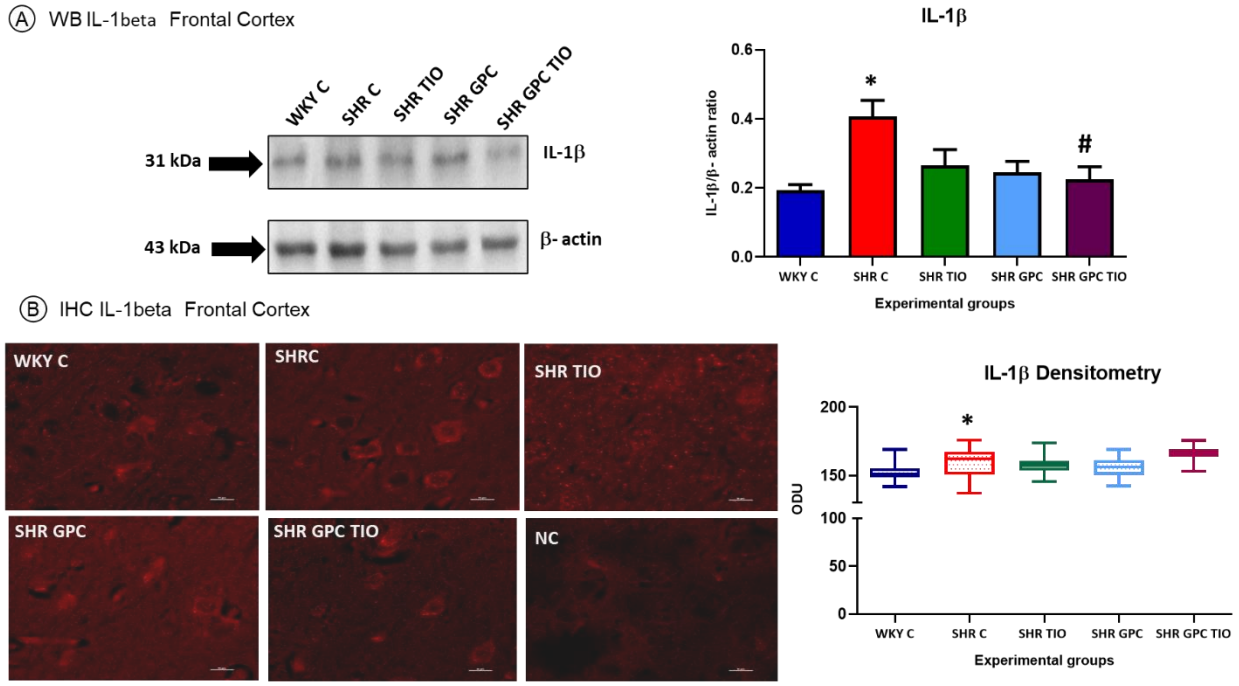

Figure 57. *Western blot analysis of the Interleukin 1 beta (Il-1β) at the region of the Frontal Cortex (A) and the relative densitometric analysis express as ratio between protein and β actin as reference. Immunohistochemical analysis for Il-1β in the v layer of the frontal cortex. Confocal image of (Il-1β) (Alexa Fluor® 594 dye in red) (left panel). The mean immune reaction area of reactive microglia in each group (right panel) was expressed as mean ± S.E.M. *= p<0.05 vs WKY; #=p<0.05 vs SHR. WKY C: Wistar Kyoto rats: SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate.; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Calibration bar: 10 μm. Data are the mean ± S.D.* ******* *= p<0.05 vs WKY; #=p<0.05 vs SHR*

Interleukin-6 (IL-6)

The exposure of the membranes at IL-6 antibody developed at 31KDa, while to β-actin developed at 43 KDa. In the case of IL-6 the expression on hippocampus the morphological and immunochemical analysis indicates a not significantly changes in the expression in the SHR treated or not compared to WKY (Figure 58A.), indicating an inflammatory process not mediated by IL-6.

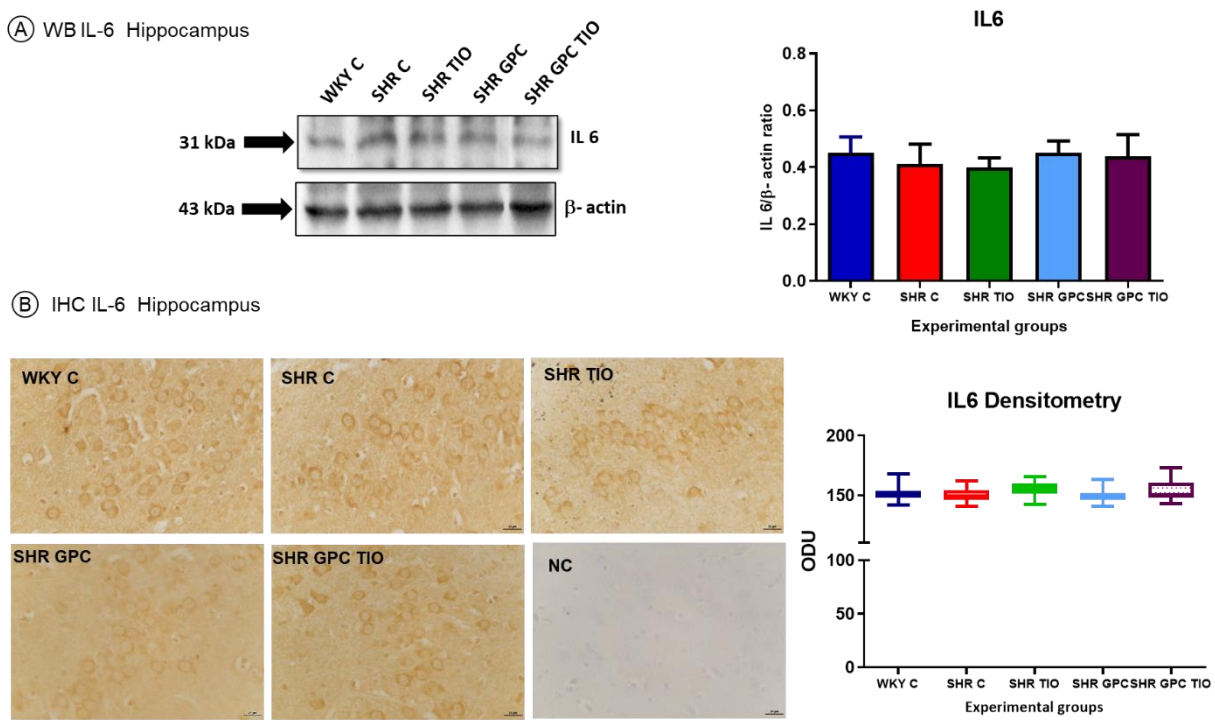

Figure 58. *Western blot analysis of the Interleukin 6 (IL-6) at the level of the Hippocampus (A) and the relative densitometric analysis express as ratio between protein and β actin as reference. Immunohistochemical analysis for IL-6 in the CA1 subfield of hippocampus (B) in the different experimental groups. WKY C: Wistar Kyoto rats: SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate.; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Calibration bar: 25 μm. Data are the mean ± S.D.* *******= p<0.05 vs WKY; #=p<0.05 vs SHR*

The IL-6/β-actin densitometric analysis of bands values suggests that IL-6 expression in the frontal cortex was considerably elevated in the hypertensive group compared to the normotensive control (Figure 59A). The IHC revealed an immunoreaction at the level cell body of the pyramidal neurons in the V layer of frontal cortex. In addition, the treatment with (+) TIO and only GPC were effective to reduce its overexpression (Figure 59B).

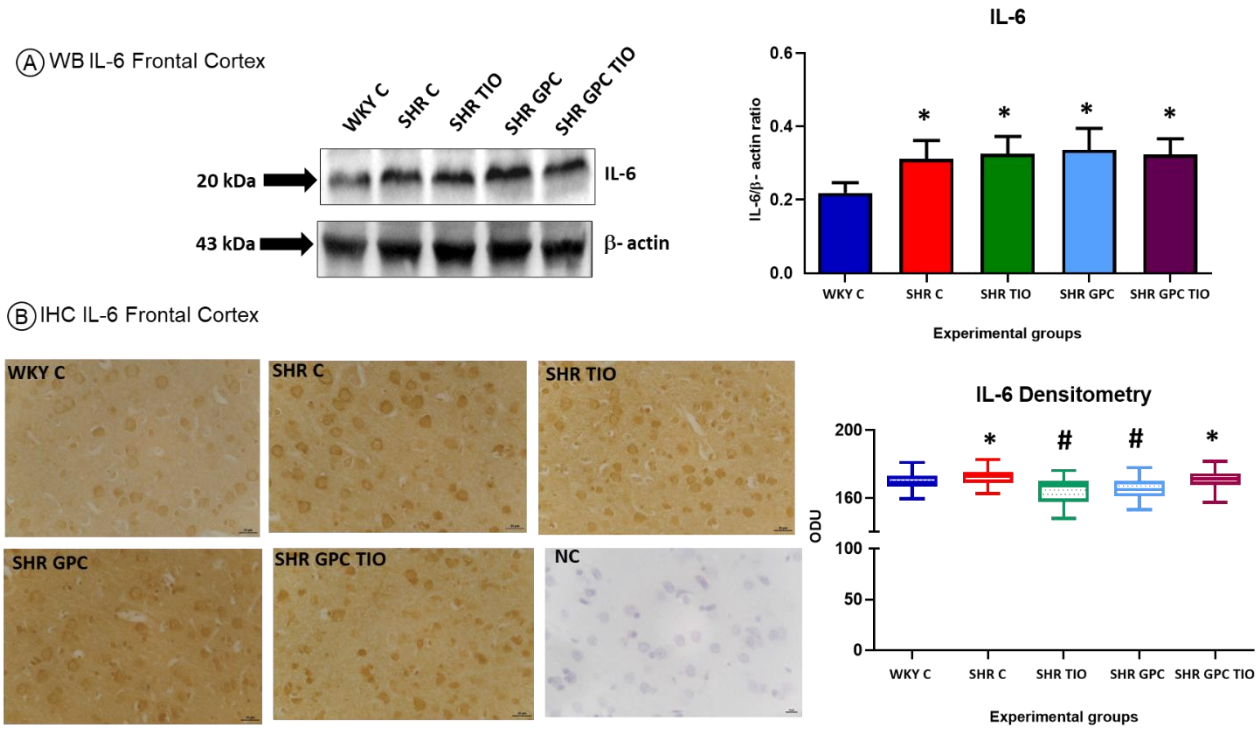

Figure 59. *Western blot analysis of the Interleukin 6 (IL-6) at the level of the Frontal Cortex (A) and the relative densitometric analysis express as ratio between protein and β actin as reference. Immunohistochemical analysis for IL-6 in the V layer of frontal cortex (B) in the different experimental groups. WKY C: Wistar Kyoto rats: SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate.; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Calibration bar: 25 μm. Data are the mean ± S.D.* ******* *= p<0.05 vs WKY; #=p<0.05 vs SHR*

Vascular cell adhesion molecule-1 (VCAM-1)

Vascular cell adhesion molecule-1 (VCAM-1) is a cell adhesion molecule that regulates inflammation-associated vascular adhesion and leukocyte migration across the endothelium. VCAM (CD-106) is normally found in stimulated endothelium, that plays a critical role in the migration of leukocytes. By stimulating VCAM-1, endothelial cells are activated to produce nicotinamide adenine dinucleotide phosphate (NADPH) oxidase.

In WB techniques, the hippocampus and the frontal cortex membrane were exposed to VCAM antibody, and they developed a band at approximately 48 KDa. Densitometric analysis of bands of different experimental groups was normalized by the intensity of reference protein band (β-

actin) (Figure 60A and B). No difference was evident for VCAM-1 in all SHR group compared to the WKY rats both in hippocampus and frontal cortex (Figure 60A and B).

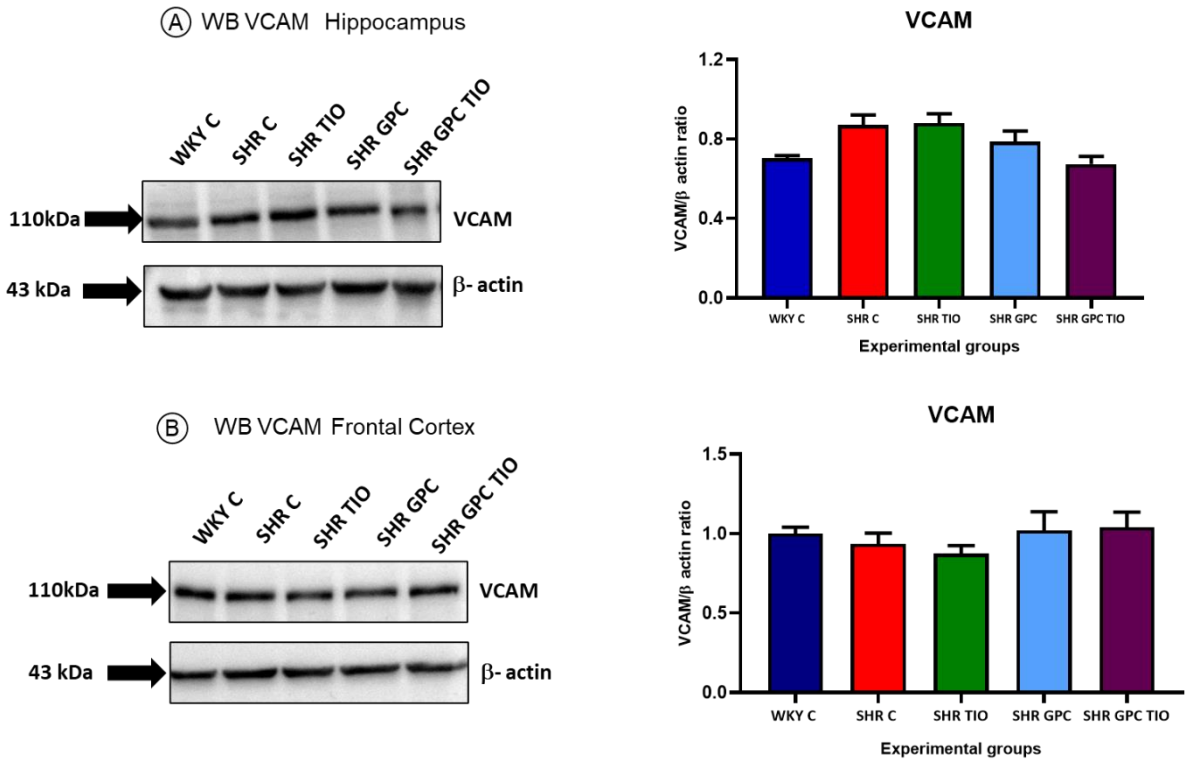

Figure 60. *Western blot analysis of the* Vascular cell adhesion molecule-1 (VCAM-1) *at the level of the Hippocampus(A) and Frontal Cortex (B) in the different experimental groups and the relative densitometric analysis express as ratio between protein and β actin as reference. WKY C: Wistar Kyoto rats; SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate.; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Data are the mean ± S.D.* ******* *= p<0.05 vs WKY; #=p<0.05 vs SHR*

4.3.4 Blood Brain Barrier Markers

Aquaporin-4 (AQP-4)

AQP-4, the most abundant water channel in the CNS, is a homotetrameric protein anchored in the plasma membrane of astrocytes by the dystroglycan protein complex. It is highly concentrated at the glial-pial and glial-endothelial interfaces in the BBB, as well as around Ranvier and paranode nodes, adjacent oligodendroglial loops, and synapses. AQP4 is associated with water balance in the brain and spinal cord, astrocyte migration, neural signal transduction, and neuroinflammation. It is responsible for the development, function, and integrity of the brain-blood interface and the brain-cerebrospinal fluid interface. An increased expression of AQP4 suggests that it may alter the fluid exchange in BBB and/or in blood-CSF barrier which promotes BBB changes causing brain damage in the animal model of hypertension.

The WB result reveals that, upon exposure to AQP-4, a band was formed at 45 KDa and the β-actin at 43 KDa (Figure 61A and 62A). The AQP-4/β-actin densitometric analysis of bands values suggests that AQP4 expression in the hippocampus was considerably elevated in the hypertensive group compared to the normotensive control. Only the treatment with (+) TIO and GPC significantly reduced the overexpression of the water channel (Figure 61A). The IHC result of AQP-4 showed immunoreaction only at the level of the wall of capillary (Figure 61B and 62B). In the hippocampus the expression of AQP-4 was substantially stronger in the SHR group compared to WKY. All three supplementation successfully brought it back to the control level (Figure 61B).

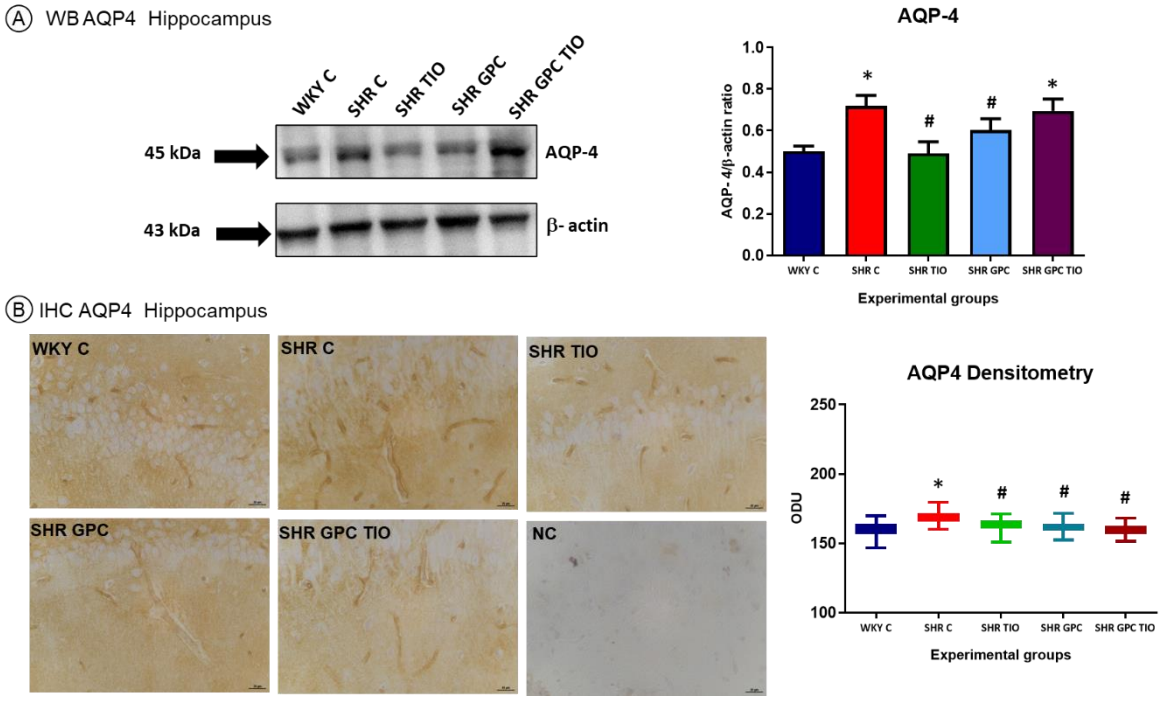

Figure 61. *Western blot analysis of the Aquaporin 4 (AQP-4) at the level of the Hippocampus (A) and the relative densitometric analysis express as ratio between protein and β actin as reference. Immunohistochemical analysis for AQP-4 in the hippocampal area (B) in the different experimental groups. WKY C: Wistar Kyoto rats; SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Calibration bar: 25 μm. Data are the mean ± S.D.* ******* *= p<0.05 vs WKY; #=p<0.05 vs SHR*

In contrast, although AQP-4 expression in SHR was significantly greater than the WKY at the frontal cortex level, all the treatment restored it to normal levels (Figure 62A). Moreover, at the level of the frontal cortex, the expression of AQP-4 was much higher in the hypertensive group as compared to the normotensive animals. In a similar way, only the treatment group were effective to reduce its overexpression (Figure 62B).

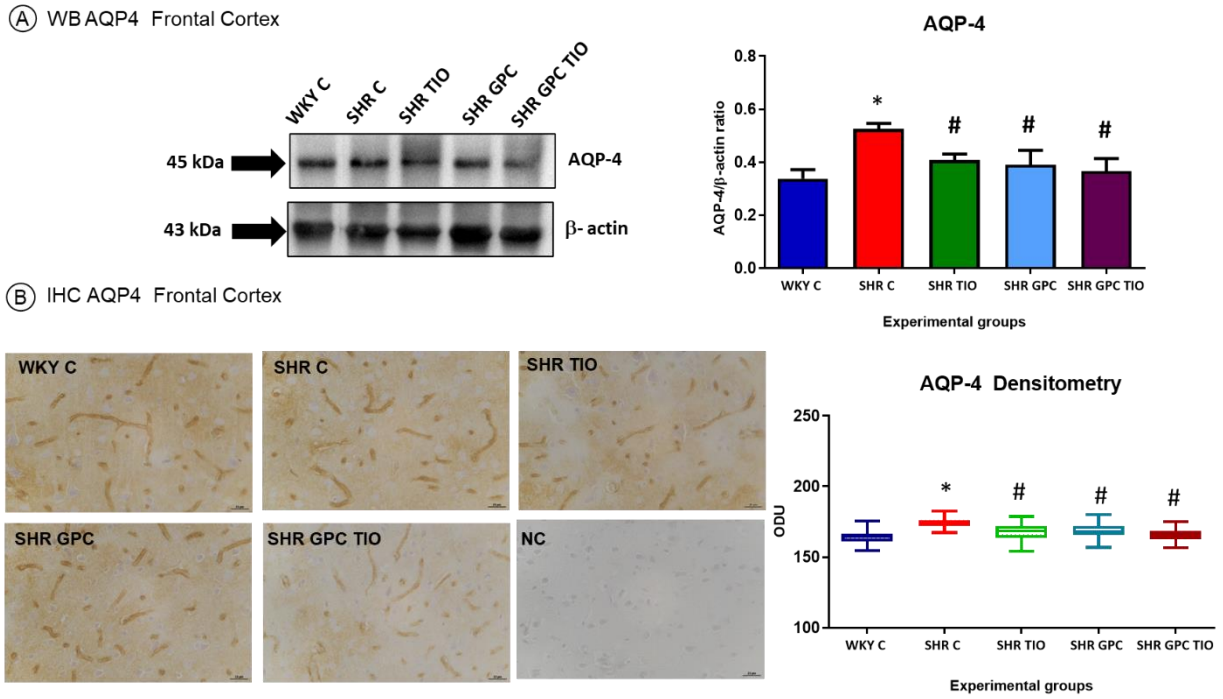

Figure 62. *Western blot analysis of the Aquaporin 4 (AQP-4) at the level of the Frontal Cortex (A) and the relative densitometric analysis express as ratio between protein and β actin as reference. Immunohistochemical analysis for AQP-4 in the region of the frontal cortex (B) in the different experimental groups. WKY C: Wistar Kyoto rats: SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate.; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Calibration bar: 25 μm. Data are the mean ± S.D.* *******= p<0.05 vs WKY; #=p<0.05 vs SHR*

Glucose transporter-1 (GLUT-1) Protein

Glucose is the principal fuel source for the brain, and the glucose transporter-1 (GLUT-1) is the only vehicle by which glucose enters the brain. Brain GLUT-1 interacts with a network of other specific GLUT-1 isoforms, mediating glucose transport into astrocytes and neurons. Moreover, specialized cerebral endothelial cells and their junctional complexes regulate the overall permeability of the BBB. Among the glucose transporter family, GLUT-1 is a unique molecule which is expressed in the cerebral endothelial cells. The junctional proteins and GLUT-1 are critical elements of the BBB. It has been suggested that GLUT-1 expression plays a significant role in the development of cerebral microvasculature with BBB properties.

The western blot findings demonstrate that GLUT-1 antibody developed a band at 54 KDa (Figure 63A and 64A). The GLUT-1/β-actin ratio of the densitometric values of bands in the hippocampus and frontal cortex defines that the expression of GLUT-1 was higher in the hypertensive animal compared to the normotensive group. Furthermore, (+) TIO and combination of GPC TIO treatment significantly reduces GLUT-1 expression in the hippocampus. Whereas the GPC didn't show either difference (Figure 63A). The IHC revealed immunoreaction at the level of the capillary in the different brain areas. In SHR the expression of GLUT-1 was significantly higher compared to the control WKY. In this case the expression of GLUT-1 was reduced by the same treatment group (Figure 63B).

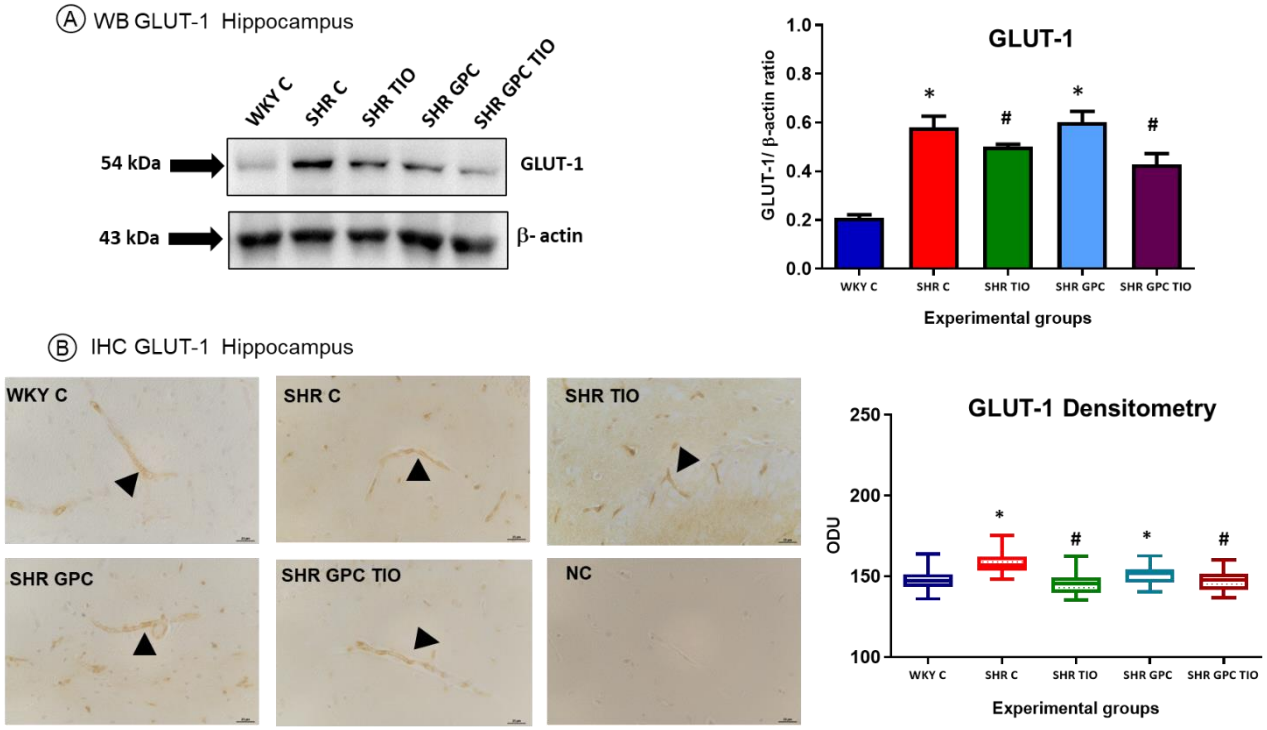

Figure 63. *Western blot analysis of the Glucose transporter Protein (GLUT-1) at the level of the Hippocampus (A) and the relative densitometric analysis express as ratio between protein and β actin as reference. Immunohistochemical analysis for GLUT-1 in the CA1 subfield of hippocampus (B) in the different experimental groups. WKY C: Wistar Kyoto rats; SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate.; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Calibration bar: 25 μm. Data are the mean ± S.D.* ******* *= p<0.05 vs WKY; #=p<0.05 vs SHR*

On the contrary, none of the treatments were as effective in reducing GLUT-1 elevated expression in the frontal cortex (Figure 64A). The IHC revealed the higher expression of GLUT-1in SHR group compared to the WKY. In a similar way (+) TIO and combination of GPC TIO treatment were significantly effective to reduce the GLUT-1 expression in frontal cortex (Figure 64B).

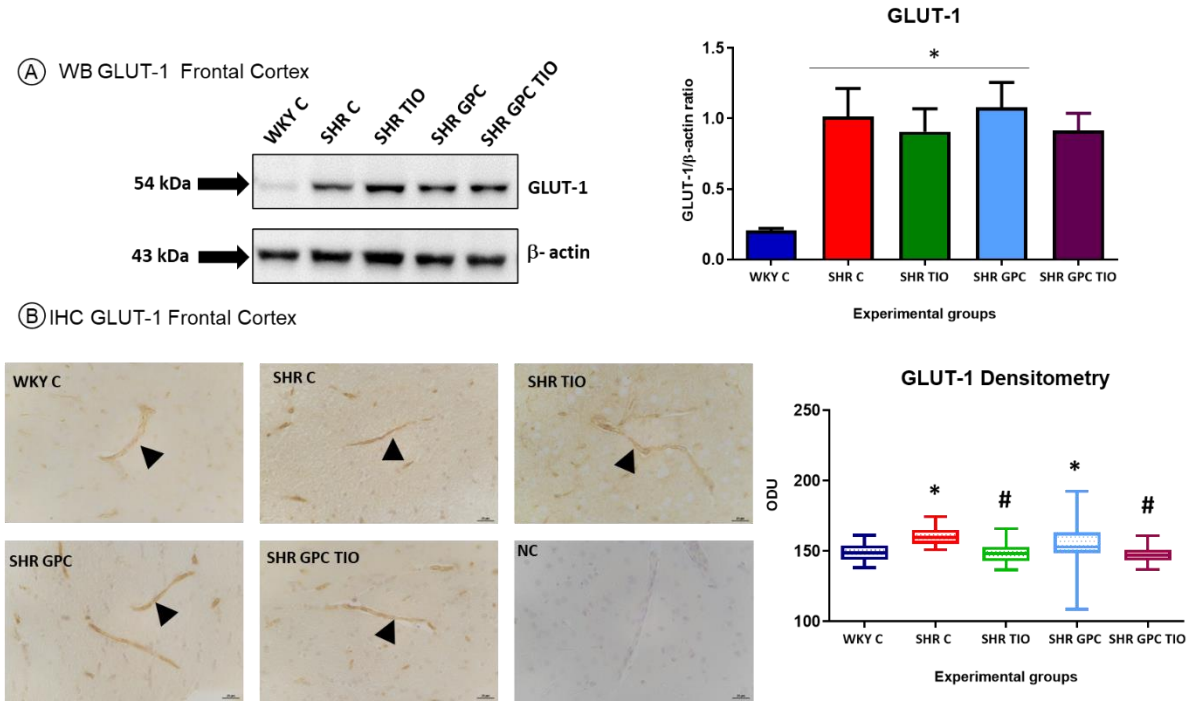

Figure 64. *Western blot analysis of the Glucose transporter Protein (GLUT-1) at the level of the Frontal Cortex (A) and the relative densitometric analysis express as ratio between protein and β actin as reference. Immunohistochemical analysis for Glucose transporter Protein (GLUT-1) in the region of the frontal cortex (B) in the different experimental groups. WKY C: Wistar Kyoto rats: SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate.; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Calibration bar: 25 μm. Data are the mean ± S.D.* *******= p<0.05 vs WKY; #=p<0.05 vs SHR*

Zonula occludens-1 (ZO-1)

Zonula occludens-1 (ZO-1) was the first tight junction protein to be cloned and is thought to serve as an important scaffold protein. There are multiple domains in this protein that bind a wide variety of junction proteins. A series of tight junction proteins in the blood-brain barrier. It is a 220 kDa

phosphoprotein found on the surface of the peripheral membrane. The protein is expressed in all epithelial and endothelial cells, as well as in cells lacking tight junctions.

ZO-1 and the reference protein β-actin developed bands at 220 KDa and 43 KDa, respectively (Figure 65A and B). Only in the frontal cortex of SHR rats were identified an increase expression of the protein (Figure 65 B).

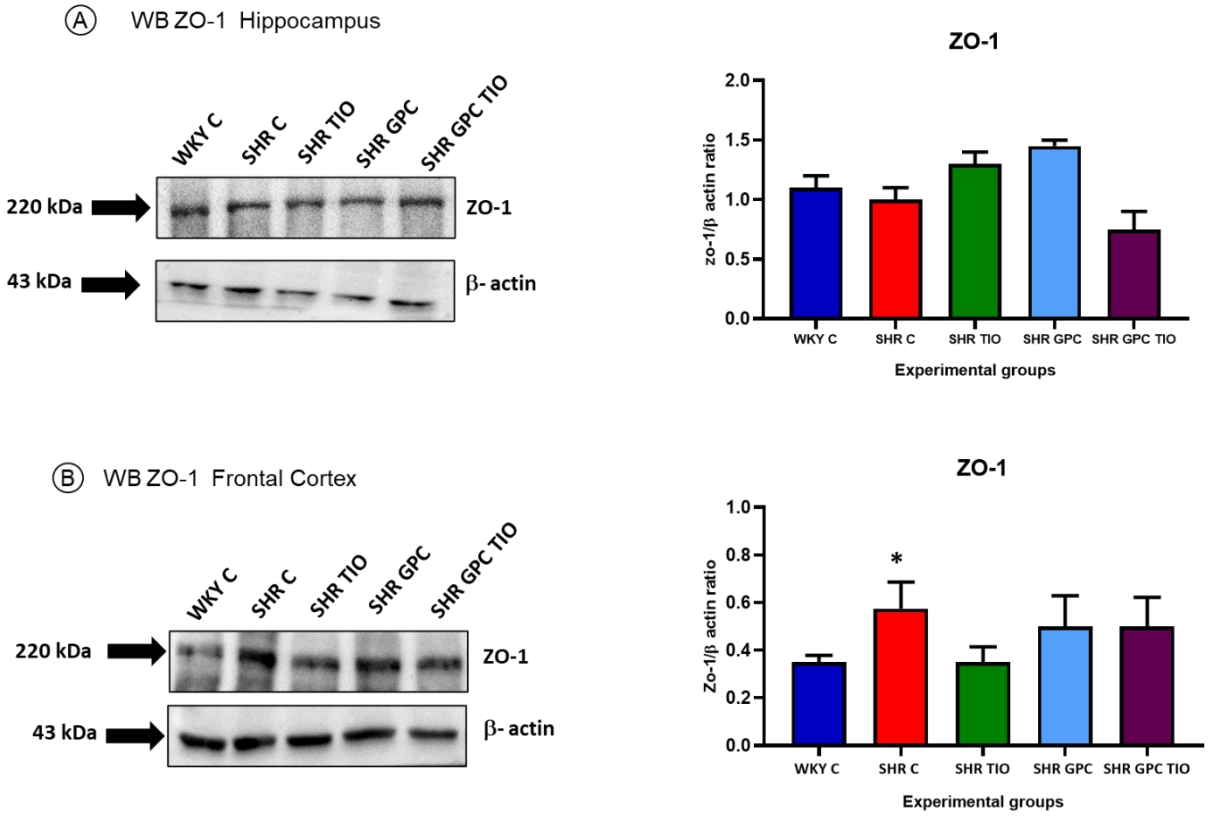

Figure 65. *Western blot analysis of the* Zonula occludens (ZO-1) *at the level of the Hippocampus(A) and Frontal Cortex (B) in the different experimental groups and the relative densitometric analysis express as ratio between protein and β actin as reference. WKY C: Wistar Kyoto rats; SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association Data are the mean ± S.D.* *******= p<0.05 vs WKY; #=p<0.05 vs SHR*

4.3.5 Cholinergic Markers

Vesicular acetyl choline transporter (VAChT)

Vesicular acethyl choline transporter (VAChT) mediates the transfer of ACh from the cytoplasm into synaptic vesicles and they uptakes the neurotransmitter into synaptic vesicles located in axon terminals and is released to the synaptic cleft upon depolarization in a process mediated by an elevation in intracellular Ca^{2+}. VAChT is an antiporter that couples ACh entry into the vesicle with proton efflux, the same mechanism used for the accumulation of other biogenic amine neurotransmitters.

The results of the western blot showed that SHR control and SHR GPC (+) TIO had significantly low expression of VAChT (approximately 80 KDa) compared to normotensive rats in hippocampus (Figure 66A). The treatment with the GPC alone restored the normal level. (Figure 66A). The IHC analysis revealed that the immunoreactivity VAChT was found to decrease in cell bodies of the V layer of frontal cortex on the SHR compared to the WKY rats. While all the treated group showed a positive intensity to improve it at normal level (Figure 66B).

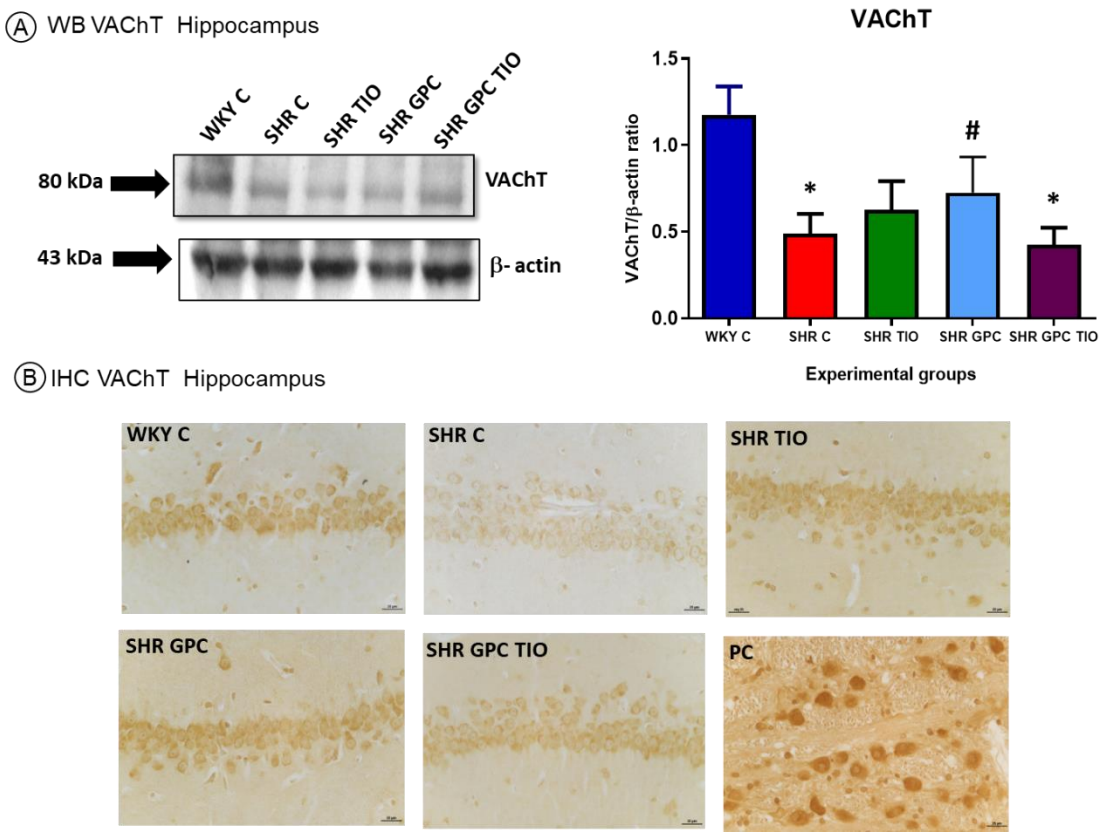

Figure 66. *Western blot analysis of the Vesicular acetylcholine transporter (VAChT)at the level of the Hippocampus (A) and the relative densitometric analysis express as ratio between protein and β actin as reference. Immunohistochemical analysis for Vesicular acetylcholine transporter (VAChT) in the CA1 subfield of hippocampus (B) in the different experimental groups. WKY C: Wistar Kyoto rats: SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate.; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Calibration bar: 25 μm. Data are the mean ± S.D.* ******* *= p<0.05 vs WKY; #=p<0.05 vs SHR*

The VAChT /β-actin densitometric analysis of bands values suggests that VAChT expression in the frontal cortex was considerably reduced in the hypertensive group compared to the normotensive control (Figure 67A). The IHC showed less expression of VAChT in the hypertensive rat compared to the normotensive rats. In contrast, the level of VAChT were restored with the treatment of only (+) TIO and GPC alone (Figure 67B).

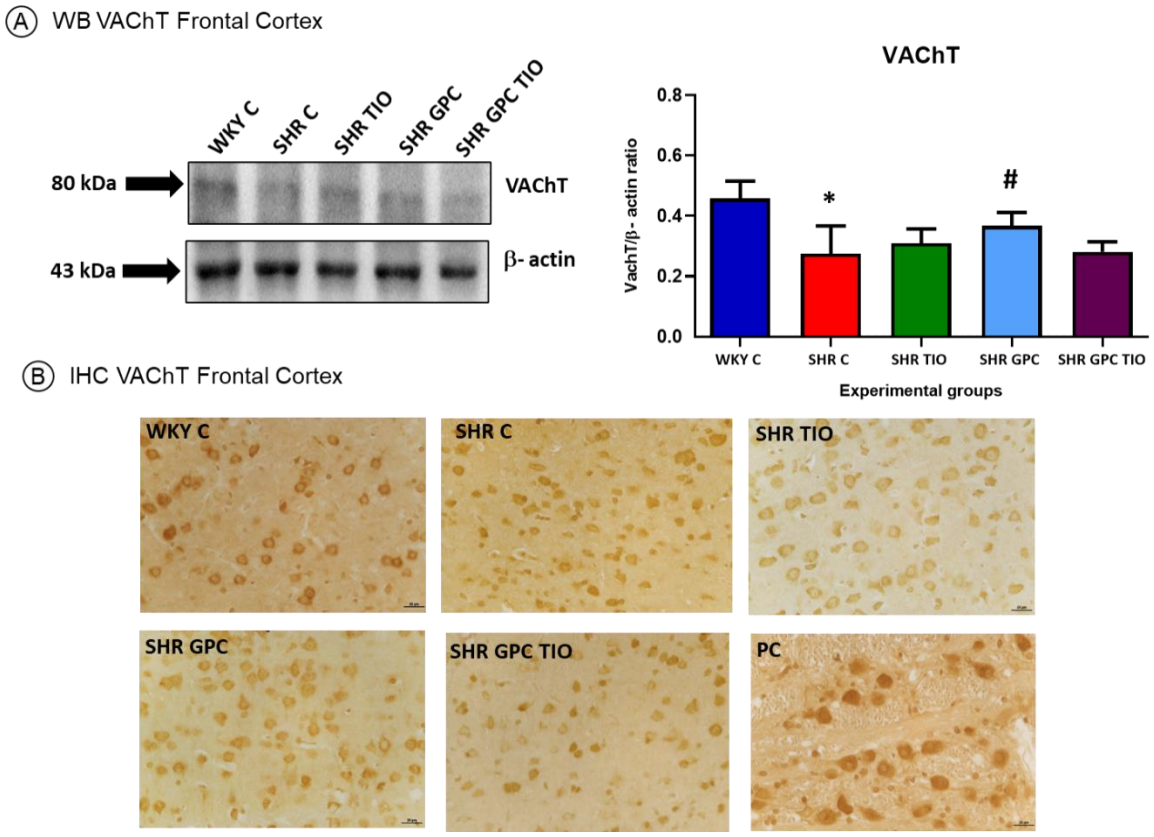

Figure 67. *Western blot analysis of the Vesicular acetylcholine transporter (VAChT)at the level of the Frontal cortex (A) and the relative densitometric analysis express as ratio between protein and β actin as reference. Immunohistochemical analysis for Vesicular acethyl choline transporter (VAChT) in the V layer of frontal cortex (B) in the different experimental groups. WKY C: Wistar Kyoto rats: SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate.; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Calibration bar: 25 μm. Data are the mean ± S.D.* ******* *= p<0.05 vs WKY; #=p<0.05 vs SHR*

Choline acetyltransferase (ChAT)

The Choline acetyltransferase (ChAT) enzyme catalyzes acetylcholine biosynthesis, which is responsible for signal transmission at the neuromuscular junction and, therefore, motor behavior as well as visceral stimulation underlying vegetative function in the autonomic nervous system through acetylcholine. ChAT and its biosynthetic product ACh are present at significant concentrations in normal plasma, so they may have a direct effect on blood pressure through their interaction with the endothelium. ACh decreases blood pressure by stimulating endothelium-dependent vasodilation in resistance arterioles through nitric oxide stimulation.

The western blot findings demonstrate that ChAT antibody developed a band at 68 KDa both in hippocampus and frontal cortex. (Figure 68A and 69A). The ChAT/β-actin ratio of the densitometric values of bands in the hippocampus and frontal cortex defines that the expression of ChAT was not indicating any difference in hypertensive animal compared to the normotensive group both in hippocampus and frontal cortex (Figure 68A and 69A).

Histochemical techniques show ChAT-immunoreaction on the hippocampus CA3 subfield. There were no differences evident in all the experimental groups (Figure 68B).

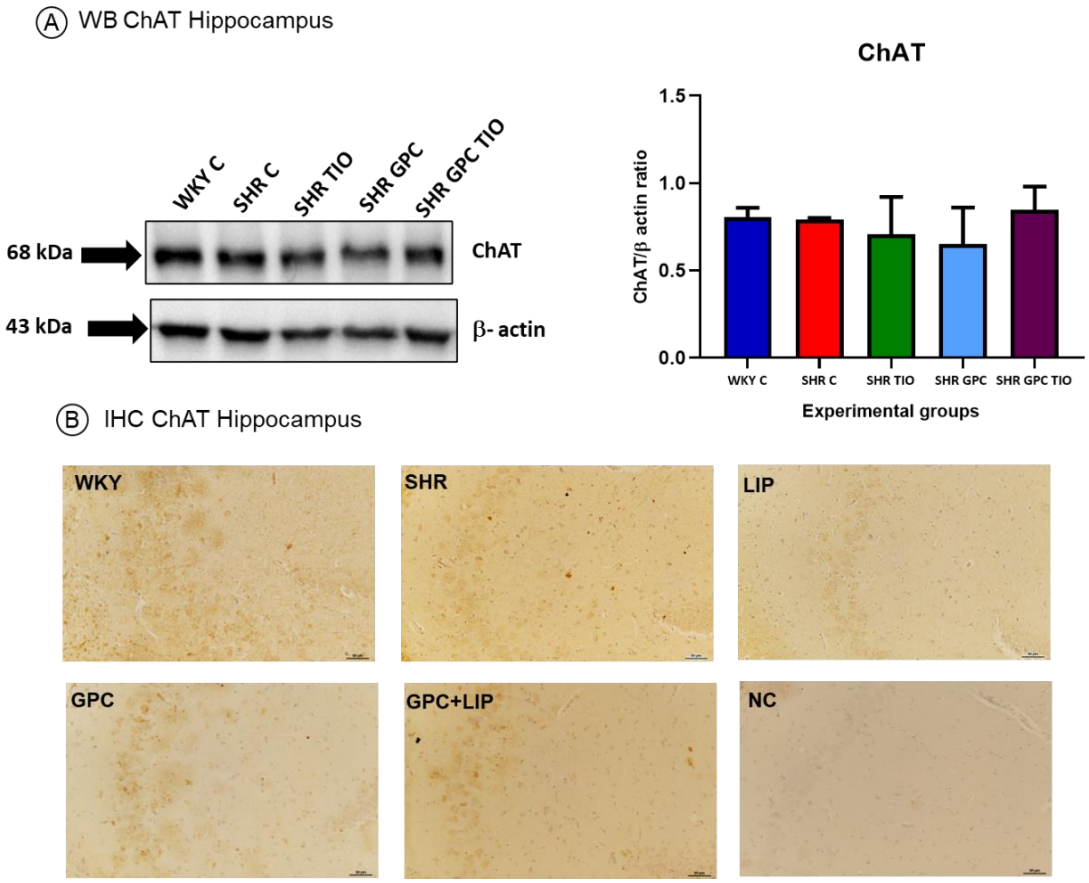

Figure 68. *Western blot analysis of the* Choline acetyltransferase (ChAT) *at the level of the Hippocampus(A) in the different experimental groups and the relative densitometric analysis express as ratio between protein and β actin as reference. WKY C: Wistar Kyoto rats: SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate.; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Calibration bar: 50 μm. Data are the mean ± S.D.* *******= p<0.05 vs WKY; #=p<0.05 vs SHR*

In the frontal cortex the IHC showed less expression of ChAT in the hypertensive rat compared to the normotensive rats. In contrast, the level of ChAT was restored with all the treatment group. (Figure 69B).

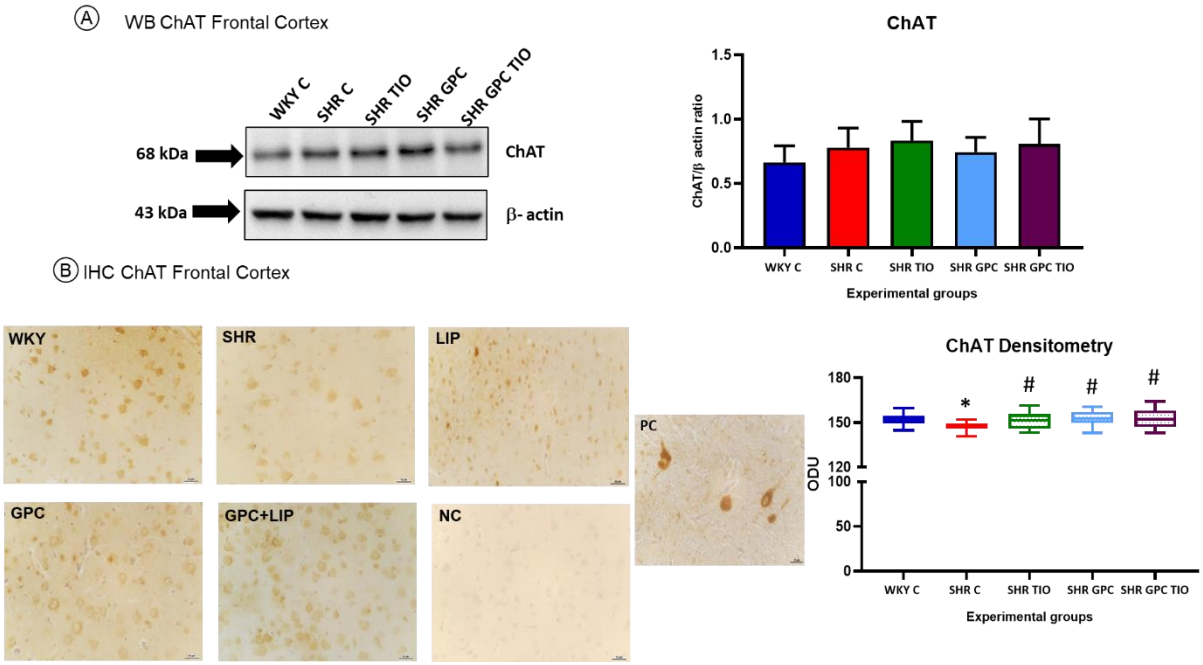

Figure 69. *Western blot analysis of the* Choline acetyltransferase (ChAT) *at the level of the Frontal Cortex (A) and the relative densitometric analysis express as ratio between protein and β actin as reference. Immunohistochemical analysis for* Choline acetyltransferase (ChAT)*) in the V layer of frontal cortex (B) in the different experimental groups. WKY C: Wistar Kyoto rats: SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate.; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Calibration bar: 25 μm. Data are the mean ± S.D.* *******= p<0.05 vs WKY; #=p<0.05 vs SHR*

Acetylcholinesterase (AChE)

The primary role of AChE is to terminate neuronal transmission and signaling between synapses to prevent ACh dispersal and activation of nearby receptors. The interaction of AChE with the substrate ACh results in the breakdown, hydrolysis, and inactivation of ACh and subsequent control of the amount of ACh at the synapse.

The results of the western blot showed a band of AChE at approximately 70 KDa both in the hippocampus and frontal cortex. (Figures 70A and 71B). No alterations in the protein expression were highlighted in the different experimental groups both in hippocampus and frontal cortex (Figures 70 and 71).

In hippocampus, IHC results showed a decrease expression of the AChE in SHR rats compared to the WKY rats whereas our treatment with GPC alone was able to improve its level (Figure 70B). In the case of frontal cortex there were no significant differences were found (Figure71B).

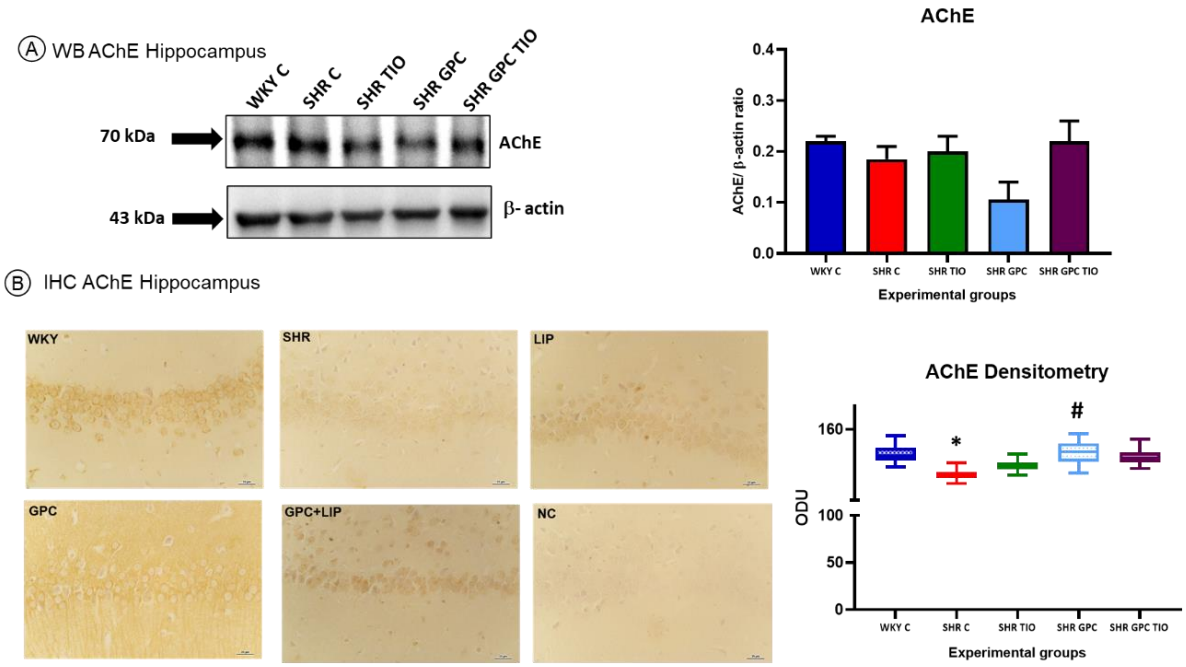

Figure 70. *Western blot analysis of the Acetylcholinesterase (AChE) at the level of the hippocampus (A) and the relative densitometric analysis express as ratio between protein and β actin as reference. Immunohistochemical analysis for acetylcholinesterase (AChE) in the CA1 subfield of hippocampus (B) in the different experimental groups. WKY C: Wistar Kyoto rats; SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Calibration bar: 25 μm. Data are the mean ± S.D.* ******* *= p<0.05 vs WKY; #=p<0.05 vs SHR*

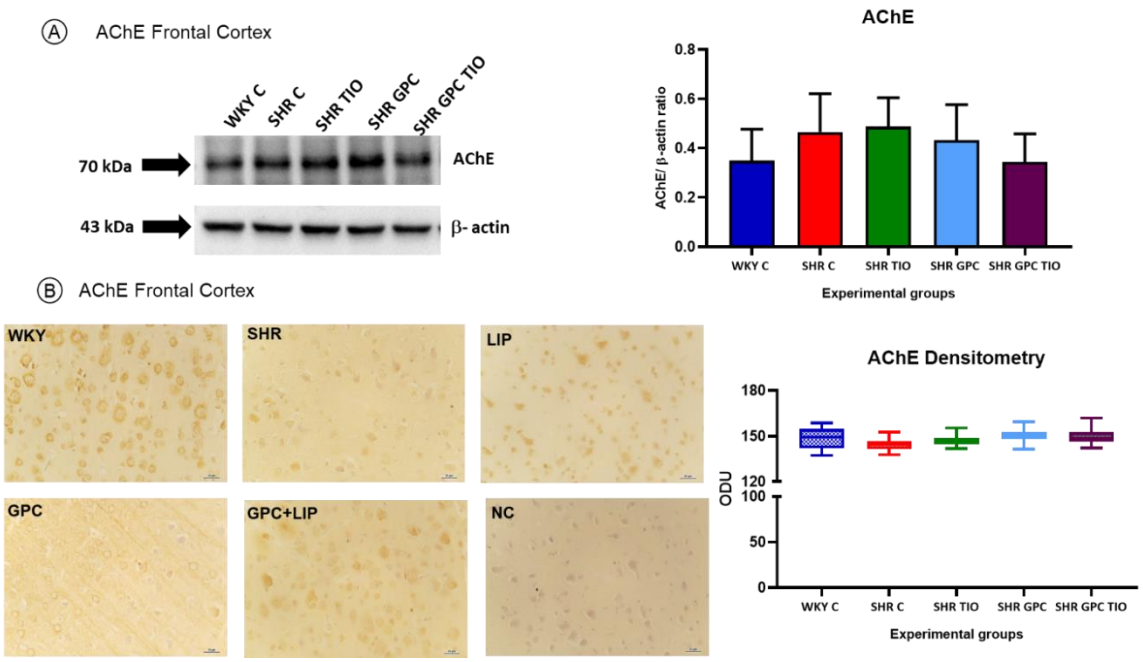

Figure 71. *Western blot analysis of the Acetylcholinesterase (AChE)at the level of the Frontal Cortex (A) and the relative densitometric analysis express as ratio between protein and β actin as reference. Immunohistochemical analysis for acetylcholinesterase (AChE)in the V layer of frontal cortex (B) in the different experimental groups. WKY C: Wistar Kyoto rats: SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate.; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Calibration bar: 25 μm. Data are the mean ± S.D.* *******= p<0.05 vs WKY; #=p<0.05 vs SHR*

Nicotinic acetylcholine receptor Alpha7 (nAChR α7)

Nicotinic acetylcholine receptor alpha7 (nAChRα7) is an important part of the cholinergic nerve system in the brain. Moreover, it is associated with a cholinergic anti-inflammatory pathway in the termination of the parasympathetic nervous system. The nAChRα7 is implicated in cognitive functions of the central nervous system. Activation of nAChRα7 attenuates neuroinflammation and oxidative stress and reduces brain injury. nAChRs have been shown to regulate inflammation, in particular via the nAChRα7 activation in macrophages which regulates the "cholinergic anti-inflammatory pathway". The transcripts for the nAChR subunits α7, α3, α5, as well as β4 have been detected in inflammatory cell types, including macrophages and microglia.

The results of western blot showed a band approximately at 55 kDa for nAChRα7 protein, both in the frontal cortex and in the hippocampus (Figure 72A and 73A). A reduction of protein expression was evident in the hippocampus of SHR compared to the WKY (Figure 72A), whereas no

alterations were evident in the frontal cortex in the different experimental groups (Figure 73A). In hippocampus an increase expression was evident in the GPC alone or in association with (+) TIO treated groups compared to the control SHR (Figure 72A).

A positive immunostaining for the nicotinic receptor nAChRα7 was observed in the CA1 and CA3 subfields of the hippocampus and the neurons of the fifth layer of the frontal cortex (Figure 72B and 73B). The expression of nAChRα7 was lower in the hippocampus of the SHR rats compared with the WKY rats. A significant increase in the expression of the nAChRα7 was evident with both the treatment of GPC alone as well as the combination of GPC and (+) TIO (Figure 72B). On the other hand, there were no significant changes in the frontal cortex in the different experimental groups (Figure 73B).

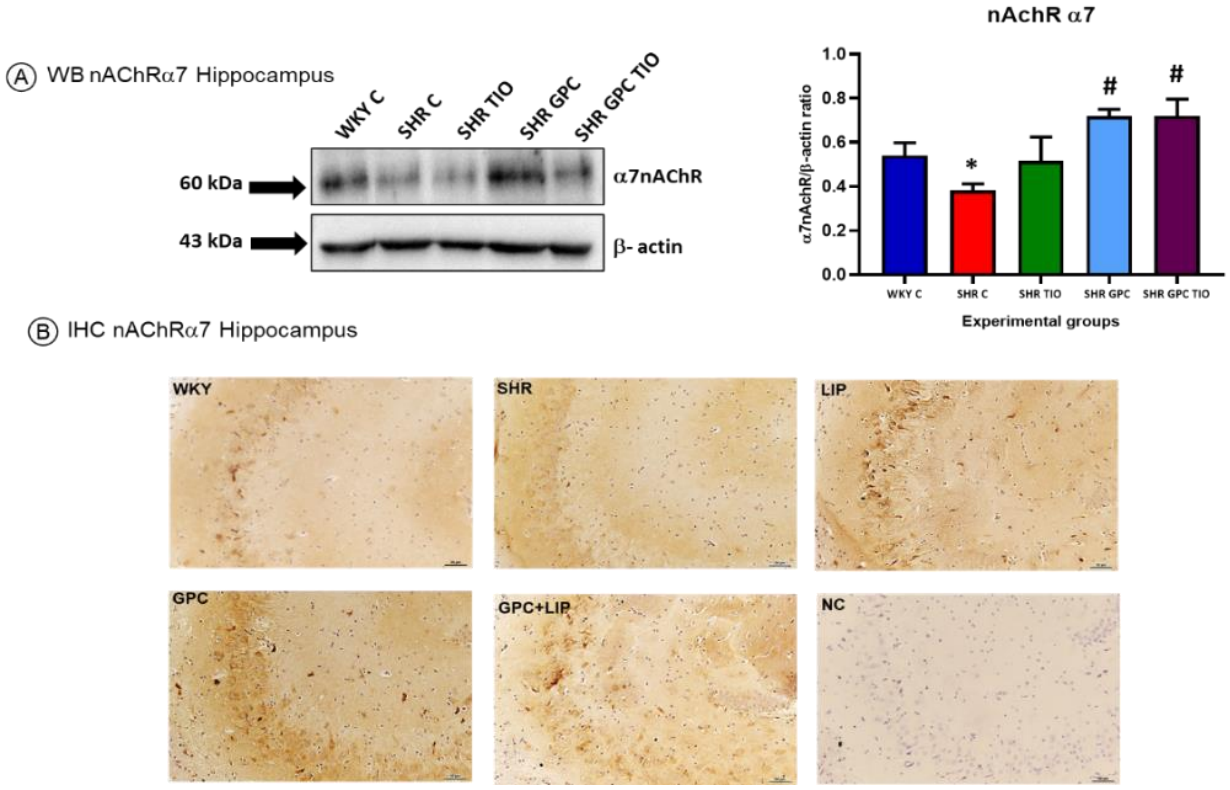

Figure 72. *Western blot analysis of the* Alpha7 nicotinic acetylcholine receptor (α7 nAChR) *at the level of the Hippocampus(A) and the relative densitometric analysis express as ratio between protein and β actin as reference. Immunohistochemical analysis for acetylcholinesterase (*α7 nAChR*) in the CA3 subfield of hippocampus (B) in the different experimental groups. WKY C: Wistar Kyoto rats; SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Calibration bar: 50 μm. Data are the mean ± S.D.* *******= p<0.05 vs WKY; #=p<0.05 vs SHR*

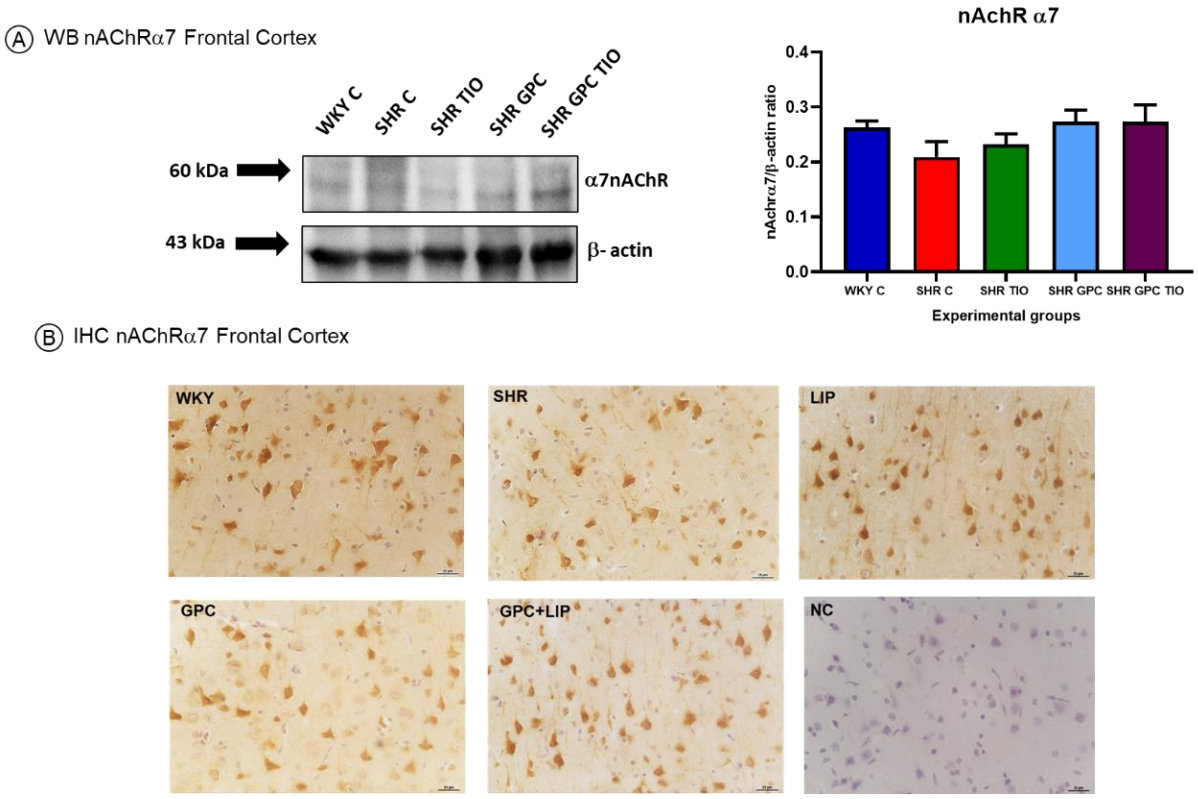

Figure 73. *Western blot analysis of the* Alpha7 nicotinic acetylcholine receptor (α7 nAChR) *at the level of the Frontal Cortex (A) and the relative densitometric analysis express as ratio between protein and β actin as reference. Immunohistochemical analysis for acetylcholinesterase (*α7 nAChR*) in the V layer of the frontal cortex (B) in the different experimental groups. WKY C: Wistar Kyoto rats; SHR C: Spontaneously hypertensive rats; SHR TIO: Spontaneously hypertensive rats treated with (+) thioctic acid; SHR GPC: Spontaneously hypertensive rats treated with choline alphoscerate; SHR GPC TIO: Spontaneously hypertensive rats treated with choline alphoscerate and (+) thioctic acid in association. Calibration bar: 25 μm. Data are the mean ± S.D.* ******* *= p<0.05 vs WKY; #=p<0.05 vs SHR.*

4.4 Results on BV-2 microglia cells experiment.

4.4.1 Lipopolysaccharide (LPS) induced morphological changes.

The normal morphology of BV-2 microglial cells cultured in normal medium for 24 h. Morphological changes of BV-2 microglia, with a reduction of filaments arborization and a more ameboide shape, were observed under 10 μg/ml LPS stimulation for 24 h (right). Arrows indicate typical control (Figure 74).

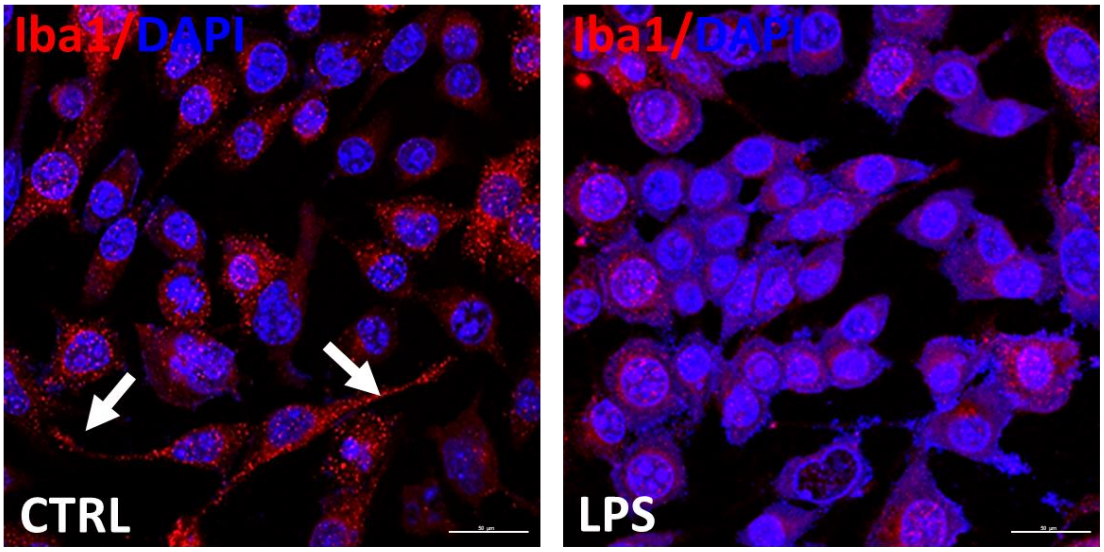

Figure.74. *Immunohistochemical analysis for Calcium Binding Adaptor Molecule 1 (IBA1) in BV-2 microglial cells treated at 10μg/ml of the LPS and in normal medium (CTRL) for 24 h. Anti rabbit Alexa Fluor® 594 conjugated was used as secondary antibodies. Nuclei were counterstained with DAPI. Calibration bar: 50 μm.*

4.4.2 IL-1beta expression in different LPS dose.

IL-1β is produced as an IL-1β precursor (pro-IL-1β) by the activated macrophages and its cleavage is caused by caspase-1, which is regulated by the inflammasomes. The protein expression and the immunofluorescence staining of IL-1β significantly increased up to 30 μg/ml LPS. Thus, BV-2 cells were exposed to 10 μg/mL of LPS as a minimal quantity able to activate the BV-2 cells (Figure 75).

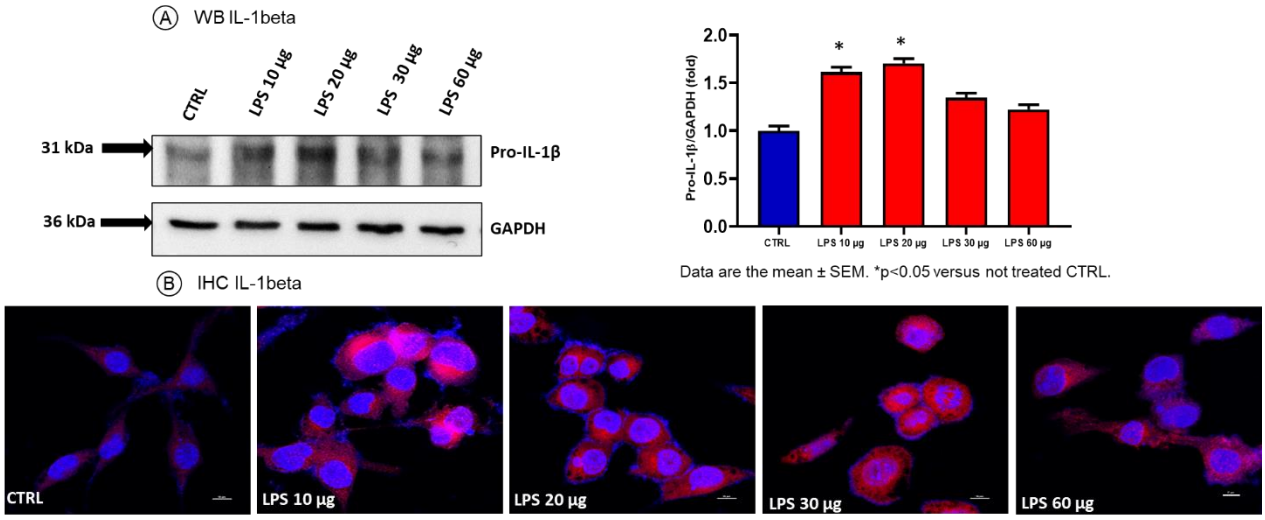

Figure.75. *(A)Western blot analysis of Interleukin-1 beta (IL-1β) in BV-2 microglial cells treated with different concentration (10,20,30,60 µg/ml) of the lipopolysaccharide (LPS). The graph shows the ratio of densitometric analysis of band and GAPDH used to normalize the data, taking not treated cells (CTRL) as reference group. (B) Immunohistochemical analysis for IL-1β in BV-2 microglial cells treated at different concentration (10,20,30,60µg/ml) of the LPS. Anti rabbit Alexa Fluor® 594 conjugated was used as secondary antibodies. Nuclei were counterstained with DAPI. Calibration bar: 10 µm. Data are the mean ± S.D. p<0.05 vs CTRL.*

4.4.3 Cytotoxicity assay: Normal condition

An MTT assay was performed 24 h after treatment with various concentrations (50,100,200 mM) of TIO and (10, 20, 40 mM) of GPC in the absence of LPS. Treatments with different concentrations of (+) TIO and GPC revealed no cytotoxic effects in the MTT assay (Figure 76).

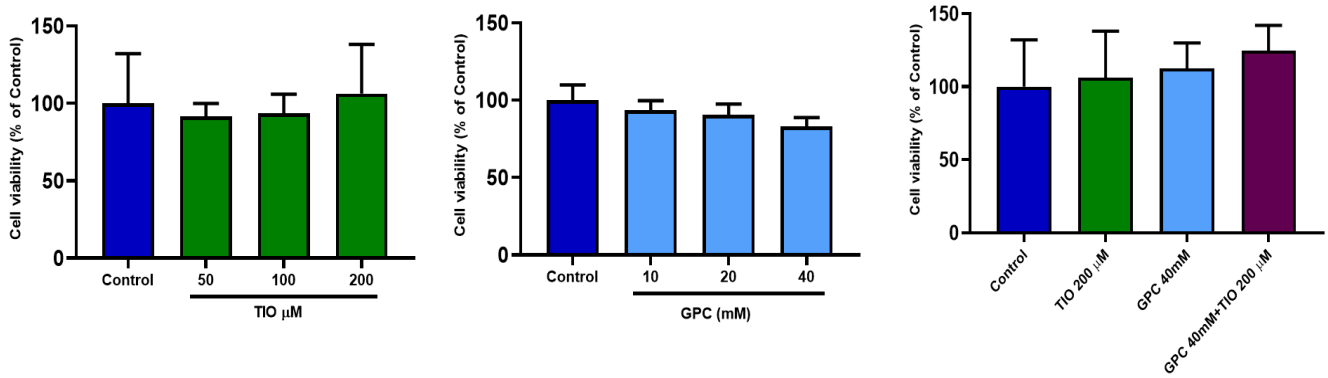

Figure.76. *Data are expressed as the percentage of surviving cells compared with control cells. The data were expressed as the mean ± S.D. CTRL: Control; LPS: Lipopolysaccharide; GPC: choline alphoscerate; TIO: (+)*

thioctic acid; GPCTIO: choline alphoscerate and (+) thioctic acid in association. Data are the mean ± S.D. ***= p<0.05** *vs CTRL; #=p<0.05 vs LPS*

4.4.4 Cytotoxicity assay: LPS condition

An MTT assay was performed 24 h after treatment with various concentrations (50,100,200 mM) of (+) TIO and (10, 20, 40 mM) of GPC in the presence of LPS (10 µg/ml). Treatments with different concentrations of (+) TIO and GPC in the presence of LPS also revealed no cytotoxic effects in the MTT assay (Figure 77).

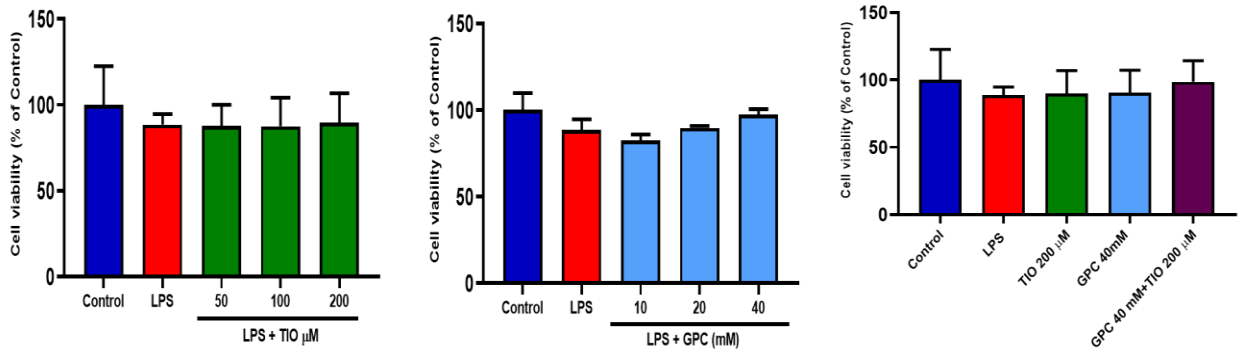

Figure.77. *Data are expressed as the percentage of surviving cells compared with control cells. The data were expressed as the mean ± S.D. CTRL: Control; LPS: Lipopolysaccharide; GPC: choline alphoscerate; TIO: (+) thioctic acid; GPCTIO: choline alphoscerate and (+) thioctic acid in association. Data are the mean ± S.D.* ***= p<0.05** *vs CTRL; #=p<0.05 vs LPS*

4.4.5 IL-1 β expression in Normal and LPS activated condition.

Western blot was performed 24h after treatment with various concentrations of GPC and CDP-choline in the absence of LPS. Predictably, no difference was found in normal condition in pro-IL-1β expression with the (+) TIO and cholinergic precursor compared to control (Figure 78A). Representative immunofluorescence images also there were no changes among the group compared with the control (Figure 78B).

After the LPS induced activation, western blot performed after treatment with high doses (40 mM) the cholinergic precursors showed a significant reduction of pro-IL-1β expression in BV-2 cells treated with LPS alone. Other treated group did not show any changes (Figure 79A). Representative immunofluorescence images showed an intense immunostaining for pro-IL-1β in LPS-stimulated BV-2 cells. However, the treatments with high doses (40 mM) of GPC reversed these phenomena (Figure 79B).

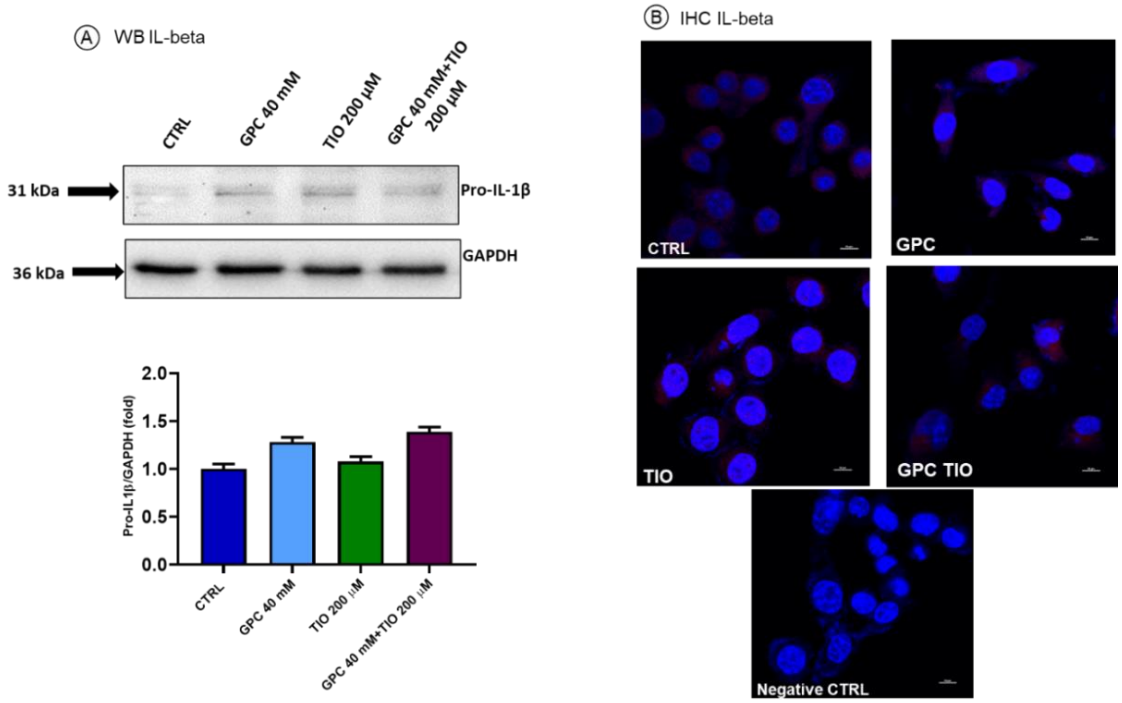

Figure 78. *(A) Western blot analysis of Interleukin 1 beta (Il-1β) in the normal BV-2 microglial cells and the graph shows the ratio of densitometric analysis of band and GAPDH used to normalize the data, taking not treated cells (CTRL) as reference group. (B). Immunohistochemical analysis for Il-1β in the BV-2 microglial cells. Anti rabbit Alexa Fluor® 594 conjugated was used as secondary antibodies. Nuclei were counterstained with DAPI. p<0.05 vs CTRL. (Alexa Fluor® 594 dye in red) and subsequent counterstained with DAPI (blue color) as nuclear dye CTRL: Control; LPS: Lipopolysaccharide; GPC: choline alphoscerate; TIO: (+) thioctic acid; GPCTIO: choline alphoscerate and (+) thioctic acid in association. Calibration bar: 10 µm. Data are the mean ± S.D.* ******* *= p<0.05 vs CTRL; #=p<0.05 vs LPS.*

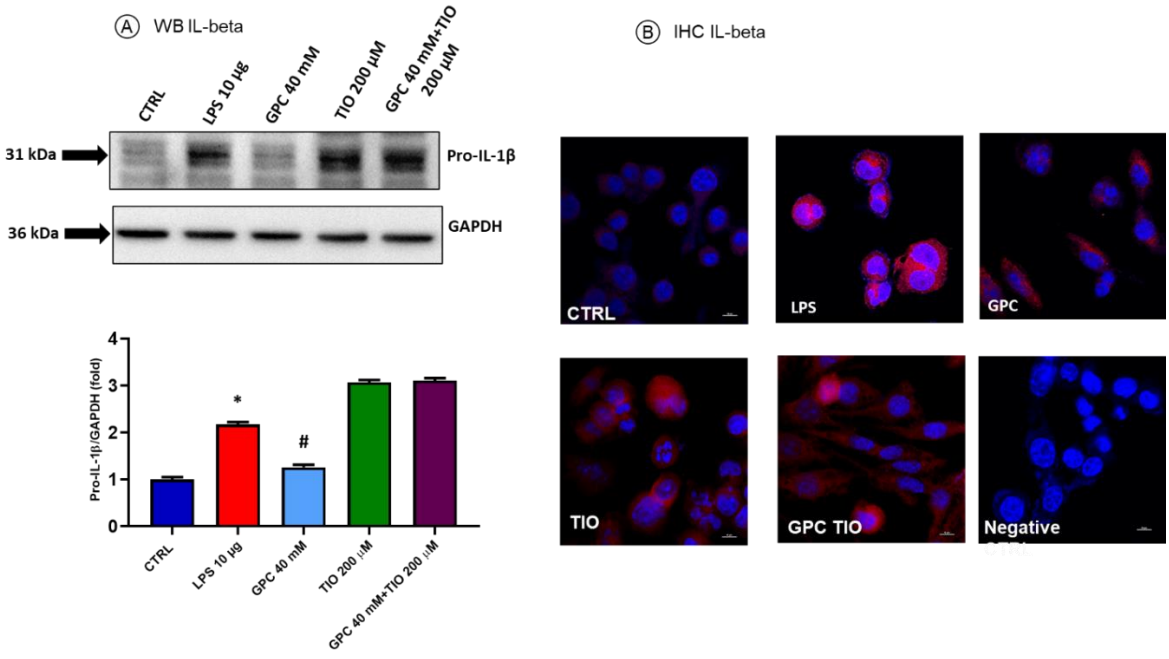

Figure 79. *(A) Western blot analysis of Interleukin 1 beta (Il-1β) in the Lipopolysaccharides (LPS) induced BV-2 microglial cells and the graph shows the ratio of densitometric analysis of band and GAPDH used to normalize the data, taking not treated cells (CTRL) as reference group. (B). Immunohistochemical analysis for Il-1β in the BV-2 microglial cells. Anti rabbit Alexa Fluor® 594 conjugated was used as secondary antibodies. Nuclei were counterstained with DAPI. p<0.05 vs CTRL. CTRL: Control; LPS: Lipopolysaccharide; GPC: choline alphoscerate; TIO: (+) thioctic acid; GPCTIO: choline alphoscerate and (+) thioctic acid in association. Calibration bar: 10 µm. Data are the mean ± S.D.* ******* *= p<0.05 vs CTRL; #=p<0.05 vs LPS.*

4.4.6 Nuclear factor kappa B (NF-κB) (p50) expression in LPS condition

Immunoblot showed a significant up-regulation of NF-κB (p50) in LPS-stimulated BV-2 cells. However, treatment with high doses (40 mM) of cholinergic precursor restored this modulation (Figure.80A). Representative immunofluorescence images showed an intense immunostaining for NF-κB (p50) and a nuclear translocation of NF-κB in LPS-stimulated BV-2 cells. However, the treatments with high doses (40 mM) of GPC and 200 µM of (+) TIO reversed both these phenomena (Figure 80B).

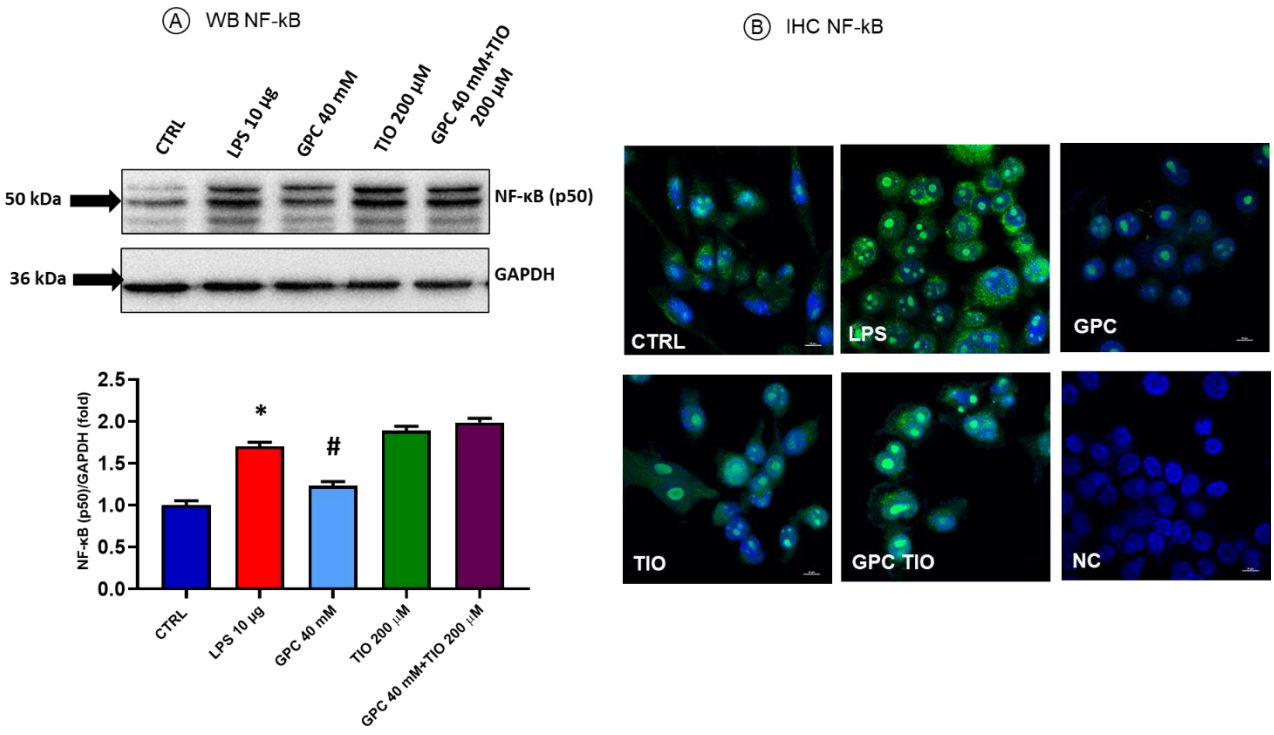

Figure 80. *(A) Western blot analysis of nuclear factor kappa B (NF-κB) in the Lipopolysaccharides (LPS) induced BV-2 microglial cells and the graph shows the ratio of densitometric analysis of band and GAPDH used to normalize the data, taking not treated cells (CTRL) as reference group. (B). Immunohistochemical analysis for NF-κB in the BV-2 microglial cells. Anti rabbit Alexa Fluor® 488 conjugated was used as secondary antibodies. Nuclei were counterstained with DAPI. p<0.05 vs CTRL. CTRL: Control; LPS: Lipopolysaccharide; GPC: choline alphoscerate; TIO: (+) thioctic acid; GPCTIO: choline alphoscerate and (+) thioctic acid in association. Data are the mean ± S.D. Calibration bar: 10 µm.* *******= p<0.05 vs CTRL; #=p<0.05 vs LPS.*

4.4.7 Ionized Calcium Binding Adaptor Molecule 1 (IBA1) expression in LPS condition

Immunoblot showed a significant up-regulation of IBA1 in LPS-stimulated BV-2 cells. However, treatment with high doses (40 mM) of cholinergic precursors restored this modulation (Figure.81A). Representative immunofluorescence images showed an intense immunostaining for IBA1 in LPS-stimulated BV-2 cells. However, the treatments with high doses (40 mM) of GPC and 200 µM (+)TIO reduced the fluorescence intensity (Figure 81 B).

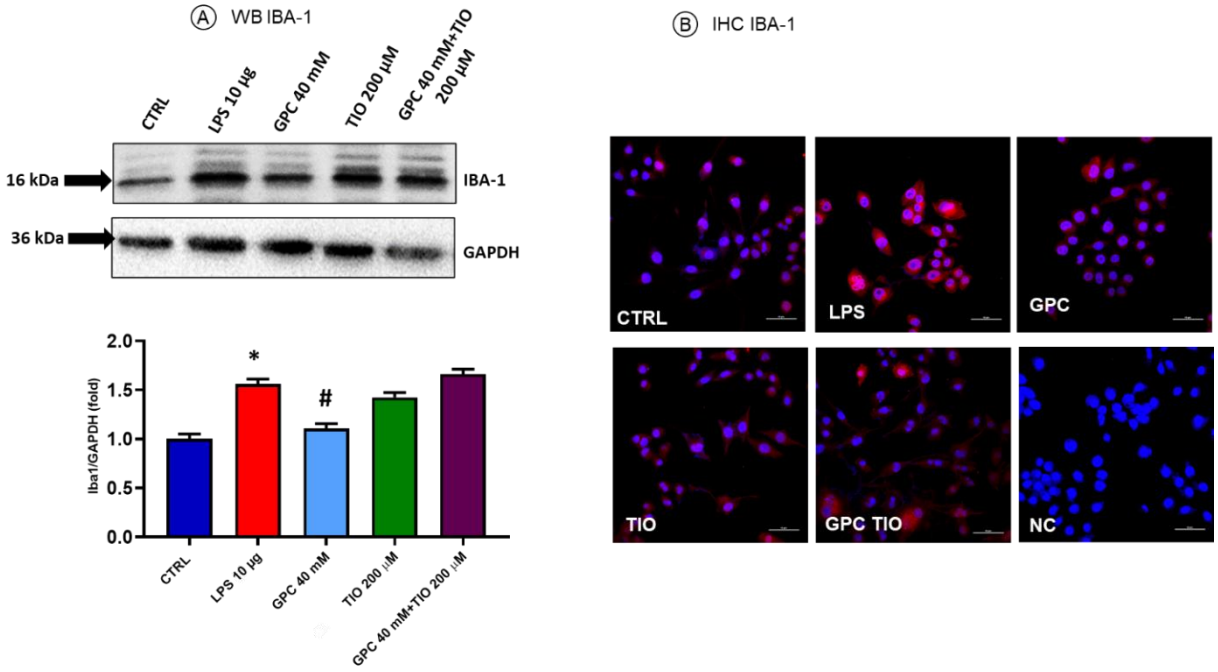

Figure 81. *(A) Western blot analysis of Ionized Calcium Binding Adaptor Molecule 1 (IBA1) in the Lipopolysaccharides (LPS) induced BV-2 microglial cells and the graph shows the ratio of densitometric analysis of band and GAPDH used to normalize the data, taking not treated cells (CTRL) as reference group. (B). Immunohistochemical analysis for IBA1 in the BV-2 microglial cells. Anti rabbit Alexa Fluor® 594 conjugated was used as secondary antibodies. Nuclei were counterstained with DAPI. $p<0.05$ vs CTRL. CTRL: Control; LPS: Lipopolysaccharide; GPC: choline alphoscerate; TIO: (+) thioctic acid; GPCTIO: choline alphoscerate and (+) thioctic acid in association. Calibration bar: 50 µm. Data are the mean ± S.D.* ******* *= $p<0.05$ vs CTRL; #=$p<0.05$ vs LPS.*

4.4.8 Oxidative stress (Oxyblot) and lipid peroxidation(4-HNE) in LPS condition

Western blot data showed an increase of oxidative state proteins in LPS-treated BV-2 cells. These alterations were reversed after the treatments with both GPC meanwhile (+) TIO is also actively reduced but not significantly (Figure 82). In case of 4-HNE western blot data showed in LPS-treated BV-2 cells an increase of 4-HNE, a well-known marker for lipid peroxidation These alterations were reversed after the treatments with both GPC but not significantly (Figure 83).

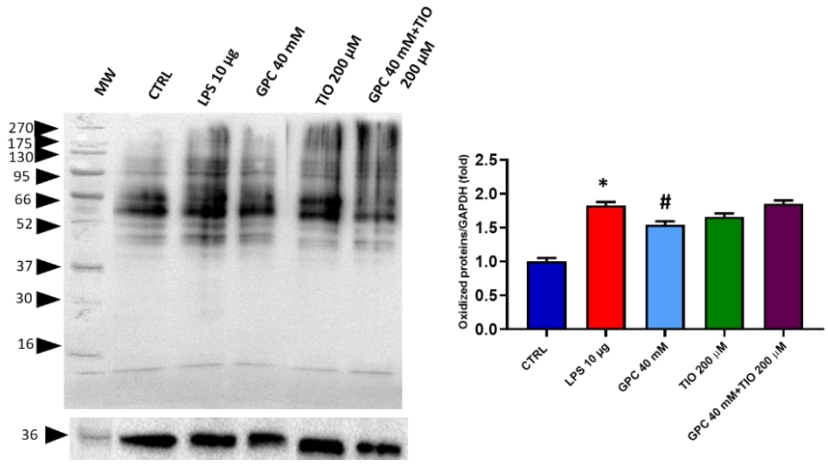

Figure 82. *Oxyblot analysis in the Lipopolysaccharides (LPS) induced BV-2 microglial cells and the relative densitometric analysis express as ratio between protein and GAPDH as reference. CTRL: Control; LPS: Lipopolysaccharide; GPC: choline alphoscerate; TIO: (+) thioctic acid; GPC TIO: choline alphoscerate and (+) thioctic acid in association. Data are the mean ± S.D.* *******= p<0.05 vs CTRL; #=p<0.05 vs LPS.*

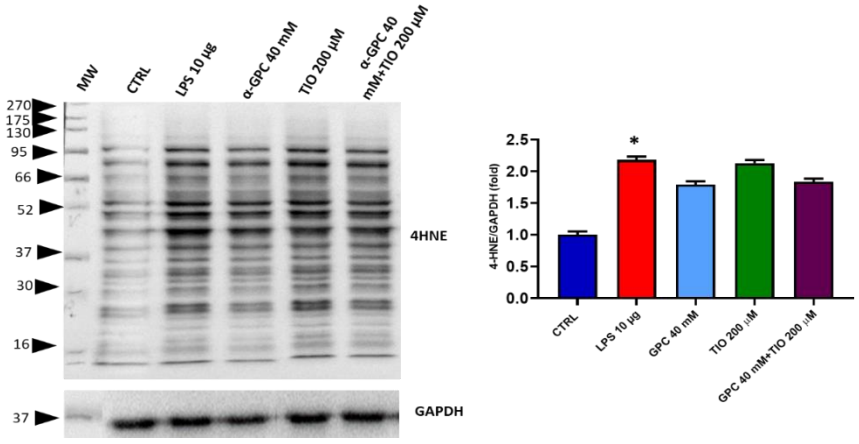

Figure 83. *Lipid peroxidation 4HNE analysis in the Lipopolysaccharides (LPS) induced BV-2 microglial cells and the relative densitometric analysis express as ratio between protein and GAPDH as reference. CTRL: Control; LPS: Lipopolysaccharid; GPC: choline alphoscerate; TIO: (+) thioctic acid; GPC TIO: choline alphoscerate and (+) thioctic acid in association. Data are the mean ± S.D.* *******= p<0.05 vs CTRL; #=p<0.05 vs LPS.*

5. DISCUSSION

The brain is one of the major targets of hypertension's mediated deleterious effects and accounts for a significant portion of the related mortality and morbidity [Dahlof, 2007]. Arterial hypertension significantly contributes to cerebrovascular and parenchymal brain alterations, thereby increasing the risk of cerebral hypoperfusion and ischemia and impairing oxygenation of the brain [Amenta et al., 2003]. Different studies in neurovascular regulation and hypertension pathophysiology have facilitated a better understanding of how hypertension impaired cerebral blood supply. There are several pathological cerebrovascular changes associated with hypertension, including arterial remodeling, impaired cerebral autoregulation, cerebral microbleeds, WML, lacunar infarctions, and Alzheimer-like changes, such as amyloid angiopathy and cerebral atrophy [Sierra et al., 2006]. Additionally, abnormally high BP stresses the blood vessel wall, causing thickening and the development of regressive changes. The result may be cerebral hemorrhage or infarction and subsequent brain damage.

Arterial hypertension is considered one of the leading risk factors for cerebrovascular disease. Cerebrovascular diseases play a vital role in the pathophysiology of cognitive impairment known as VaD. In VaD, the underlying cause may be cerebral small vessel disease (CSVD), leading to extensive leucoencephalopathy because of lacunes occurring in large vessels or infarcts in strategic locations. In addition, it may also result from an intracerebral hemorrhage, global cerebral hypoperfusion, or other causes, such as vasculitis [Amenta et al., 2003; Caruso et al., 2019]. Studies also find that histological changes associated with leukoaraiosis include localized oedema, damage to axons, oligodendrocytes, and activation of astrocytes and microglia. Additionally, baseline cerebral blood flow and autoregulation are decreased in CSVD and several lines of research point to endothelial dysfunction as being a key factor. It has been proposed that this could result in damage due to hypoperfusion and/or increased BBB permeability [Hainsworth and Markus., 2008].

The cholinergic system of the forebrain base plays an important role both in the elaboration of attention, memory and behavior, that in the regulation of cerebral blood flow [Everitt and Robins et al., 1999]. The cholinergic structures of the forebrain base, as well as numerous brain areas involved in cognitive activities, are particularly sensitive to ischemia. This consideration would

explain the strong cholinergic deficit that is observed in the associated neurodegenerative and vascular forms of dementia [Everitt and Robins et al., 1999; Roman et al., 2003].

It has been established that essential hypertension results in an alteration of the BBB. Histological studies on hypertensive individuals with CSVD observed arterial wall thickening, collagen deposition, degeneration of smooth muscle cells, and lumen narrowing, which are frequently associated with localized BBB impairment. The integrity of the BBB is important for central autonomic control, which may be involved in the pathophysiology of hypertension. Conversely, evidence from experimental research indicates that BBB disruption can be present in both hypertensive disease and dementia syndromes, implying that loss of BBB integrity may play a significant role in the pathophysiological pathways linking arterial hypertension with cognitive decline [Hainsworth and Markus., 2008; Katsi et al., 2020]. Endothelial dysfunction has been linked to the pathogenesis of various forms of cardiovascular disease, including hypertension. It is characterized by a shift in endothelial actions toward decreased vasodilation, a proinflammatory state, and a prothrombotic setting.

These factors can trigger vascular inflammation, which may be mediated in part by ROS generated by activated mononuclear cells [González et al., 2014]. The primary enzymatic source of cardiovascular ROS is nonphagocytic NOXes, which are upregulated in hypertension in a variable manner. Emerging evidence suggests that, in addition to NOX hyperactivation, NOS uncoupling, endoplasmic reticulum (ER) stress, and mitochondrial oxidative stress contribute to hypertension-related redox changes. In hypertension, these processes are redox-sensitive and can be considered secondary mechanisms involved in oxidative stress. Activation of eNOS typically generates NO, but when an oxidizing environment is present, NOS is uncoupled, generating Vaso injurious $O2^{-}$ instead of Vaso protective NO [Griendling et al., 2021]. This in turn not only has detrimental effects on vascular tone but also has the capacity to activate key pathways that are known to play a crucial part in the pathophysiology of hypertensive target organ damage, including platelet activity, leukocyte adhesion, vascular smooth muscle cell proliferation, and adhesion molecule expression [Portaluppi et al., 2004].

In our study, we investigated in SHR the effects of the GPC and the antioxidant activity of (+) TIO alone or in association, after 4 weeks of treatment, to analyze the possible protective effects by evaluating the neuronal and glial cell involvements and the BBB structure. In fact, all the pathological neurological alterations related to hypertension determine a decrease in the vascular tone, with a release of the BBB permeability, internal vascular remodeling, and neuroinflammation.

SHR represents an animal model of chronic hypertension which shares several similarities with essential hypertension in humans. This rat strain is also distinguished by brain injury mimics of VaD. Nerve cell loss, astrogliosis, cytoskeletal breakdown and atrophy both in the cerebral cortex and in the hippocampus are common traits of middle-age SHR [Amenta et al., 2010; Tayebati et al., 2012]. Moreover, young and aged SHRs exhibit reduced nicotinic acetylcholine receptors (nAChRs) compared to age matched normotensive WKY rats in important brain regions for cognitive functions such as the cerebral cortex, hippocampus and thalamus [Amenta et al., 2010; Tayebati et al., 2012].

Starting from 24 weeks of age, in which the systolic BP is higher in SHR compared to the normotensive WKY, after 4 weeks of treatment with GPC and (+) TIO alone or in association the values of blood pressure were significantly decreased in SHR.

Starting from 24 weeks of age, in which the systolic blood pressure is higher in SHR compared to the normotensive WKY, after four weeks of treatment with (+)-TIO, the values of blood pressure were significantly decreased in SHR.

This is possibly related to the increase of the ACh levels that determine vasodilation or possible interaction of the GPC with vasoconstrictor molecules. In a model of ischemic reperfusion of the superior mesenteric artery GPC treatment moderates the vasoconstrictive effects of ischemic reperfusion. In this regard, not only the microcirculation but also the microcirculatory changes in the small intestine are influenced. In the event of an occlusion of a main perfusing artery, the microcirculation in the tissues was impaired, leading to longer recovery times after re-establishing the blood supply. In contrast, the micro perfusion was significantly improved in the GPC treated animals [Tokes et al., 2014].

The positive effect of (+) TIO on BP is possibly related to the effects at the levels of the endothelial cells in the vessel. In fact, (+) TIO appears to improve endothelial function by increasing the bioavailability of endothelium-derived nitric oxide, decreasing oxidative stress and inflammation [Hajizadeh and Sharifi., 2022]. Endothelial cells are important constituents of blood vessels that play key roles in cardiovascular homeostasis [Konukoglu et al., 2017]. Endothelial dysfunction implicated in the pathophysiology of hypertension is characterized by a reduction of vasodilation, a pro-thrombotic setting, and a pro-inflammatory state. Excessive ROS formation by vascular walls can mediate these events in the vessels [Bengtsson et al., 2013].

The vasculature is a major source of NADPH-oxidase-derived ROS, which has a prominent role in vascular damage under pathological conditions [Park et al., 2001]. Additionally, endothelium-derived vasoconstricting factors, such as endothelin, urotensin II, vasoconstrictor prostaglandins, angiotensin II, and thromboxane A2, can be released by endothelial cells and contribute to the vasoconstrictor effects. Conversely, reduced NO bioavailability, a well-known endothelium derived, relaxing factor, is considered a hallmark of endothelial dysfunction [Tain and Hsu et al., 2022]. Additionally, endothelium-derived vasoconstricting factors, such as endothelin, urotensin II, vasoconstrictor prostaglandins, angiotensin II, and thromboxane A2, can be released by endothelial cells and contribute to the vasoconstrictor effects. Conversely, reduced NO bioavailability, a well-known endothelium-derived, relaxing factor, is considered a hallmark of endothelial dysfunction [Li et al., 2015].

As previously demonstrated, dietary (+) TIO acid supplementation in SHRs lowered the systolic blood pressure, cytosolic Ca2+ blood glucose and insulin levels, and tissue aldehyde conjugates, and attenuated adverse vascular changes [Vasdev et al., 2000].

In contrast with our previous results [Martinelli et al., 2021, Pacini et al.,2021], data of the present study, showed that GPC and (+) TIO treatment can reduce systolic blood pressure. This effect could be due to the lower basal blood pressure of the SHR strain used in this study. This evidence may represent an important indication of how a therapy with antioxidants or cholinergic precursor compounds, can show antihypertensive properties in the early stages of disease development as suggested by Kizhakekuttu et al. [Hajizadeh-Sharafabad and Sharifi Zahabi 2022]. In an animal model of obesity associated with other diseases, including hypertension, (+) TIO decreased the blood pressure at the standard levels. As was previously reported, the antihypertensive role of (+)

TIO depends on initial hypertension, and in the case of early hypertension or non-dramatic hypertension, it is effective as a hypotensive drug [Konukoglu and Uzun., 2017]. Furthermore, as previously demonstrated, dietary (+) TIO acid supplementation in SHRs lowered the systolic blood pressure, cytosolic Ca2+, blood glucose and insulin levels, tissue aldehyde conjugates, and attenuated adverse vascular changes [Bengtsson et al.,2003]. (+) TIO has also effects on vascular relaxation in SHR at the level of the aortic smooth muscle cells [Li et al., 2015].

Previous study found that, the contribution of antioxidant substances in metabolic pathways related to mitochondria, in cell signaling that may improve coupling of eNOS, and in anti-inflammatory actions [Zhang and Frei., 2001]. The supplementation of (+)TIO as a natural antioxidant has already been demonstrated to be safe in clinical trials [Fogacci et al., 2020], and to have multiple beneficial effects [Lucarini et al., 2020]. In particular, the active enantiomer of (+) TIO showed the most pronounced activity, with extremely few acute and subchronic toxicities, compared to the racemic or levorotatory forms of (+)TIO [Lucarini et al., 2020].

TIO has neuroprotective effects, probably due to its antioxidant properties. The treatment reduced the level of oxidized proteins [Roy et al., 2021; Martinelli et al., 2021]. A previous study demonstrated in brain of SHR a decrease of Oxo-2'-deoxyguanosine immunoreactions, a marker of oxidized DNA, in the nuclei of cortical neurons and a decrease of neurofilament expression, which were counteracted with thioctic acid administration [Tomassoni et al., 2013]. These findings were consistent with the demonstration of neuroprotective properties of thioctic acid in animal models of brain injury [Rocamonde et al., 2012].

The eutomer (+) TIO represents the active form of the thioctic acid, whereas it is normally produced as a racemic form. In fact, the dextrorotatory enantiomer is better recognized by enzymes [Streeper et al., 1997], and maximum plasma concentration (Cmax) is approximately 40–50% higher with (+) TIO than with the racemic form of thioctic acid at the same dose [Carlson and Kingston, 2007]. Moreover, a recent study demonstrated the beneficial effects of (+)TIO were related to its antioxidant properties, like the ability to restore the intrinsic antioxidant systems, supporting their production or cell accessibility [Salehi et al., 2019]. The compound lowered the oxidation status of proteins; it might be, in a positive loop, linked to effects on astrocyte cells since treatments with (+)TIO formulation reduced the size of astrocytes [Tomassoni et al., 2013].

Preclinical studies reported a neuroprotective effect of inhibitors of AChE / ChE, which could slow the progressive evolution of the disease in question [Francis et al., 2005]. However, these benefits in front a low cost / benefit ratio (especially in terms of adverse reactions) are modest and of doubtful clinical significance [Kavirajan and Schneider., 2007]. One of the more negative aspects of therapy with inhibitors of AChE / ChE lies in the loss of efficacy of treatment with time. Another problem is the treatment of specific patient groups in which inhibitors of AChE/ChE are contraindicated; particularly the very elderly (over 85 years) or those suffering from bradycardia or obstructive pulmonary diseases, such as bronchial asthma and CPOD [Parnetti et al., 2007].

There is a reciprocal relationship between the cholinergic system and cerebral blood flow. It has been well documented that patients with vascular dementia have cholinergic deficits in their brains and cerebrospinal fluids. As cholinergic structures and specific brain areas such as the hippocampus are particularly vulnerable to ischemic damage, impaired cholinergic neurotransmission may play a role in the pathophysiology of VaD [Brashear., 2003]. For instance, 40% of VaD patients were found to have a loss of cholinergic neurons, accompanied by decreased ACh activity in the cortex, hippocampus, and striatum [Court and Perry., 2003]. Moreover, previous research also documented the concept of the “cholinergic anti-inflammation pathway”. According to these findings, ACh released from cholinergic axon terminals can interact nAChR-α7 on vicinal immune cells. Upon receiving the cholinergic signal, the nicotinic receptors suppress the release of cytokines, which are involved in the inflammatory cascade [Pavlov and Tracey., 2006].

Cholinergic agents include ACh precursors, which elevate ACh synthesis. GPC is a cholinergic precursor probably among the most active and certainly more effective respect CDP-choline in increasing brain levels of ACh [Amenta et al., 2001]. A recent metanalysis showed that GPC alone or in combination with donepezil improved cognition, behavior, and functional outcomes among patients with neurological conditions associated with cerebrovascular injury [Sagaro et al., 2023]. The author found a significant effects of GPC in combination with donepezil on cognition [4 RCTs, mean difference (MD):1.72, 95% confidence interval (CI): 0.20 to 3.25], functional outcomes [3 RCTs, MD:0.79, 95% CI: 0.34 to 1.23], and behavioral outcomes [4 RCTs; MD: -7.61, 95% CI: -10.31 to -4.91]. Where also demonstrated that patients who received GPC had significantly better

cognition than those who received either placebo or [MD: 3.50, 95% CI: 0.36 to 6.63] other medications [Sagaro et al., 2023].

Moreover, enhanced cholinergic neurotransmission by GPC may prevent glutamate neurotoxicity via activation of nicotinic acetylcholine receptors and the phosphatidylinositol 3-kinase cascade [Takada et al., 2006]. Stimulation of α7AChR may interfere with inflammatory responses in the central nervous system that could be regulated by astrocytes [Shytle et al., 2004]. It cannot be excluded that enhanced brain cholinergic activity documented for GPC may contribute to interference with mechanisms of neuroprotection mediated via nicotinic receptors [Amenta et al., 2006]. In fact, it is widely accepted that AChR stimulation reduces hypoxic-ischemic brain damage in rat pups as well as cytokine production as a result of hypoxia-ischemia damage [Alkondon and Albuquerque, 2004].

A protective effect may be raised from the activation of the anti-inflammatory pathway involving the astroglial nAChRα7 [Patel et al.,2017, Piovesana et al.,2021]. Cholinergic pathways project widely to different brain areas: basal-forebrain, cerebral cortex and hippocampus, as well as some intrinsic cholinergic hippocampal interneurons, show a decisive part in cognitive activity [González et al., 2014]. Among the consequences of AD, the presynaptic cholinergic hypofunction is the most relevant; thus, cholinergic replacement therapy is beneficial in alleviating cognitive dysfunction.

Our study, in an animal model of obesity correlated with an increase of BP value, showed an alteration of cholinergic pathways. Particularly as reported in the paper:

"Western blot results for the α7nAChR showed a 55 kDa band in the two brain areas considered (Figure 4A,C). In the frontal cortex, protein quantification reported a less expression of α7nAChR in obese animals fed for 17 weeks with HFD compared with lean rats (Figure 4A), while in the hippocampus, a remarkable reduction was reported both after 5 and 17 weeks of hypercaloric diet (Figure 4C). The immunostaining for the nicotinic receptor α7nAChR was present in the neurons of the V layer of the frontal cortex (Figure 4B), the CA1, and the CA3 subfields (Figure 4D). The intensities of immunoreaction for α7nAChR confirmed the immunochemical results in these two brain areas analyzed (Figure 4B, D).

In the discussion we highlighted that:

Different anti-inflammatory pathways control the HFD-induced inflammation, including the cholinergic pathway [62], through the activation of the α7nAChR [63]. A stimulatory reaction of dietary fat has been reported on α7nAChR in the lateral and ventromedial hypothalamus [56]. Unlike other studies also in the hypothalamus, liver, spleen, and adipose tissue, we sustained that HFD reduced the expression of α7nAChR [64,65]. In accordance, our data demonstrated a reduction of this receptor both in the frontal cortex and hippocampus in rats fed with HFD, as we described recently in the same regions of older OZRs [22]. Since the stimulation of α7nAChR both in microglia as well as in astrocytes can induce neuroprotection resulting from anti-inflammatory activities [66], we could speculate that the reactive microglia, astrogliosis, and vascular inflammation, characterized by an increase of endothelial inflammatory markers (cell adhesion molecules) in DIO rats after 17 weeks of HFD [17], may be related to the low expression of α7nAChR in obese phenotype [***Martinelli et al.,2021Appendix 7****].*

The present work confirms and extends the results of previous studies, further supporting the hypothesis that GPC may act as a neuroprotectant in an animal model of VaD. In fact, 4 weeks of GPC treatment can reduce the decreased expression of the neuronal proteins in the hippocampus that represent the area more sensitive to the alterations of BP.

Moreover, the two treatments are efficient counteract the astrogliosis, particularly in hippocampus. Astrocytes play an active role in maintaining the structure, metabolism, and function of the brain [Barcia et al., 2008] and become hypertrophic in response to diverse brain injuries. Reactive astrocytes are recognized by their increased size, upregulation of GFAP expression and immunoreactivity [Faulkner et al., 2004], and arterial hypertension increases astrocyte activation [Amenta and Tomassoni, 2011]. Oxidative stress is most likely contributing to the brain's suffering status accompanied by increased astrocytes immunoreaction. Treatment with (+) TIO and GPC decreases the area occupied by glial cells and therefore counters brain injury accompanied by increased oxidative stress. These data, similar to those found in another animal model of brain injury [Rocamonde et al., 2012], confirm a more pronounced activity of dextrorotatory forms of TIO.

Gliosis in the SHR is also characterized by microgliosis. Microglia, just like macrophages, are phagocytes of the CNS, capable of engulfing microorganisms, whole neurons [Brown and Neher,

2014] or parts of blood vessels [Jolivel et al., 2015] depending on their different activation state. The countered action of the combination with GPC and (+) TIO on microglial activation and astrogliosis could be related to the decreased expression of cytokines in particularly IL-β beta and TNF-alpha. In fact, previous results indicate in the same animal model a decrease expression of TNF-alpha both in the frontal cortex and hippocampus of animals treated with GPC and (+)TIO [Roy et al., 2021].

The results of *in vitro* study on LPS activated BV-2 cells support the evidence of a possible anti-inflammatory effects of the cholinergic precursor drug on the activation at the M1 phenotype of microglial cells, associated with the release of proinflammatory cytokines. The (+) TIO in our experiments didn't modulate the inflammatory pathways, probably it could be related to the concentrations. Evidence suggests an anti-inflammatory property of (+)TIO on LPS activated BV-2 at higher dose (500 and 1000 μM) [Kim et al., 2019]. Another study will be performed to identify the specific activity of the antioxidant compound and the possible effects in combination with GPC.

Our results showed in the cerebral area of SHR rats, an increase in AQP-4 and GLUT-1, in accordance with the previous study. An increased expression of AQP-4 protein was also found in the cerebral cortex of stroke-prone SHR at 20 weeks and in SHR at 32 weeks compared to age-matched WKY rats [Ishida et al., 2006; Tayebati et al., 2009; Tomassoni et al., 2010]. It has been suggested that AQP-4 plays an important role in BBB function and in the pathogenesis of hypertensive cerebral injury [Ishida et al., 2006; Tayebati et al., 2009]. Despite the above data, the role of AQP-4 in keeping BBB integrity has not been clarified yet. AQP-4 deletion alters BBB integrity and glial fibrillary acidic protein (GFAP) immunoreactivity in AQP-4 null mice [Zhou et al., 2008]. Hypoxia conditions in brain capillary endothelial cells culture increase some BBB transporters' expression (GLUT-1, P-gp, SLC7A5 and TFRC) via hypoxia-inducible factor-1 (HIF-1α) stabilization, without compromising monolayer integrity. This may in part explain why brain capillaries show early maturation, in terms of barrier tightness and protein expression [Ozgür et al., 2022].

The increased expression of AQP-4 and GLUT-1 was not correlated with the astrogliosis developed in the brain of SHR rats. Data indicated an increased expression of glial fibrillary acidic protein and increasing size of astrocytes. This phenomenon did not involve the perivascular

astrocytes that selectively expressed AQP-4. The effect of the administration of two compounds was demonstrated by the reduction of blood pressure directly related to the benefits at the level of the cerebrovascular tree. This could restore the normal permeability and/or integrity of the BBB.

Based on the results of the present thesis, we are unable to say if the protective effects of GPC are due to the phospholipid nature of the compound, to its interference with cholinergic neurotransmission mechanisms, or to both possibilities. As previous pointed out, GPC is able to increase the expression level of vesicular acetylcholine transporter (VAChT) in brain areas of SHR modulating the cholinergic system [Roy et al., 2021]. Further studies are necessary to clarify the positive effects of GPC and its mechanism of action on neuroinflammation. However, the results represent an important step towards exploring and clarifying the potential cerebrovascular activity of GPC as shown in randomized clinical studies [Sagaro et al., 2023].

The cholinergic system is one of the excitatory pathways participating in the parasympathetic, sympathetic, and the central nervous system using acetylcholine as a neurotransmitter. Acetylcholine and acetylcholine receptors are known to be present on many cell types including endothelial cells and cells of the immune system.The activation of the “cholinergic anti-inflammatory pathway” mediated by ACh binding to nAChR α7 as one of the possible mechanisms for controlling inflammation, have restarted interest in cholinergic-mediated pathological processes and in the new potential therapeutic target for neuro-inflammatory-degenerative diseases.

6. CONCLUSION

In conclusion, the data confirm that SHRs are an interesting model for screening substances with therapeutic properties for treating brain injury of vascular origin. Moreover, our finding indicates that treatment with GPC and (+) TIO attenuates neural damage and glial reactions, particularly in the hippocampus of SHR. This suggests that the two compounds, alone or in association, may afford neuroprotection in the animal model investigated. The modulation of cholinergic enzymes and/or receptors might be a useful therapeutic approach in cerebral dysfunction related to hypertension.

Moreover, BV-2 microglial cells data demonstrate that GPC alone attenuate LPS-induced neuroinflammatory responses and suggest insights to explain their therapeutic role in brain disorders characterized by vascular impairment. Further investigation needs to clarify the aspect of neuroinflammation processes and the possible protective effects of the drugs in other i*n-vitro* model or BBB organoids.

These data already presented at different Congress [***Appendices 8-11***], may have a pharmacological relevance and suggest that the two compounds, although they are not hypertensive drugs, could represent a new prospective strategy to prevent hypertension-associated cerebrovascular alterations that could be studied by controlled clinical trial.

REFFERENCES

1. Kearney PM, Whelton M, Reynolds K. et al. Global burden of hypertension: analysis of worldwide data. *Lancet*. **2005**;*365(9455):217-223*. doi:10.1016/S0140-6736(05)17741-1.

2. Piskorz D. Hypertensive Mediated Organ Damage and Hypertension Management. How to Assess the Beneficial Effects of Antihypertensive Treatments? *High Blood Press Cardiovasc Prev*.**2020**;*27(1):9-17*. doi:10.1007/s40292-020-00361-6.

3. NCD Risk Factor Collaboration (NCD-RisC). Worldwide trends in blood pressure from 1975 to 2015: a pooled analysis of 1479 population-based measurement studies with 19·1 million participants. *Lancet*. *Lancet.* **2020;** Sep, *26;396(10255):886.* doi:10.1016/S0140-6736(16)31919-5.

4. Giles TD, Materson BJ, Cohn JN, Kostis JB. Definition and classification of hypertension: an update. *J Clin Hypertens* (Greenwich). **2009;** *Nov;11(11):611-4.* doi: 10.1111/j.1751-7176.2009.00179. x.

5. www.who.int/news-room/fact-sheets/detail/hypertension. Hypertension. *World health organization*. **2021;** *August 25*, Accessed July 14, 2022.

6. Rimoldi SF, Scherrer U, Messerli FH. Secondary arterial hypertension: when, who, and how to screen? *Eur Heart J*. **2014**;*35(19):1245-1254*. doi:10.1093/eurheartj/eht534.

7. Muntner P, Shimbo D, Carey RM, Charleston JB, Gaillard T, Misra S, Myers MG, Ogedegbe G, Schwartz JE, Townsend RR, Urbina EM, Viera AJ, White WB, Wright JT Jr. Measurement of Blood Pressure in Humans: A Scientific Statement from the American Heart Association. *Hypertension*. **2019;** May;73(5): e35-e66. doi: 10.1161/HYP.0000000000000087.

8. Carey RM, Muntner P, Bosworth HB, Whelton PK. Prevention and Control of Hypertension: JACC Health Promotion Series. *J Am Coll Cardiol.* **2018***; Sep 11;72(11):1278-1293*. doi: 10.1016/j.jacc.2018.07.008.

9. Gaziano TA, Bitton A, Anand S, Weinstein MC; International Society of Hypertension. The global cost of nonoptimal blood pressure. *J Hypertens*. **2009;** *Jul;27(7):1472-7*. doi: 10.1097/HJH.0b013e32832a9ba3.

10. Vasan RS, Beiser A, Seshadri S, Larson MG, Kannel WB, D'Agostino RB, Levy D. Residual lifetime risk for developing hypertension in middle-aged women and men: The Framingham Heart Study. *JAMA.* **2002**; Feb *27;287(8):1003-10*. doi: 10.1001/jama.287.8.1003.

11. NCD Risk Factor Collaboration (NCD-RisC). Worldwide trends in hypertension prevalence and progress in treatment and control from 1990 to 2019: a pooled analysis of 1201 population-representative studies with 104 million participants. *Lancet*. **2022;** *Feb 5;399(10324):520*. doi:10.1016/S0140-6736(21)01330-1.

12. Whelton PK, He J, Appel LJ, Cutler JA, Havas S, Kotchen TA, Roccella EJ, Stout R, Vallbona C, Winston MC, Karimbakas J; National High Blood Pressure Education Program Coordinating Committee. Primary prevention of hypertension: clinical and public health

advisory from The National High Blood Pressure Education Program. *JAMA*. **2002; Oct** 16;288(15):1882-8. doi: 10.1001/jama.288.15.1882.

13. Hruby A, Hu FB. The Epidemiology of Obesity: A Big Picture. *Pharmacoeconomics*. **2015**; Jul;33(7):673-89. doi: 10.1007/s40273-014-0243-x.

14. Lee IM, Shiroma EJ, Lobelo F, et al. Effect of physical inactivity on major non-communicable diseases worldwide: an analysis of burden of disease and life expectancy. *Lancet*. **2012**;*380(9838):219-229*. doi:10.1016/S0140-6736(12)61031-9.

15. Forouzanfar MH, Liu P, Roth GA, et al. Global Burden of Hypertension and Systolic Blood Pressure of at Least 110 to 115 mm Hg, 1990-2015. *JAMA*. **2017**; Jan *10;317(2):165-182*. doi: 10.1001/jama.2016.19043.

16. Meissner A. Hypertension and the Brain: A Risk Factor for More Than Heart Disease. *Cerebrovasc Dis*. **2016***;42(3-4):255-62*. doi: 10.1159/000446082.

17. Amenta F, Di Tullio MA, Tomassoni D. Arterial hypertension and brain damage--evidence from animal models (review). *Clin Exp Hypertens*. **2003;** *Aug;25(6):359-80*. doi: 10.1081/ceh-120023545.

18. Yu JG, Zhou RR, Cai GJ. From hypertension to stroke: mechanisms and potential prevention strategies. *CNS Neurosci Ther*. **2011;** *Oct;17(5):577-84*. doi: 10.1111/j.1755-5949.2011.00264. x.

19. Sharp SI, Aarsland D, Day S, Sønnesyn H; Alzheimer's Society Vascular Dementia Systematic Review Group; Ballard C. Hypertension is a potential risk factor for vascular dementia: systematic review. Int J Geriatr Psychiatry. 2011 Jul;26(7):661-9. doi: 10.1002/gps.2572. Epub 2010 Dec 29.

20. Amenta F, Ferrante F, Sabbatini M, Ricci A. Quantitative image analysis study of the cerebral vasodilatory activity of nicardipine in spontaneously hypertensive rats. *Clin Exp Hypertens*. **1994;** *May;16(3):359-71*. doi: 10.3109/10641969409072222.

21. Kalra M, al-Khaffaf H, Farrell A, Wallbank WA, Charlesworth D. Comparison of measurement of stump pressure and transcranial measurement of flow velocity in the middle cerebral artery in carotid surgery. *Ann Vasc Surg*. **1994;** *May;8(3):225-31*. doi: 10.1007/BF02018168.

22. Sierra C. Early brain damage in essential hypertension: "to have and have not"--is it important? *Curr Hypertens Rep*. **2006;** *Aug;8(4):269-70*. doi: 10.1007/s11906-006-0061-4.

23. Price RS, Kasner SE. Hypertension and hypertensive encephalopathy. *Handb Clin Neurol*. **2014**; *119:161-7*. doi: 10.1016/B978-0-7020-4086-3.00012-6.

24. Andrea M. Maresca, Anna Realdi, FrancoDel Piccolo, Roberta Rocchi, Pietro Amodio, Daniela Mapelli, Achille C. Pessina, Andrea Semplicini, P-215: Impaired cerebral vasoreactivity in hypertensives: An early marker of vascular dementia? *American Journal of Hypertension,* Volume 15, Issue S3, April **2002**; *Page 107A*, https://doi.org/10.1016/S0895-7061(02)02566-9.

25. Veglio F, Paglieri C, Rabbia F, Bisbocci D, Bergui M, Cerrato P. Hypertension and cerebrovascular damage. *Atherosclerosis*. **2009;** *Aug;205(2):331-41*. doi: 10.1016/j.atherosclerosis.2008.

26. Schwartz RB. Hyperperfusion encephalopathies: hypertensive encephalopathy and related conditions. *Neurologist.* **2002***; Jan;8(1):22-34.* doi: 10.1097/00127893-200201000-00003.

27. Miller JB, Suchdev K, Jayaprakash N, Hrabec D, Sood A, Sharma S, Levy PD. New Developments in Hypertensive Encephalopathy. *Curr Hypertens Rep.* **2018**; Feb *26;20(2):13.* doi: 10.1007/s11906-018-0813-y.

28. Rovira A, Grivé E, Rovira A, Alvarez-Sabin J. Distribution territories and causative mechanisms of ischemic stroke. *Eur Radiol*. **2005**; *Mar;15(3):416-26.* doi: 10.1007/s00330-004-2633-5.

29. Lammie GA, Brannan F, Slattery J, Warlow C. Nonhypertensive cerebral small-vessel disease. *An autopsy studies. Stroke.* **1997**; Nov*;28(11):2222-9.* doi: 10.1161/01.str.28.11.2222.

30. Kato H, Izumiyama M, Izumiyama K, Takahashi A, Itoyama Y. Silent cerebral microbleeds on T2*-weighted MRI: correlation with stroke subtype, stroke recurrence, and leukoaraiosis. *Stroke.* **2002**; *Jun;33(6):1536-40*. doi: 10.1161/01.str.0000018012.65108.86.

31. Spence JD. Blood Pressure Gradients in the Brain: Their Importance to Understanding Pathogenesis of Cerebral Small Vessel Disease. *Brain Sci*. **2019;** *Jan 23;9(2):21*. doi: 10.3390/brainsci9020021.

32. Faraco G, Iadecola C. Hypertension: a harbinger of stroke and dementia. *Hypertension*. **2013;** *Nov;62(5):810-7.* doi: 10.1161/HYPERTENSIONAHA.113.01063.

33. Iadecola C, Davisson RL. Hypertension and cerebrovascular dysfunction. *Cell Metab*. **2008;** *Jun;7(6):476-84.* doi: 10.1016/j.cmet.2008.03.010.

34. Henskens LH, Kroon AA, van Oostenbrugge RJ, Gronenschild EH, Fuss-Lejeune MM, Hofman PA, Lodder J, de Leeuw PW. Increased aortic pulse wave velocity is associated with silent cerebral small-vessel disease in hypertensive patients. *Hypertension*. **2008;** *Dec;52(6):1120-6.* doi: 10.1161/HYPERTENSIONAHA.108.119024.

35. Sakamoto H, Aikawa M, Hill CC, Weiss D, Taylor WR, Libby P, Lee RT. Biomechanical strain induces class a scavenger receptor expression in human monocyte/macrophages and THP-1 cells: a potential mechanism of increased atherosclerosis in hypertension. *Circulation*. **2001;** *Jul 3;104(1):109-14.* doi: 10.1161/hc2701.091070.

36. Sörös P, Whitehead S, Spence JD, Hachinski V. Antihypertensive treatment can prevent stroke and cognitive decline. *Nat Rev Neurol*. **2013;** *Mar;9(3):174-8.* doi: 10.1038/nrneurol.2012.255.

37. Faraco G, Iadecola C. Hypertension: a harbinger of stroke and dementia. *Hypertension*. **2013;** *Nov;62(5):810-7.* doi: 10.1161/HYPERTENSIONAHA.113.01063.

38. Immink RV, van den Born BJ, van Montfrans GA, Koopmans RP, Karemaker JM, van Lieshout JJ. Impaired cerebral autoregulation in patients with malignant hypertension. *Circulation*. **2004**; *Oct 12;110(15):2241-5*. doi: 10.1161/01.CIR.0000144472.08647.40.

39. Touyz RM. Reactive oxygen species, vascular oxidative stress, and redox signaling in hypertension: what is the clinical significance*? Hypertension*. **2004;** *Sep;44(3):248-52.* doi: 10.1161/01.HYP.0000138070.47616.9d.

40. Prince M, Bryce R, Albanese E, Wimo A, Ribeiro W, Ferri CP. The global prevalence of dementia: a systematic review and metaanalysis. *Alzheimers Dement*. **2013;** *Jan;9(1):63-75. e2.* doi: 10.1016/j.jalz.2012.11.007.

41. Tian Z, Ji X, Liu J. Neuroinflammation in Vascular Cognitive Impairment and Dementia: Current Evidence, Advances, and Prospects. *Int J Mol Sci*. **2022;** *Jun 2;23(11):6224*. doi: 10.3390/ijms23116224.

42. Gorelick PB, Scuteri A, Black SE, et al. Vascular contributions to cognitive impairment and dementia: a statement for healthcare professionals from the American heart association/American stroke association. *Stroke*. **2011;** *Sep;42(9):2672-713.* doi: 10.1161/STR.0b013e3182299496.

43. Singh Sidhu N, Kaur S. Cerebrovascular Disease and Hypertension. *Cerebrovascular Diseases*. **2021**; *November 9*. doi:10.5772/intechopen.101180.

44. Faraco G, Iadecola C. Hypertension: a harbinger of stroke and dementia. *Hypertension*. **2013;** *Nov;62(5):810-7.* doi: 10.1161/HYPERTENSIONAHA.113.01063.

45. Presa JL, Saravia F, Bagi Z, Filosa JA. Vasculo-Neuronal Coupling and Neurovascular Coupling at the Neurovascular Unit: Impact of Hypertension. *Front Physiol*. **2020;** *Sep 25; 11:584135.* doi: 10.3389/fphys.2020.584135.

46. Levit A, Cheng S, Hough O, Liu Q, Agca Y, Agca C, Hachinski V, Whitehead SN. Hypertension and Pathogenic hAPP Independently Induce White Matter Astrocytosis and Cognitive Impairment in the Rat. *Front Aging Neurosci*. **2020;** *Apr 15; 12:82.* doi: 10.3389/fnagi.2020.00082.

47. Roy A, Guatimosim S, Prado VF, Gros R, Prado MA. Cholinergic activity as a new target in diseases of the heart. *Mol Med*. **2015;** *Jan 26;20(1):527-37.* doi: 10.2119/molmed.2014.00125.

48. Fujii N, McGarr GW, Ghassa R, Schmidt MD, McCormick JJ, Nishiyasu T, Kenny GP. Sex-differences in cholinergic, nicotinic, and β-adrenergic cutaneous vasodilation: Roles of nitric oxide synthase, cyclooxygenase, and K+ channels. *Microvasc Res*. **2020;** *Sep; 131:104030*. doi: 10.1016/j.mvr.2020.104030.

49. Fujii N, Louie JC, McNeely BD, Amano T, Nishiyasu T, Kenny GP. Mechanisms of nicotine-induced cutaneous vasodilation and sweating in young adults: roles for KCa, KATP, and KV channels, nitric oxide, and prostanoids. *Appl Physiol Nutr Metab*. **2017;** *May;42(5):470-478.* doi: 10.1139/apnm-2016-0615.

50. Lazartigues E, Brefel-Courbon C, Tran MA, Montastruc JL, Rascol O. Spontaneously hypertensive rats cholinergic hyper-responsiveness: central and peripheral pharmacological mechanisms. *Br J Pharmacol.* **1999;** *Aug;127(7):1657-65*. doi: 10.1038/sj.bjp.0702678.

51. Parnetti L, Mignini F, Tomassoni D, Traini E, Amenta F. Cholinergic precursors in the treatment of cognitive impairment of vascular origin: ineffective approaches or need for re-evaluation? *J Neurol Sci.* **2007;** *Jun 15;257(1-2):264-9.* doi: 10.1016/j.jns.2007.01.043.

52. Erkinjuntti T, Román G, Gauthier S. Treatment of vascular dementia--evidence from clinical trials with cholinesterase inhibitors. *J Neurol Sci.* **2004;** *Nov 15;226(1-2):63-6*. doi: 10.1016/j.jns.2004.09.018.

53. Tomimoto H, Ohtani R, Shibata M, Nakamura N, Ihara M. Loss of cholinergic pathways in vascular dementia of the Binswanger type. *Dement Geriatr Cogn Disord.* **2005***;19(5-6):282-8*. doi: 10.1159/000084553.

54. Liu Q, Zhu Z, Teipel SJ, Yang J, Xing Y, Tang Y, Jia J. White Matter Damage in the Cholinergic System Contributes to Cognitive Impairment in Subcortical Vascular Cognitive Impairment, No Dementia. *Front Aging Neurosci.* ***2017;*** *Feb 27;9:47*. doi: 10.3389/fnagi.2017.00047.

55. Zhang LC, Jin X, Huang Z, Yan ZN, Li PB, Duan RF, Feng H, Jiang JH, Peng H, Liu W. Protective effects of choline against hypoxia-induced injuries of vessels and endothelial cells. *Exp Ther Med.* **2017;** *May;13(5):2316-2324.* doi: 10.3892/etm.2017.4276.

56. Kaplan L, Chow BW, Gu C. Neuronal regulation of the blood-brain barrier and neurovascular coupling. *Nat Rev Neurosci*. **2020**; *Aug;21(8):416-432*. doi: 10.1038/s41583-020-0322-2.

57. Iadecola C. The Neurovascular Unit Coming of Age: A Journey through Neurovascular Coupling in Health and Disease. *Neuron.* **2017;** *Sep 27;96(1):17-42.* doi: 10.1016/j.neuron.2017.07.030.

58. De Silva TM, Faraci FM. Microvascular Dysfunction and Cognitive Impairment. *Cell Mol Neurobiol*. **2016;** *Mar;36(2):241-58*. doi: 10.1007/s10571-015-0308-1.

59. Zlokovic BV. Neurovascular pathways to neurodegeneration in Alzheimer's disease and other disorders. *Nat Rev Neurosci.* **2011;** *Nov 3;12(12):723-38.* doi: 10.1038/nrn3114.

60. Yu X, Ji C, Shao A. Neurovascular Unit Dysfunction and Neurodegenerative Disorders. *Front Neurosci*. **2020**; Apr *29;14:334.* doi: 10.3389/fnins.2020.00334.

61. Gordon GR, Howarth C, MacVicar BA. Bidirectional control of arteriole diameter by astrocytes. *Exp Physiol*. **2011;** *Apr;96(4):393-9*. doi: 10.1113/expphysiol.2010.053132.

62. Quick S, Moss J, Rajani RM, Williams A. A Vessel for Change: Endothelial Dysfunction in Cerebral Small Vessel Disease. *Trends Neurosci*. **2021;** *Apr;44(4):289-305*. doi: 10.1016/j.tins.2020.11.003.

63. Howarth C, Gleeson P, Attwell D. Updated energy budgets for neural computation in the neocortex and cerebellum. *J Cereb Blood Flow Metab*. **2012;** *Jul;32(7):1222-32.* doi: 10.1038/jcbfm.2012.35.

64. Hill-Eubanks DC, Gonzales AL, Sonkusare SK, Nelson MT. Vascular TRP channels: performing under pressure and going with the flow. *Physiology (Bethesda)*. **2014;** *Sep;29(5):343-60*. doi: 10.1152/physiol.00009.2014.

65. Hill MA, Davis MJ, Meininger GA, Potocnik SJ, Murphy TV. Arteriolar myogenic signaling mechanisms: Implications for local vascular function. *Clin Hemorheol Microcirc*. **2006**;*34(1-2):67-79*. PMID: 16543619.

66. Chirinos JA, Segers P, Hughes T, Townsend R. Large-Artery Stiffness in Health and Disease: JACC State-of-the-Art Review. *J Am Coll Cardiol***. 2019;** *Sep 3;74(9):1237-1263.* doi: 10.1016/j.jacc.2019.07.012.

67. Vaziri ND. Causal link between oxidative stress, inflammation, and hypertension. *Iran J Kidney Dis.* **2008;** *Jan;2(1):1-10*. PMID: 19367002.

68. Roberts CK, Barnard RJ, Sindhu RK, Jurczak M, Ehdaie A, Vaziri ND. Oxidative stress and dysregulation of NAD(P)H oxidase and antioxidant enzymes in diet-induced metabolic syndrome. *Metabolism*. **2006;** *Jul;55(7):928-34.* doi: 10.1016/j.metabol.2006.02.022.

69. Vaziri ND. Roles of oxidative stress and antioxidant therapy in chronic kidney disease and hypertension. *Curr Opin Nephrol Hypertens*. **2004;** *Jan;13(1):93-9.* doi: 10.1097/00041552-200401000-00013.

70. Vaziri ND, Wang XQ, Oveisi F, Rad B. Induction of oxidative stress by glutathione depletion causes severe hypertension in normal rats. *Hypertension*. **2000;** *Jul;36(1):142-6.* doi: 10.1161/01.hyp.36.1.142.

71. Ranadive SM, Dillon GA, Mascone SE, Alexander LM. Vascular Health Triad in Humans with Hypertension-Not the Usual Suspects. *Front Physiol*. **2021;** *Oct 1; 12:746278.* doi: 10.3389/fphys.2021.746278.

72. Rodríguez-Iturbe B, Ferrebuz A, Vanegas V, Quiroz Y, Mezzano S, Vaziri ND. Early and sustained inhibition of nuclear factor-kappaB prevents hypertension in spontaneously hypertensive rats. *J Pharmacol Exp Ther*. **2005;** *Oct;315(1):51-7.* doi: 10.1124/jpet.105.088062.

73. Wadley AJ, Veldhuijzen van Zanten JJ, Aldred S. The interactions of oxidative stress and inflammation with vascular dysfunction in ageing: the vascular health triad. *Age (Dordr)*. **2013;** *Jun;35(3):705-18*. doi: 10.1007/s11357-012-9402-1.

74. Pizzino G, Irrera N, Cucinotta M, Pallio G, Mannino F, Arcoraci V, Squadrito F, Altavilla D, Bitto A. Oxidative Stress: Harms and Benefits for Human Health. *Oxid Med Cell Longev*. **2017**; *2017:8416763*. doi: 10.1155/2017/8416763.

75. Lip GY, Edmunds E, Nuttall SL, Landray MJ, Blann AD, Beevers DG. Oxidative stress in malignant and non-malignant phase hypertension. *J Hum Hypertens*. **2002;** *May;16(5):333-6.* doi: 10.1038/sj.jhh.1001386.

76. González J, Valls N, Brito R, Rodrigo R. Essential hypertension and oxidative stress: New insights. *World J Cardiol*. **2014;** *Jun 26;6(6):353-66*. doi: 10.4330/wjc.v6.i6.353.

77. Schulz E, Gori T, Münzel T. Oxidative stress and endothelial dysfunction in hypertension. *Hypertens Res*. **2011** *Jun;34(6):665-73*. doi: 10.1038/hr.2011.39.

78. Flammer AJ, Lüscher TF. Human endothelial dysfunction: EDRFs. *Pflugers Arch*. **2010;** *May;459(6):1005-13*. doi: 10.1007/s00424-010-0822-4.

79. Napoli C, Ignarro LJ. Nitric oxide and pathogenic mechanisms are involved in the development of vascular diseases. *Arch Pharm Res.* **2009;** *Aug;32(8):1103-8.* doi: 10.1007/s12272-009-1801-1.

80. Deanfield JE, Halcox JP, Rabelink TJ. Endothelial function and dysfunction: testing and clinical relevance. *Circulation*. **2007;** *Mar 13;115(10):1285-95*. doi: 10.1161/CIRCULATIONAHA.106.652859.

81. Montezano AC, Touyz RM. Oxidative stress, Noxs, and hypertension: experimental evidence and clinical controversies. *Ann Med*. **2012;** *Jun;44 Suppl 1: S2-16*. doi: 10.3109/07853890.2011.653393.

82. Lambeth JD. NOX enzymes and the biology of reactive oxygen. *Nat Rev Immunol*. **2004;** *Mar;4(3):181-9*. doi: 10.1038/nri1312.

83. Nuyt AM. Mechanisms underlying developmental programming of elevated blood pressure and vascular dysfunction: evidence from human studies and experimental animal models. *Clin Sci (Lond)*. **2008;** *Jan;114(1):1-17*. doi: 10.1042/CS20070113.

84. Schiffrin EL. Oxidative stress, nitric oxide synthase, and superoxide dismutase: a matter of imbalance underlies endothelial dysfunction in the human coronary circulation. *Hypertension*. **2008;** *Jan;51(1):31-2*. doi: 10.1161/HYPERTENSIONAHA.107.103226.

85. Ungvari Z, Toth P, Tarantini S, Prodan CI, Sorond F, Merkely B, Csiszar A. Hypertension-induced cognitive impairment: from pathophysiology to public health. *Nat Rev Nephrol*. **2021;** *Oct;17(10):639-654*. doi: 10.1038/s41581-021-00430-6.

86. Allen CL, Bayraktutan U. Oxidative stress and its role in the pathogenesis of ischaemic stroke. Int J Stroke. **2009;** *Dec;4(6):461-70*. doi: 10.1111/j.1747-4949.2009.00387.x.

87. Wevers NR, de Vries HE. Morphogens and blood-brain barrier function in health and disease. *Tissue Barriers*. **2015;** *Sep 11;4(1):e1090524*. doi: 10.1080/21688370.2015.1090524.

88. Shah GN, Morofuji Y, Banks WA, Price TO. High glucose-induced mitochondrial respiration and reactive oxygen species in mouse cerebral pericytes is reversed by pharmacological inhibition of mitochondrial carbonic anhydrases: Implications for cerebral microvascular disease in diabetes. *Biochem Biophys Res Commun*. **2013**; Oct *18;440(2):354-8.* doi: 10.1016/j.bbrc.2013.09.086.

89. Nelson AR, Sweeney MD, Sagare AP, Zlokovic BV. Neurovascular dysfunction and neurodegeneration in dementia and Alzheimer's disease. *Biochim Biophys Acta*. **2016**; *May;1862(5):887-900*. doi: 10.1016/j.bbadis.2015.12.016.

90. Bell RD, Zlokovic BV. Neurovascular mechanisms and blood-brain barrier disorder in Alzheimer's disease. *Acta Neuropathol*. **2009**; *Jul;118(1):103-13*. doi: 10.1007/s00401-009-0522-3.

91. Carvalho C, Moreira PI. Oxidative Stress: A Major Player in Cerebrovascular Alterations Associated to Neurodegenerative Events. *Front Physiol*. **2018**; *Jul 3;9:806*. doi: 10.3389/fphys.2018.00806.

92. Agita A, Alsagaff MT. Inflammation, Immunity, and Hypertension. *Acta Med Indones*. **2017**; *Apr;49(2):158-165.*

93. Tayebati SK, Tomassoni D, Amenta F. Neuroinflammatory Markers in Spontaneously Hypertensive Rat Brain: An Immunohistochemical Study. *CNS Neurol Disord Drug Targets*. **2016**;*15(8):995-1000*. doi: 10.2174/1871527315666160527155014.

94. Harrison DG, Guzik TJ, Lob HE, et al. Inflammation, immunity, and hypertension. *Hypertension*. **2011**;*57(2):132-140.* doi:10.1161/hypertensionaha.110.163576.

95. Winklewski PJ, Radkowski M, Wszedybyl-Winklewska M, Demkow U. Brain inflammation and hypertension: the chicken or the egg? *J Neuroinflammation*. **2015;** *May 3; 12:85*. doi: 10.1186/s12974-015-0306-8.

96. Youwakim J, Girouard H. Inflammation: A Mediator Between Hypertension and Neurodegenerative Diseases. Am *J Hypertens*. **2021**; *Oct 27;34(10):1014-1030*. doi: 10.1093/ajh/hpab094.

97. Liu LR, Liu JC, Bao JS, Bai QQ, Wang GQ. Interaction of Microglia and Astrocytes in the Neurovascular Unit. *Front Immunol*. **2020**; *Jul 8; 11:1024.* doi: 10.3389/fimmu.2020.01024.

98. Liu X, Nemeth DP, McKim DB, Zhu L, DiSabato DJ, Berdysz O, Gorantla G, Oliver B, Witcher KG, Wang Y, Negray CE, Vegesna RS, Sheridan JF, Godbout JP, Robson MJ, Blakely RD, Popovich PG, Bilbo SD, Quan N. Cell-Type-Specific Interleukin 1 Receptor 1 Signaling in the Brain Regulates Distinct Neuroimmune Activities. *Immunity*. **2019;** *Feb 19;50(2):317-333.e6*. doi: 10.1016/j.immuni.2018.12.012.

99. Yenari MA, Xu L, Tang XN, Qiao Y, Giffard RG. Microglia potentiates damage to blood-brain barrier constituents: improvement by minocycline in vivo and in vitro. *Stroke*. **2006;** *Apr;37(4):1087-93*. doi: 10.1161/01.STR.0000206281.77178.ac.

100. Evans LE, Taylor JL, Smith CJ, Pritchard HAT, Greenstein AS, Allan SM. Cardiovascular comorbidities, inflammation, and cerebral small vessel disease. *Cardiovasc Res*. **2021;** *Nov 22;117(13):2575-2588*. doi: 10.1093/cvr/cvab284.

101. Kumar A, Barrett JP, Alvarez-Croda DM, Stoica BA, Faden AI, Loane DJ. NOX2 drives M1-like microglial/macrophage activation and neurodegeneration following experimental traumatic brain injury. Brain *Behav Immun*. **2016**; *Nov; 58:291-309*. doi: 10.1016/j.bbi.2016.07.158.

102. Faraco G, Brea D, Garcia-Bonilla L, Wang G, Racchumi G, Chang H, Buendia I, Santisteban MM, Segarra SG, Koizumi K, Sugiyama Y, Murphy M, Voss H, Anrather J, Iadecola C. Dietary salt promotes neurovascular and cognitive dysfunction through a gut-initiated TH17 response. *Nat Neurosci*. **2018;** *Feb;21(2):240-249*. doi: 10.1038/s41593-017-0059-z.

103. Kazama K, Anrather J, Zhou P, Girouard H, Frys K, Milner TA, Iadecola C. Angiotensin II impairs neurovascular coupling in neocortex through NADPH oxidase-derived radicals. *Circ Res.* **2004;** *Nov 12;95(10):1019-26.* doi: 10.1161/01.RES.0000148637. 85595.c5.

104. Kandalam U, Palanisamy M, Clark MA. Angiotensin II induces cell growth and IL-6 mRNA expression through the JAK2-STAT3 pathway in rat cerebellar astrocytes. *JAKSTAT.* **2012;** *Apr 1;1(2):83-9.* doi: 10.4161/jkst.19688.

105. Amenta F, Mignini F, Rabbia F, Tomassoni D, Veglio F. Protective effect of anti-hypertensive treatment on cognitive function in essential hypertension: analysis of published clinical data. *J Neurol Sci.* **2002;** *Nov 15;203-204:147-51*. doi: 10.1016/s0022-510x (02)00281-2.

106. Vega E, Gómez-Villalobos Mde J, Flores G. Alteration in dendritic morphology of pyramidal neurons from the prefrontal cortex of rats with renovascular hypertension. *Brain Res.* **2004***; Sep 17;1021(1):112-8.* doi: 10.1016/j.brainres.2004.06.042.

107. Joseph AT, Bhardwaj SK, Srivastava LK. Role of Prefrontal Cortex Anti- and Pro-inflammatory Cytokines in the Development of Abnormal Behaviors Induced by Disconnection of the Ventral Hippocampus in Neonate Rats. *Front Behav Neurosci.* **2018;** *Oct 22; 12:244.* doi: 10.3389/fnbeh.2018.00244.

108. Fuster JM. Frontal lobe and cognitive development. *J Neurocytol.* **2002;** *Mar-Jun;31(3-5):373-85.* doi: 10.1023/a:1024190429920.

109. Wood JN. Social cognition and the prefrontal cortex. *Behav Cogn Neurosci Rev.* **2003;** *Jun;2(2):97-114*. doi: 10.1177/1534582303253625.

110. Goto Y, O'Donnell P. Network synchrony in the nucleus accumbens in vivo. *J Neurosci***.** **2001;** *Jun 15;21(12):4498-504.* doi: 10.1523/JNEUROSCI.21-12-04498.2001.

111. Silva-Gómez AB, Rojas D, Juárez I, Flores G. Decreased dendritic spine density on prefrontal cortical and hippocampal pyramidal neurons in postweaning social isolation rats. *Brain Res. **2003**; Sep 5;983(1-2):128-36*. doi: 10.1016/s0006-8993(03)03042-7.

112. Konur S, Rabinowitz D, Fenstermaker VL, Yuste R. Systematic regulation of spine sizes and densities in pyramidal neurons. *J Neurobiol***.** **2003***;Aug;56(2):95-112*. doi: 10.1002/neu.10229.

113. Al-Sarraf H, Philip L. Effect of hypertension on the integrity of blood brain and blood CSF barriers, cerebral blood flow and CSF secretion in the rat. *Brain Res.* **2003;** *Jun 13;975(1-2):179-88.* doi: 10.1016/s0006-8993(03)02632-5.

114. Fiala JC, Spacek J, Harris KM. Dendritic spine pathology: cause or consequence of neurological disorders? *Brain Res Rev.* **2002***; Jun;39(1):29-54*. doi: 10.1016/s0165-0173(02)00158-3.

115. Feng XY, Hu HD, Chen J, Long C, Yang L, Wang L. Acute neuroinflammation increases excitability of prefrontal parvalbumin interneurons and their functional recruitment during novel object recognition. *Brain Behav Immun.* **2021***; Nov; 98:48-58.* doi: 10.1016/j.bbi.2021.08.216.

116. Bitzer-Quintero OK, González-Burgos I. Immune system in the brain: a modulatory role on dendritic spine morphophysiology? *Neural Plast.* **2012***; 2012:348642.* doi: 10.1155/2012/348642.

117. Joseph AT, Bhardwaj SK, Srivastava LK. Role of Prefrontal Cortex Anti- and Pro-inflammatory Cytokines in the Development of Abnormal Behaviors Induced by Disconnection of the Ventral Hippocampus in Neonate Rats. *Front Behav Neurosci.* **2018***; Oct 22; 12:244.* doi: 10.3389/fnbeh.2018.00244.

118. Nwaubani P, Cercignani M, Colasanti A. In vivo quantitative imaging of hippocampal inflammation in autoimmune neuroinflammatory conditions: a systematic review. *Clin Exp Immunol.* **2022;** *Oct 21;210(1):24-38.* doi: 10.1093/cei/uxac058.

119. Chesnokova V, Pechnick RN, Wawrowsky K. Chronic peripheral inflammation, hippocampal neurogenesis, and behavior. *Brain Behav Immun.* **2016***; Nov; 58:1-8.* doi: 10.1016/j.bbi.2016.01.017.

120. Vereker E, O'Donnell E, Lynch MA. The inhibitory effect of interleukin-1beta on long-term potentiation is coupled with increased activity of stress-activated protein kinases. *J Neurosci.* **2000***; Sep 15;20(18):6811-9.* doi: 10.1523/JNEUROSCI.20-18-06811.2000.

121. Kelly A, Vereker E, Nolan Y, Brady M, Barry C, Loscher CE, Mills KH, Lynch MA. Activation of p38 plays a pivotal role in the inhibitory effect of lipopolysaccharide and interleukin-1 beta on long term potentiation in rat dentate gyrus. *J Biol Chem.* **2003***; May 23;278(21):19453-62*. doi: 10.1074/jbc.M301938200.

122. Minogue AM, Schmid AW, Fogarty MP, Moore AC, Campbell VA, Herron CE, Lynch MA. Activation of the c-Jun N-terminal kinase signaling cascade mediates the effect of amyloid-beta on long term potentiation and cell death in hippocampus: a role for interleukin-1beta? *J Biol Chem.* **2003***; Jul 25;278(30):27971-80*. doi: 10.1074/jbc.M302530200.

123. Seger R, Krebs EG. The MAPK signaling cascade. *FASEB J.* **1995;** *Jun;9(9):726-35*. PMID: 7601337.

124. Williamson LL, Bilbo SD. Chemokines and the hippocampus: a new perspective on hippocampal plasticity and vulnerability. *Brain Behav Immun.* **2013***; May;30:186-94.* doi: 10.1016/j.bbi.2013.01.077.

125. Barrientos RM, Kitt MM, Watkins LR, Maier SF. Neuroinflammation in the normal aging hippocampus. *Neuroscience***.** **2015***; Nov 19;309:84-99.* doi: 10.1016/j.neuroscience.2015.03.007.

126. Bartsch T, Wulff P. The hippocampus in aging and disease: From plasticity to vulnerability. *Neuroscience.* **2015** *Nov 19;309:1-16.* doi: 10.1016/j.neuroscience.2015.07.084.

127. Olson JK, Miller SD. Microglia initiate central nervous system innate and adaptive immune responses through multiple TLRs. *J Immunol.* **2004***; Sep 15;173(6):3916-24.* doi: 10.4049/jimmunol.173.6.3916.

128. Glezer I, Simard AR, Rivest S. Neuroprotective role of the innate immune system by microglia. *Neuroscience.* **2007;** *Jul 29;147(4):867-83.* doi: 10.1016/j.neuroscience.2007.02.055.

129. Kim SH, Oh KW, Jin HK, Bae JS. Immune inflammatory modulation as a potential therapeutic strategy of stem cell therapy for ALS and neurodegenerative diseases. *BMB Rep.* **2018***; Nov;51(11):545-546.* doi: 10.5483/BMBRep.2018.51.11.255.

130. Henn A, Lund S, Hedtjärn M, Schrattenholz A, Pörzgen P, Leist M. The suitability of BV2 cells as alternative model system for primary microglia cultures or for animal experiments examining brain inflammation. *ALTEX*. **2009***;26(2):83-94.* doi: 10.14573/altex.2009.2.83.

131. He BP, Wen W, Strong MJ. Activated microglia (BV-2) facilitation of TNF-alpha-mediated motor neuron death in vitro. *J Neuroimmunol.* **2002***; Jul;128(1-2):31-8.* doi: 10.1016/s0165-5728(02)00141-8.

132. Luan W, Li M, Wu C, Shen X, Sun Z. Proteomic dissimilarities of primary microglia and BV2 cells under stimuli. *Eur J Neurosci.* **2022;** *Apr;55(7):1709-1723.* doi: 10.1111/ejn.15637.

133. Stansley B, Post J, Hensley K. A comparative review of cell culture systems for the study of microglial biology in Alzheimer's disease. J Neuroinflammation. 2012 May 31;9:115. doi: 10.1186/1742-2094-9-115.

134. Lim JY, Sul D, Hwang BY, Hwang KW, Yoo KY, Park SY. Suppression of LPS-induced inflammatory responses by inflexanin B in BV2 microglial cells. *Can J Physiol Pharmacol.* **2013***; Feb;91(2):141-8.* doi: 10.1139/cjpp-2012-0242.

135. Dansokho C, Heneka MT. Neuroinflammatory responses in Alzheimer's disease. *J Neural Transm (Vienna).* **2018;** *May;125(5):771-779*. doi: 10.1007/s00702-017-1831-7.

136. Subedi L, Kwon OW, Pak C, Lee G, Lee K, Kim H, Kim SY. N, N-disubstituted azines attenuate LPS-mediated neuroinflammation in microglia and neuronal apoptosis via inhibiting MAPK signaling pathways. *BMC Neurosci.* **2017***; Dec 28;18(1):82.* doi: 10.1186/s12868-017-0399-3.

137. Fellner A, Barhum Y, Angel A, Perets N, Steiner I, Offen D, Lev N. Toll-Like Receptor-4 Inhibitor TAK-242 Attenuates Motor Dysfunction and Spinal Cord Pathology in an Amyotrophic Lateral Sclerosis Mouse Model. Int *J Mol Sci.* **2017***; Aug 1;18(8):1666.* doi: 10.3390/ijms18081666.

138. Hines DJ, Choi HB, Hines RM, Phillips AG, MacVicar BA. Prevention of LPS-induced microglia activation, cytokine production and sickness behavior with TLR4 receptor interfering peptides. *PLoS One.* **2013***;8(3):e60388.* doi: 10.1371/journal.pone.0060388.

139. Lester SN, Li K. Toll-like receptors in antiviral innate immunity. *J Mol Biol.* **2014***; Mar 20;426(6):1246-64.* doi: 10.1016/j.jmb.2013.11.024.

140. Park T, Chen H, Kevala K, Lee JW, Kim HY. N-Docosahexaenoylethanolamine ameliorates LPS-induced neuroinflammation via cAMP/PKA-dependent signaling. J Neuroinflammation. **2016** *Nov 4;13(1):284*.

141. Kim EJ, Kwon KJ, Park JY, Lee SH, Moon CH, Baik EJ. Neuroprotective effects of prostaglandin E2 or cAMP against microglial and neuronal free radical mediated toxicity associated with inflammation. J Neurosci Res. **2002** *Oct 1;70(1):97-107*.

142. Woo MS, Jang PG, Park JS, Kim WK, Joh TH, Kim HS. Selective modulation of lipopolysaccharide-stimulated cytokine expression and mitogen-activated protein kinase pathways by dibutyryl-cAMP in BV2 microglial cells. Brain Res Mol Brain Res. **2003** *May 12;113(1-2):86-96.*

143. Zhong H, Voll RE, Ghosh S. Phosphorylation of NF-kappa B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300. Mol Cell. **1998** *Apr;1(5):661-71.*

144. Lopes PC. LPS and neuroinflammation: a matter of timing. Inflammopharmacology. **2016** *Oct;24(5):291-293.*

145. Schmidt AF, Kannan PS, Chougnet CA, Danzer SC, Miller LA, Jobe AH, Kallapur SG. Intra-amniotic LPS causes acute neuroinflammation in preterm rhesus macaques. J Neuroinflammation. **2016** *Sep 6;13(1):238.*

146. Brito R, Castillo G, González J, Valls N, Rodrigo R. Oxidative stress in hypertension: mechanisms and therapeutic opportunities. *Exp Clin Endocrinol Diabetes***. 2015***; Jun;123(6):325-35.* doi: 10.1055/s-0035-1548765.

147. Martinelli I, Tomassoni D, Roy P, Di Cesare Mannelli L, Amenta F, Tayebati SK. Antioxidant Properties of Alpha-Lipoic (Thioctic) Acid Treatment on Renal and Heart Parenchyma in a Rat Model of Hypertension. *Antioxidants (Basel).* **2021***; Jun 23;10(7):1006.* doi: 10.3390/antiox10071006.

148. Canavan M, O'Donnell MJ. Hypertension and Cognitive Impairment: A Review of Mechanisms and Key Concepts. *Front Neurol.* **2022***; Feb 4;13:821135.* doi: 10.3389/fneur.2022.821135.

149. Tomassoni D, Avola R, Mignini F, Parnetti L, Amenta F. Effect of treatment with choline alphoscerate on hippocampus microanatomy and glial reaction in spontaneously hypertensive rats. *Brain Res.* **2006***; Nov 20;1120(1):183-90.* doi: 10.1016/j.brainres.2006.08.068.

150. Gomes MB, Negrato CA. Alpha-lipoic acid as a pleiotropic compound with potential therapeutic use in diabetes and other chronic diseases. *Diabetol Metab Syndr.* **2014***; Jul 28;6(1):80.* doi: 10.1186/1758-5996-6-80.

151. Tomassoni D, Amenta F, Amantini C, Farfariello V, Di Cesare Mannelli L, Nwankwo IE, Marini C, Tayebati SK. Brain activity of thioctic Acid enantiomers: in vitro and in vivo studies in an animal model of cerebrovascular injury. *Int J Mol Sci.* **2013***; Feb 26;14(3):4580-95.* doi: 10.3390/ijms14034580.

152. Manna P, Jain SK. Obesity, Oxidative Stress, Adipose Tissue Dysfunction, and the Associated Health Risks: Causes and Therapeutic Strategies. *Metab Syndr Relat Disord.* **2015***; Dec;13(10):423-44.* doi: 10.1089/met.2015.0095.

153. Salinthone S, Yadav V, Bourdette DN, Carr DW. Lipoic acid: a novel therapeutic approach for multiple sclerosis and other chronic inflammatory diseases of the CNS. *Endocr Metab Immune Disord Drug Targets.* **2008** *;Jun;8(2):132-42.* doi: 10.2174/187153008784534303.

154. Roy P, Tomassoni D, Traini E, Martinelli I, Micioni Di Bonaventura MV, Cifani C, Amenta F, Tayebati SK. Natural Antioxidant Application on Fat Accumulation: Preclinical Evidence. *Antioxidants (Basel).* **2021** *May 27;10(6):858*. doi: 10.3390/antiox10060858.

155. Seifar F, Khalili M, Khaledyan H, Amiri Moghadam S, Izadi A, Azimi A, Shakouri SK. α-Lipoic acid, functional fatty acid, as a novel therapeutic alternative for central nervous system

diseases: A review. *Nutr Neurosci.* **2019***; May;22(5):306-316.* doi: 10.1080/1028415X.2017.1386755.

156. Bush AI. Metal complexing agents as therapies for Alzheimer's disease. *Neurobiol Aging.* **2002***; Nov-Dec;23(6):1031-8.* doi: 10.1016/s0197-4580(02)00120-3.

157. Kim HS, Kim HJ, Park KG, Kim YN, Kwon TK, Park JY, Lee KU, Kim JG, Lee IK. Alpha-lipoic acid inhibits matrix metalloproteinase-9 expression by inhibiting NF-kappaB transcriptional activity. *Exp Mol Med.* **2007***; Feb 28;39(1):106-13.* doi: 10.1038/emm.2007.12.

158. Seifar F, Khalili M, Khaledyan H, Amiri Moghadam S, Izadi A, Azimi A, Shakouri SK. α-Lipoic acid, functional fatty acid, as a novel therapeutic alternative for central nervous system diseases: A review. *Nutr Neurosci.* **2019***; May;22(5):306-316.* doi: 10.1080/1028415X.2017.1386755.

159. Sancheti H, Akopian G, Yin F, Brinton RD, Walsh JP, Cadenas E. Age-dependent modulation of synaptic plasticity and insulin mimetic effect of lipoic acid on a mouse model of Alzheimer's disease. *PLoS One***. 2013***; Jul 17;8(7):e69830.* doi: 10.1371/journal.pone.0069830.

160. Tayebati SK, Di Tullio MA, Tomassoni D, Amenta F. Neuroprotective effect of treatment with galantamine and choline alphoscerate on brain microanatomy in spontaneously hypertensive rats. *J Neurol Sci.* **2009***; Aug 15;283(1-2):187-94.* doi: 10.1016/j.jns.2009.02.349.

161. Tomassoni D, Catalani A, Cinque C, Di Tullio MA, Tayebati SK, Cadoni A, Nwankwo IE, Traini E, Amenta F. Effects of cholinergic enhancing drugs on cholinergic transporters in the brain and peripheral blood lymphocytes of spontaneously hypertensive rats. *Curr Alzheimer Res.* **2012***; Jan;9(1):120-7.* doi: 10.2174/156720512799015118.

162. Traini E, Bramanti V, Amenta F. Choline alphoscerate (alpha-glyceryl-phosphoryl-choline) an old choline- containing phospholipid with a still interesting profile as cognition enhancing agent. *Curr Alzheimer Res.* **2013***; Dec;10(10):1070-9.* doi: 10.2174/15672050113106660173.

163. Amenta F, Parnetti L, Gallai V, Wallin A. Treatment of cognitive dysfunction associated with Alzheimer's disease with cholinergic precursors. Ineffective treatments or inappropriate approaches? *Mech Ageing Dev.* **2001***; Nov;122(16):2025-40.* doi: 10.1016/s0047-6374(01)00310-4.

164. Sagaro GG, Traini E, Amenta F. Activity of Choline Alphoscerate on Adult-Onset Cognitive Dysfunctions: A Systematic Review and Meta-Analysis. *J Alzheimers Dis.* **2023***;92(1):59-70.* doi: 10.3233/JAD-221189.

165. Tayebati SK, Martinelli I, Moruzzi M, Amenta F, Tomassoni D. Choline and Choline alphoscerate Do Not Modulate Inflammatory Processes in the Rat Brain. *Nutrients.* **2017***; Sep 29;9(10):1084.* doi: 10.3390/nu9101084.

166. Ren C, Tong YL, Li JC, Lu ZQ, Yao YM. The Protective Effect of Alpha 7 Nicotinic Acetylcholine Receptor Activation on Critical Illness and Its Mechanism. *Int J Biol Sci.* **2017***; Jan 1;13(1):46-56.* doi: 10.7150/ijbs.16404.

167. Roy P, Tomassoni D, Nittari G, Traini E, Amenta F. Effects of choline containing phospholipids on the neurovascular unit: A review. *Front Cell Neurosci.* **2022***; Sep 23;16:988759.* doi: 10.3389/fncel.2022.988759.

168. Leong XF, Ng CY, Jaarin K. Animal Models in Cardiovascular Research: Hypertension and Atherosclerosis. *Biomed Res Int.* **2015***; 2015:528757*. doi: 10.1155/2015/528757.

169. Lerman LO, Kurtz TW, Touyz RM, Ellison DH, Chade AR, Crowley SD, Mattson DL, Mullins JJ, Osborn J, Eirin A, Reckelhoff JF, Iadecola C, Coffman TM. Animal Models of Hypertension: A Scientific Statement from the American Heart Association. *Hypertension.* **2019;** *Jun;73(6):e87-e120.* doi: 10.1161/HYP.0000000000000090.

170. Risler NR, Cruzado MC, Miatello RM. Vascular remodeling in experimental hypertension. *Scientific World Journal.* **2005** *Dec 12; 5:959-71*. doi: 10.1100/tsw.2005.122.

171. Amenta F, Tomassoni D. Spontaneously Hypertensive Rat (SHR): An Animal Model of Vascular Brain Disorder. In: De Deyn, P., Van Dam, D. (eds) Animal Models of Dementia. *Neuromethods.* **2011***:48 (30)*. Humana Press, NM,volume 48.

172. Tayebati SK, Tomassoni D, Amenta F. Spontaneously hypertensive rat as a model of vascular brain disorder: microanatomy, neurochemistry and behavior. *J Neurol Sci.* **2012***; Nov 15;322(1-2):241-9.* doi: 10.1016/j.jns.2012.05.047.

173. Tayebati SK, Amenta F, Tomassoni D. Cerebrovascular and blood-brain barrier morphology in spontaneously hypertensive rats: effect of treatment with choline alphoscerate. *CNS Neurol Disord Drug Targets.* **2015***;14(3):421-9.* doi: 10.2174/1871527314666150225140855.

174. Sabbatini M, Strocchi P, Vitaioli L, Amenta F. The hippocampus in spontaneously hypertensive rats: a quantitative microanatomical study. *Neuroscience***.** **2000***;100(2):251-8.* doi: 10.1016/s0306-4522(00)00297-9.

175. Amenta F, Tayebati SK, Tomassoni D. Spontaneously hypertensive rat neuroanatomy: applications to pharmacological research. *Ital J Anat Embryol.* **2010***;115(1-2):13-7.* PMID: 21072984.

176. Tomassoni D, Avola R, Di Tullio MA, Sabbatini M, Vitaioli L, Amenta F. Increased expression of glial fibrillary acidic protein in the brain of spontaneously hypertensive rats. *Clin Exp Hypertens.* **2004***; May;26(4):335-50.* doi: 10.1081/ceh-120034138.

177. Sun L, Gao YH, Tian DK, Zheng JP, Zhu CY, Ke Y, Bian K. Inflammation of different tissues in spontaneously hypertensive rats. *Sheng Li Xue Bao.* **2006***; Aug 25;58(4):318-23.* PMID: 16906331.

178. Arika WM, Kibiti CM, Njagi JM, Ngugi MP. Effects of DCM Leaf Extract of Gnidia glauca (Fresen) on Locomotor Activity, Anxiety, and Exploration-Like Behaviors in High-Fat Diet-Induced Obese Rats. *Behav Neurol*. **2019**, *Dec 20;2019:7359235*. doi: 10.1155/2019/7359235

179. Branchi, I., & Ricceri, L. (**2013**). Active and passive avoidance. In W. Crusio, F. Sluyter, R. Gerlai, & S. Pietropaolo (Eds.), Behavioral Genetics of the Mouse (Cambridge Handbooks

in Behavioral Genetics, pp. 291-298). Cambridge: Cambridge University Press. doi:10.1017/CBO9781139541022.029.

180. Stankowski J, Codreanu S, Liebler D, et al. Analysis of Protein Targets by Oxidative Stress Using the Oxyblot and Biotin−Avidin-Capture Methodology; Human Press: New York**, 2011.** DOI: 10.1007/978-1-61779-077-5_18.

181. Dalle-Donne I, Rossi R, Giustarini D, Milzani A, Colombo R. Protein carbonyl groups as biomarkers of oxidative stress. *Clin Chim Acta.* **2003***; Mar;329(1-2):23-38.* doi: 10.1016/s0009-8981(03)00003-2.

182. Mahmood T, Yang PC. Western blot: technique, theory, and trouble shooting. *N Am J Med Sci.* **2012***; Sep;4(9):429-34.* doi: 10.4103/1947-2714.100998.

183. Kurien BT, Scofield RH. Western blotting: an introduction. *Methods Mol Biol.* **2015***; 1312:17-30*. doi: 10.1007/978-1-4939-2694-7_5.

184. Ramos-Vara JA. Technical aspects of immunohistochemistry. *Vet Pathol.* **2005***; Jul;42(4):405-26*. doi: 10.1354/vp.42-4-405.

185. Dahlöf B. Prevention of stroke in patients with hypertension. *Am J Cardiol.* **2007***; Aug 6;100(3A):17J-24J*. doi: 10.1016/j.

186. Caruso P, Signori R, Moretti R. Small vessel disease to subcortical dementia: a dynamic model, which interfaces aging, cholinergic dysregulation and the neurovascular unit. *Vasc Health Risk Manag.* **2019***; Aug 7;15:259-281*. doi: 10.2147/VHRM.S190470.

187. Hainsworth AH, Markus HS. Do in vivo experimental models reflect human cerebral small vessel disease? A systematic review. *J Cereb Blood Flow Metab.* **2008***; Dec;28(12):1877-91*. doi: 10.1038/jcbfm.2008.91.

188. Everitt BJ, Robbins TW. Central cholinergic systems and cognition. *Annu Rev Psychol.* **1997***; 48:649-84*. doi: 10.1146/annurev.psych.48.1.649.

189. Román GC. Vascular dementia: distinguishing characteristics, treatment, and prevention. *J Am Geriatr Soc.* **2003***; May;51(5 Suppl Dementia):S296-304.* doi: 10.1046/j.1532-5415.5155.x.

190. Katsi V, Marketou M, Maragkoudakis S, Didagelos M, Charalambous G, Parthenakis F, Tsioufis C, Tousoulis D. Blood-brain barrier dysfunction: the undervalued frontier of hypertension. *J Hum Hypertens.* **2020***; Oct;34(10):682-691*. doi: 10.1038/s41371-020-0352-2.

191. Griendling KK, Camargo LL, Rios FJ, Alves-Lopes R, Montezano AC, Touyz RM. Oxidative Stress and Hypertension. *Circ Res.* **2021***; Apr 2;128(7):993-1020.* doi: 10.1161/CIRCRESAHA.121.318063.

192. Portaluppi F, Boari B, Manfredini R. Oxidative stress in essential hypertension. *Curr Pharm Des.* **2004***;10(14):1695-8.* doi: 10.2174/1381612043384619.

193. Zhang WJ, Frei B. Alpha-lipoic acid inhibits TNF-alpha-induced NF-kappaB activation and adhesion molecule expression in human aortic endothelial cells. *FASEB J.* **2001***; Nov;15(13):2423-32.* doi: 10.1096/fj.01-0260com.

194. Fogacci F, Rizzo M, Krogager C, Kennedy C, Georges CMG, Knežević T, Liberopoulos E, Vallée A, Pérez-Martínez P, Wenstedt EFE, Šatrauskienė A, Vrablík M, Cicero AFG. Safety Evaluation of α-Lipoic Acid Supplementation: A Systematic Review and Meta-Analysis of Randomized Placebo-Controlled Clinical Studies. *Antioxidants (Basel).* **2020***; Oct 19;9(10):1011*. doi: 10.3390/antiox9101011.

195. Lucarini E, Trallori E, Tomassoni D, Amenta F, Ghelardini C, Pacini A, Di Cesare Mannelli L. Toxicological Profile of the Pain-Relieving Antioxidant Compound Thioctic Acid in Its Racemic and Enantiomeric Forms. *Antioxidants (Basel).* **2020***; Aug 14;9(8):749.* doi: 10.3390/antiox9080749.

196. Rocamonde B, Paradells S, Barcia JM, Barcia C, García Verdugo JM, Miranda M, Romero Gómez FJ, Soria JM. Neuroprotection of lipoic acid treatment promotes angiogenesis and reduces the glial scar formation after brain injury. *Neuroscience.* **2012***; Nov 8; 224:102-15.* doi: 10.1016/j.neuroscience.2012.08.028.

197. Streeper RS, Henriksen EJ, Jacob S, Hokama JY, Fogt DL, Tritschler HJ. Differential effects of lipoic acid stereoisomers on glucose metabolism in insulin-resistant skeletal muscle. *Am J Physiol.* **1997***; Jul;273(1 Pt 1):E185-91*. doi: 10.1152/ajpendo.1997.273.1.E185.

198. Carlson BA, Kingston JD. Docosahexaenoic acid biosynthesis and dietary contingency: Encephalization without aquatic constraint. *Am J Hum Biol***. 2007** *Jul-Aug;19(4):585-8.* doi: 10.1002/ajhb.20683.

199. Salehi B, Berkay Yılmaz Y, Antika G, Boyunegmez Tumer T, Fawzi Mahomoodally M, Lobine D, Akram M, Riaz M, Capanoglu E, Sharopov F, Martins N, Cho WC, Sharifi-Rad J. Insights on the Use of α-Lipoic Acid for Therapeutic Purposes. *Biomolecules.* **2019***; Aug 9;9(8):356.* doi: 10.3390/biom9080356.

200. Francis PT, Nordberg A, Arnold SE. A preclinical view of cholinesterase inhibitors in neuroprotection: do they provide more than symptomatic benefits in Alzheimer's disease? *Trends Pharmacol Sci.* **2005***; Feb;26(2):104-11*. doi: 10.1016/j.tips.2004.12.010.

201. Kavirajan H, Schneider LS. Efficacy and adverse effects of cholinesterase inhibitors and memantine in vascular dementia: a meta-analysis of randomised controlled trials. *Lancet Neurol.* **2007***; Sep;6(9):782-92.* doi: 10.1016/S1474-4422(07)70195-3.

202. Parnetti L, Mignini F, Tomassoni D, Traini E, Amenta F. Cholinergic precursors in the treatment of cognitive impairment of vascular origin: ineffective approaches or need for re-evaluation? *J Neurol Sci.* **2007***; Jun 15;257(1-2):264-9.* doi: 10.1016/j.jns.2007.01.043.

203. Brashear HR. Galantamine in the treatment of vascular dementia. *Int Psychogeriatr.* **2003***;15 Suppl 1:187-93.* doi: 10.1017/S1041610203009189.

204. Court JA, Perry EK. Neurotransmitter abnormalities in vascular dementia. *Int Psychogeriatr.* **2003***;15 Suppl 1:81-7*. doi: 10.1017/S1041610203009013.

205. Pavlov VA, Tracey KJ. Controlling inflammation: the cholinergic anti-inflammatory pathway. *Biochem Soc Trans.* **2006***; Dec;34(Pt 6):1037-40.* doi: 10.1042/BST0341037.

206. Takada-Takatori Y, Kume T, Sugimoto M, Katsuki H, Sugimoto H, Akaike A. Acetylcholinesterase inhibitors used in treatment of Alzheimer's disease prevent glutamate

neurotoxicity via nicotinic acetylcholine receptors and phosphatidylinositol 3-kinase cascade. *Neuropharmacology.* **2006;** *Sep;51(3):474-86.* doi: 10.1016/j.neuropharm.2006.04.007.

207. Shytle RD, Mori T, Townsend K, Vendrame M, Sun N, Zeng J, Ehrhart J, Silver AA, Sanberg PR, Tan J. Cholinergic modulation of microglial activation by alpha 7 nicotinic receptors. *J Neurochem.* **2004***; Apr;89(2):337-43.* doi: 10.1046/j.1471-4159.2004.02347.x.

208. Alkondon M, Albuquerque EX. The nicotinic acetylcholine receptor subtypes and their function in the hippocampus and cerebral cortex. *Prog Brain Res.* **2004***; 145:109-20.* doi: 10.1016/S0079-6123(03)45007-3.

209. Patel H, McIntire J, Ryan S, Dunah A, Loring R. Anti-inflammatory effects of astroglial α7 nicotinic acetylcholine receptors are mediated by inhibition of the NF-κB pathway and activation of the Nrf2 pathway. *J Neuroinflammation.* **2017***; Sep 26;14(1):192.* doi: 10.1186/s12974-017-0967-6.

210. Piovesana R, Salazar Intriago MS, Dini L, Tata AM. Cholinergic Modulation of Neuroinflammation: Focus on α7 Nicotinic Receptor. *Int J Mol Sci.* **2021***; May 6;22(9):4912.* doi: 10.3390/ijms22094912.

211. Tőkés T, Tuboly E, Varga G, Major L, Ghyczy M, Kaszaki J, Boros M. Protective effects of L-alpha-glycerylphosphorylcholine on ischaemia-reperfusion-induced inflammatory reactions. *Eur J Nutr.* **2015***; Feb;54(1):109-18*. doi: 10.1007/s00394-014-0691-2.

212. Hajizadeh-Sharafabad F, Sharifi Zahabi E. Role of alpha-lipoic acid in vascular function: A systematic review of human intervention studies. *Crit Rev Food Sci Nutr.* **2022***;62(11):2928-2941*. doi: 10.1080/10408398.2020.1861425.

213. Konukoglu D, Uzun H. Endothelial Dysfunction and Hypertension. *Adv Exp Med Biol.* **2017***; 956:511-540*. doi: 10.1007/5584_2016_90.

214. Bengtsson SH, Gulluyan LM, Dusting GJ, Drummond GR. Novel isoforms of NADPH oxidase in vascular physiology and pathophysiology. *Clin Exp Pharmacol Physiol.* **2003***; Nov;30(11):849-54.* doi: 10.1046/j.1440-1681.2003.03929.x.

215. Park JB, Charbonneau F, Schiffrin EL. Correlation of endothelial function in large and small arteries in human essential hypertension. *J Hypertens.* **2001***; Mar;19(3):415-20.* doi: 10.1097/00004872-200103000-00009.

216. Tain YL, Hsu CN. Oxidative Stress-Induced Hypertension of Developmental Origins: Preventive Aspects of Antioxidant Therapy. *Antioxidants (Basel).* **2022***; Mar 7;11(3):511*. doi: 10.3390/antiox11030511.

217. Li Q, Youn JY, Cai H. Mechanisms and consequences of endothelial nitric oxide synthase dysfunction in hypertension. *J Hypertens.* **2015** *; Jun;33(6):1128-36*. doi: 10.1097/HJH.0000000000000587.

218. Vasdev S, Ford CA, Parai S, Longerich L, Gadag V. Dietary alpha-lipoic acid supplementation lowers blood pressure in spontaneously hypertensive rats. *J Hypertens.* **2000***; May;18(5):567-73*. doi: 10.1097/00004872-200018050-00009.

219. Pacini A, Tomassoni D, Trallori E, Micheli L, Amenta F, Ghelardini C, Di Cesare Mannelli L, Traini E. Comparative Assessment of the Activity of Racemic and Dextrorotatory Forms of Thioctic (Alpha-Lipoic) Acid in Low Back Pain: Preclinical Results and Clinical Evidences From an Open Randomized Trial. *Front Pharmacol.* **2021***; Feb 24;12:607572.* doi: 10.3389/fphar.2021.607572.

220. Barcia C, Sanderson NS, Barrett RJ, Wawrowsky K, Kroeger KM, Puntel M, Liu C, Castro MG, Lowenstein PR. T cells' immunological synapses induce polarization of brain astrocytes in vivo and in vitro: a novel astrocyte response mechanism to cellular injury. *PLoS One***. 2008** *;Aug 20;3(8):e2977.* doi: 10.1371/journal.pone.0002977.

221. Faulkner JR, Herrmann JE, Woo MJ, Tansey KE, Doan NB, Sofroniew MV. Reactive astrocytes protect tissue and preserve function after spinal cord injury. *J Neurosci.* **2004** *;Mar 3;24(9):2143-55*. doi: 10.1523/JNEUROSCI.3547-03.2004.

222. Brown GC, Neher JJ. Microglial phagocytosis of live neurons. *Nat Rev Neurosci.* **2014;** *Apr;15(4):209-16*. doi: 10.1038/nrn3710.

223. Jolivel V, Bicker F, Binamé F, Ploen R, Keller S, Gollan R, Jurek B, Birkenstock J, Poisa-Beiro L, Bruttger J, Opitz V, Thal SC, Waisman A, Bäuerle T, Schäfer MK, Zipp F, Schmidt MHH. Perivascular microglia promote blood vessel disintegration in the ischemic penumbra. *Acta Neuropathol.* **2015***; Feb;129(2):279-95.* doi: 10.1007/s00401-014-1372-1.

224. Kim SM, Ha JS, Han AR, Cho SW, Yang SJ. Effects of α-lipoic acid on LPS-induced neuroinflammation and NLRP3 inflammasome activation through the regulation of BV-2 microglial cells activation.*BMBRep.***2019***;Oct;52(10):613-618*. doi: 10.5483/BMBRep.2019.52.10.026.

225. Ishida H, Takemori K, Dote K, Ito H. Expression of glucose transporter-1 and aquaporin-4 in the cerebral cortex of stroke-prone spontaneously hypertensive rats in relation to the blood-brain barrier function. *Am J Hypertens.* **2006;** *Jan;19(1):33-9.* doi: 10.1016/j.amjhyper.2005.06.023.

226. Tomassoni D, Bramanti V, Amenta F. Expression of aquaporins 1 and 4 in the brain of spontaneously hypertensive rats. *Brain Res.* **2010***; Apr 14; 1325:155-63.* doi: 10.1016/j.brainres.2010.02.023.

227. Zhou J, Kong H, Hua X, Xiao M, Ding J, Hu G. Altered blood-brain barrier integrity in adult aquaporin-4 knockout mice. *Neuroreport.* **2008; Jan** *8;19(1):1-5.* doi: 10.1097/WNR.0b013e3282f2b4eb.

228. Ozgür B, Helms HCC, Tornabene E, Brodin B. Hypoxia increases expression of selected blood-brain barrier transporters GLUT-1, P-gp, SLC7A5 and TFRC, while maintaining barrier integrity, in brain capillary endothelial monolayers. *Fluids Barriers CNS***. 2022***; Jan 4;19(1):1.* doi: 10.1186/s12987-021-00297-6.

Summary of PhD Activities and List of publications

The PhD activity focused on cerebrovascular disease related to hypertension and the effects of antioxidant and cholinergic precursor molecules.

Hypertension represents a risk factor for the development of cerebrovascular disease and cognitive impairment. Based on this hypothesis, I designed my study to assess if a cholinergic precursors drug, choline alphoscerate or alpha-glycerylphosphorylcholine (GPC), causes an increase in acetylcholine levels improving the cholinergic system countering cognitive impairment, associated with cerebrovascular damage, and could targeting neuroinflammation. (+) Thioctic acid [(+) TIO] is the naturally occurring eutomer that has been shown to anti-inflammatory and antioxidant effects in the brain.

Moreover, in the meanwhile I assessed these molecules effects on the heart of spontaneously hypertensive rats. This works results are going to be published by the Journal of hypertension.

In the beginning of my PhD activities, I was continuing the work followed during my thesis on master's degree on the characterization of transient receptor potential (TRP) cation channels on the brain of rats with Diet Induced Obesity (DIO) and the effects of a supplementation with natural antioxidant compounds. Moreover, the analysis of the alterations of heart in spontaneously hypertensive rats (SHR) and DIO rats were performed, to identify the protective effects of (+) TIO and of a supplementation with tart cherry seeds and juice.

During the aboard period followed at the Medical University of Vienna, under the supervision of the prof. Sigismund Huck, we characterized the role of the Ca^{2+} on the, nAChRα7 signaling using neuronal cell culture obtained from superior cervical ganglion (SCG).

He wants to investigate that some people claim that the nAChRα7 signals via metabotropic, in addition to ionotropic, mechanisms. In my hands, the ionotropic mechanism (outlined above) seems to predominate. Still, we will have to test PLC inhibitors like U73122.

For this experiment he isolated the superior cervical ganglion (SCG) neurons from mouse pups in short term-cultures (1-3 days in vitro, DIV). Effects by just PNU282 are more often seen after 1 DIV than in older cultures. The concentration range is narrow, I see maximal effects with 0.1 µM

PNU282. Lower concentrations are sub-threshold, and with higher concentration, receptors enter the desensitized state.

There we indirectly monitor nAChRα7 activity via intracellular Ca^{2+} we must consider Ca^{2+} pathways. The activated nAChRα7 receptor passes Ca^{2+} which in turn activates Ca^{2+} induced Ca^{2+} release from the ER via ryanodine receptors. Ryanodine at low concentrations activates and at high concentrations inhibits Ca^{2+}-induced Ca^{2+} release. Hence, the high responders in nAChRα7 receptor activation could also be the result of a particular sensitive Ca^{2+}-induced Ca^{2+} release.

We saw maximal response in virtually all cells when adding 10 mM caffeine (which also activates the ryanodine receptor) in 1 DIV SCG neurons. So, for the next experiments we will initially reduce the caffeine concentration to 10.0, 3.0, and 1.0 µM. After having determined the threshold, I will use the lowest effective caffeine concentration for observations with 0.1 µM PNU282 to see whether the same cells respond to the two test compounds. These experiments will be done with both 1 and 2 DIV cultures. The time course the elevated Ca^{2+} caused by PNU282 with and without PNU120. Is of interest. The Fura2 signal declines more rapidly in the presence of caffeine than upon nAChRα7 receptor activation. Ca^{2+} levels decline if Ca^{2+} is more effectively reduced (by SERCA, smooth endoplasmic reticulum (ER) Ca^{2+} ATPase and by PMCA, the plasma membrane Ca^{2+} ATPase) than released (via ryanodine receptors).

Without extracellular Ca^{2+} (with added 0.5 µM EGTA), nAChRα7 activation does not induce any Fura2 signal. Pretreatment of cultures with 2 µM xestospongin C also completely suppresses any Fura2 signal. Xestospongin C is mostly considered an inhibitor of IP3 receptors. However, some people claim that it depletes ER of Ca^{2+} by inhibiting SERCA, just as thapsigargin.

However, when 2 DIV cultures were treated with 5 µM thapsigargin I did not observe large inhibition of nAChRα7 receptor mediated Fura2 transients. We must repeat the thapsigargin experiment with higher concentrations, and we will check whether xestospongin C does in fact deplete the ER. This can be easily monitored with Fura2. Given the complete inhibition of Fura2 transients by xestospongin C we also need to check whether it directly affects nAChRα7 receptors. Further studies need to understand the variable effects of Caffeine and the bradykinin effects in the absence of Ca^{2+}.

During my PhD period I was a supervisor of students enrolled on the bachelor's degree in Bioscience and Biotechnology and master's degree in Biological Sciences of the University of Camerino. I helped them as a co supervisor to complete their Stage and master's thesis activity.

In the list the name of the students and the thesis title:

Bachelor's degree student: Sofia Christina Gkolfinopoulou

Immunohistochemistry to identify the TRP channels expression in kidney of obese rats.

Bachelor's degree student: Saqib Hussain

Morphological techniques to evaluate the neuroinflammation in hypertensive rats.

Bachelor's degree student: Emmanuella Onosetale Ojuromi

Morphological evaluation of the small intestine in an animal model of binge-eating disorder.

Master's degree student: MD Abdullah Al Kafi

Brain Alterations in an animal model of cerebrovascular disease: protective effects of choline alphoscerate and thioctic acid.

The data that were obtained in the laboratory of Human Anatomy of University of Camerino during the PhD activities were published in different papers (list of publications) and were presented at different National and International Conference (Abstract conferences).

List of Publications

1. **Proshanta Roy** , Daniele Tomassoni , Ilenia Martinelli, Vincenzo Bellitto, Giulio Nittari, Francesco Amenta, Seyed Khosrow Tayebati. **Protective effects of the (+)-thioctic acid treatment: possible anti-inflammatory activity on heart of hypertensive rats. Submitted in BMC Complementary Medicine and Therapies, 2023 July.**

2. Giulio Nittari, **Proshanta Roy,** Ilenia Martinelli, Vincenzo Bellitto, Daniele Tomassoni, EneaTraini, Seyed Khosrow Tayebati1, Francesco Amenta. **Rodent models of huntington's disease: an overview. In preparation for Journal of Neurosciences Research.**

3. Giulio Nittari **,** Daniele Tomassoni, **Proshanta Roy**, Ilenia Martinelli , Seyed Khosrow Tayebati , Francesco Amenta**. Batten disease through different in vivo and in vitro models: A review.** *J Neurosci Res* **2022**, *Mar;101(3):298-315. doi: 10.1002/jnr.25147.*

4. **Proshanta Roy**, Daniele Tomassoni, Giulio Nittari, Enea Traini, Francesco Amenta. **Effects of choline containing phospholipids on the neurovascular unit**. *Front. Cell. Neurosci.Cellular Neurophysiology*, **2022** ,*Sep 23;16:988759. doi: 10.3389/fncel.2022.988759*.

5. Ilenia Martinelli, Daniele Tomassoni, Vincenzo Bellitto, **Proshanta Roy**, Maria Vittoria Micioni Di Bonaventura, Francesco Amenta, Consuelo Amantini, Carlo Cifani 1, and Seyed Khosrow Tayebati. **Anti Inflammatory and Antioxidant Properties of Tart Cherry Consumption in the Heart of Obese Rats.** *Biology*, **2022**, *Apr 23;11(5):646. doi: 10.3390/biology11050646.*

6. Ilenia Martinelli, Seyed Khosrow Tayebati, Daniele Tomassoni, Giulio Nittari, **Proshanta Roy**, Francesco Amenta. **Brain and Retinal Organoids for Disease Modeling: The Importance of In Vitro Blood-Brain and Retinal Barriers Studies**. *Cells*, **2022,** *Mar 25;11(7):1120. doi: 10.3390/cells11071120.*

7. Ilenia Martinelli , Seyed Khosrow Tayebati , **Proshanta Roy** , Maria Vittoria Micioni Di Bonaventura , Michele Moruzzi , Carlo Cifani , Francesco Amenta , Daniele Tomassoni .**Obesity-Related Brain Cholinergic System Impairment in High-Fat-Diet-Fed Rats.** *Nutrients***. 2022**, *Mar 15;14(6):1243. doi: 10.3390/nu14061243.*

8. **Proshanta Roy**, Ilenia Martinelli, Michele Moruzzi, Maria Vittoria Micioni Di Bonaventura , Carlo Cifani, Consuelo Amantini , Federica Maggi , Seyed Khosrow Tayebati , Francesco Amenta , Daniele Tomassoni. **Ion channels alteration in the forebrain of high fat diet-fed rats**. *European Journal of Histochemistry*, **2021**, *Nov 23;65(s1):3305. doi: 10.4081/ejh.2021.3305.*

9. Ilenia Martinelli , Daniele Tomassoni, **Proshanta Roy**, Francesco Amenta, Seyed Khosrow Tayebati. **Altered Brain Cholinergic and Synaptic Markers in Obese Zucker Rats**. *Cells*, **2021,** *Sep 24;10(10):2528. doi: 10.3390/cells10102528.*

10. **Proshanta Roy**, Daniele Tomassoni, EneaTraini, Ilenia Martinelli, Francesco Amenta, Seyed Khosrow Tayebati. **Natural Antioxidants Application on Fat Accumulation: Preclinical Evidence**. *Antioxidant*, **2021**, *May 27;10(6):858. doi: 10.3390/antiox10060858.*

11. Ilenia Martinelli, Daniele Tomassoni, **Proshanta Roy**, Lorenzo Di Cesare Mannelli, Francesco Amenta, Seyed Khosrow Tayebati.**Antioxidant properties of alpha-lipoic (thioctic) acid treatment on renal and heart parenchyma in a rat model of hypertension**. *Antioxidants*, **2021,** *Jun 23;10(7):1006. doi: 10.3390/antiox10071006.*

12. Camilla Ceccarelli, Valeria Zeni, Roberto Rizzo, Gabriella Lo Verde Milko Sinacori, Maria C. Boukouvala, Nickolas G. Kavalieratos, Massimo Ubaldi, Daniele Tomassoni, Federica Benvenuti, **Proshanta Roy**, Riccardo Petrelli, Loredana Cappellacci, Eleonora Spinozzi, Filippo Maggi, Angelo Canale. **Lethal and behavioral effects of a green insecticide against an invasive polyphagous fruit fly pest and its safety to mammals.** *Chemosphere*, **2022**, *Jan;287(Pt 2):132089. doi: 10.1016/j.chemosphere.2021.132089.*

13. Michele Moruzzi, Nora Klöeting, Matthias Blüher, Ilenia Martinelli, Seyed Khosrow Tayebati, Maria Gabriella Gabrielli, **Proshanta Roy**, Maria Vittoria Micioni Di Bonaventura, Carlo Cifani, Giulio Lupidi, Francesco Amenta, Daniele Tomassoni. **Tart Cherry Juice and seeds affect Pro-Inflammatory Markers in Visceral Adipose Tissue of High-fat Diet Obese Rats**. *Molecules*, **2021**, *Mar 5;26(5):1403. doi: 10.3390/molecules26051403.*

14. Ilenia Martinelli, Daniele Tomassoni, Michele Moruzzi, **Proshanta Roy**, Carlo Cifani , Francesco Amenta and Seyed Khosrow Tayebati. **Cardiovascular changes related to metabolic syndrome: evidence in obese Zucker rats**. *International journal of molecular sciences*, **2020**, *Mar 16;21(6):2035. doi: 10.3390/ijms21062035.*

Abstract conferences

1. **Neuroinflammation on hippocampus of hypertensive rats: possible protective activity of antioxidant and cholinergic cognitive enhancing compounds.**
   V. Bellitto**, P. Roy**, I Martinelli, S.K. Tayebati, F Amenta, D Tomassoni.
   76 Congress of the Italian Society of Anatomy and Histology. Modena 11-13 September 2023.

2. **Choline alphoscerate and thioctic acid effects neuroinflammation in lipopolysaccharide stimulated BV2 microglial cells.**
   I. Martinelli, S. K.Tayebati , V. Bellitto, **P. Roy**, F. Amenta, D. Tomassoni.
   76 Congress of the Italian Society of Anatomy and Histology. Modena 11-13 September 2023

3. **Morphological study of colonic mucosa in mice with dextran sulfate sodium-induced colitis: the impact of probiotic supplementation.**
   D. Tomassoni, M.G. Gabrielli, V. Bellitto, I. Martinelli, **P. Roy**, C. Salvesi, S. Silvi, A.M. Tambella, C. Miceli.
   European Journal of Histochemistry, 2023, 67, suppl2, P2.10. XXXIX Congress of the Italian Society of Histochemistry 14-16 June 2023 Vulcano Island, Italy

4. **Protective role of tart cherry against whitening of brown adipose tissue of obese rats.**
   V. Bellitto, S. K. Tayebati, I. Martinelli, P. Roy, G. Nittari, P. Cocci, F. A. Palermo, F.Amenta, D. Tomassoni, M.G. Gabrielli.
   European Journal of Histochemistry, 2023, 67, suppl2, P2.9. XXXIX Congress of the Italian Society of Histochemistry 14-16 June 2023 Vulcano Island, Italy.

5. **Neuroplasticity of enteric nervous system in animal model of obesity.**
   V. Bellitto[2], I. Martinelli [2], **P. Roy[1]**, D. Tomassoni [1], M.G. Gabrielli[1], F. Amenta[2], S.K. Tayebati[2].
   [1]School of Biosciences and Veterinary Medicine, University of Camerino; [2]School of Medicinal and Health Products Sciences, University of Camerino, Italy.
   Proceedings of the 32nd National Conference of the Italian Group for the Study of Neuromorphology "Gruppo Italiano per lo Studio della Neuromorfologia" G.I.S.N.
   Naples, November 25-26, 2022, European journal of histochemistry a journal of functional cytology, ISSN 1121-760X volume 66/ supplement 2, 2022 https://doi.org/10.4081/ejh.2022.3619 Oral presentation.

6. **Acetylcholine precursors attenuate neuroinflammation in LPS-stimulated BV2cells.**
   I.Martinelli[1], S.K. Tayebati[1], V. Bellitto[1], **P. Roy[2]**, F. Amenta [1], D. Tomassoni [2].
   Proceedings of the 32nd National Conference of the Italian Group for the Study of Neuromorphology "Gruppo Italiano per lo Studio della Neuromorfologia" G.I.S.N.
   Naples, November 25-26, 2022, European journal of histochemistry a journal of functional cytology, ISSN 1121-760X volume 66/ supplement 2, 2022 https://doi.org/10.4081/ejh.2022.3619 Poster presentation.

7. **Obesity and enteric nervous system modulation: evidence from preclinical animal models.**

   Martinelli I[1], Tayebati SK[1], Bellitto V[1], Gabrielli MG[2], **Roy P**[2], Micioni Di Bonaventura MV[1], Cifani C[1], Amenta F[1], Tomassoni D[2].

   [1]School of Pharmacy, University of Camerino, Camerino, Italy; [2]School of Biosciences and Veterinary Medicine, University of Camerino, Camerino, Italy.

   6th International Symposium in Enteric Nervous System (ENS 2022), 24-27 April 2022, Ferrara. Oral presentation.

8. **Cardiovascular changes in dietary-induced obese rats: anti-inflammatory and antioxidant effect of tart cherry supplementation.**

   Daniele Tomassoni[1], Ilenia Martinelli[2], Vincenzo Bellitto[2], **Proshanta Roy[2]**, Maria Vittoria Micioni Di Bonaventura[2], Seyed Khosrow Tayebati[2], Consuelo Amantini[1], Carlo Cifani[2] and Francesco Amenta[2]. [1]School of Biosciences and Veterinary Medicine, University of Camerino, 62032 Camerino, Italy [2] School of Medicinal and Health Products Sciences, University of Camerino, 62032 Camerino, Italy.

   75th Meeting of the Italian Society of Anatomy and Histology, Padova, 14-16 September 2022. IJAE Vol. 126, n. 1 (Supplement): 68, 2022.

9. **Choline alphoscerate and thioctic acid activity on brain injury in spontaneously hypertensive rats.**

   Seyed Khosrow Tayebati[1], **Proshanta Roy[2]**, Ilenia Martinelli[1], Daniele Tomassoni[2], Vincenzo Bellitto[1], Francesco Amenta[1].

   [1]Clinical Research Centre, School of Medicinal and Health Products Sciences, University of Camerino, 62032 Camerino, Italy; [2]School of Biosciences and Veterinary Medicine, University of Camerino, 62032 Camerino, Italy.

   75th Meeting of the Italian Society of Anatomy and Histology, Padova, 14-16 September 2022. IJAE. Vol. 126, n. 1 (Supplement): 166, 2022. Oral presentation.

10. **Heart alterations in spontaneously hypertensive rats: immunochemical and immunohistochemical assessment of the activity of dextrorotatory form of thioctic acid.**

    S. K. Tayebati, **P. Roy**, D. Tomassoni, I. Martinelli, F. Amenta,.

    74° Congress of the "Società Italiana di Anatomia e Istologia" (SIAI) Bologna 24 25 September 2021 IJAE. Vol. 125, n. 1 (Supplement): 196, 2021.

11. **Do choline alphoscerate and thioctic acid prevent cerebrovascular alterations in spontaneously hypertensive rats?**

    Ilenia Martinelli, **Proshanta Roy**, Seyed Khosrow Tayebati, EneaTraini, Francesco Amenta, Daniele Tomassoni.

    74° Congress of the "Società Italiana di Anatomia e Istologia" (SIAI) Bologna 24-25 September 2021. IJAE. Vol. 125, n. 1 (Supplement): 127, 2021.

12. **Effects of tart cherry supplementation on brown adipose tissue of high-fat diet obese rats.**

    M.G. Gabrielli, V. Bellitto, **P. Roy**, I. Martinelli, P. Cocci, F. A. Palermo, M.V. Micioni di Bonaventura, C. Cifani, S. K. Tayebati, F. Amenta, D. Tomassoni.

    74° Congress of the "Società Italiana di Anatomia e Istologia" (SIAI) Bologna 24 25 September 2021. IJAE. Vol. 125, n. 1 (Supplement): 197, 2021.

13. **Ion channels expression in the brain areas of high-fat diet fed rats.**

    P. Roy, I. Martinelli, M. Moruzzi , M. V. Micioni Di Bonaventura , C.Cifani, C. Amantini , S. K. Tayebati, F. Amenta , D. Tomassoni.

30th National Conference of the Italian Group for the Study of Neuromorphology (GISN). 25-26 November 2020.

14. Effects of choline-alphoscerate and thiocticacid on the brain of hypertensive rats

Roy P.[1], Martinelli I.[2], Tomassoni D.[1], Traini E.[2], Tayebati S.K.[2], Amenta F.[2]
[1]School of Biosciences and Veterinary Medicine, University of Camerino; [2]School of Pharmacy, University of Camerino, Camerino.
Italy Proceedings of the 31nd National Conference of the Italian Group for the Study of Neuromorphology "Gruppo Italiano per lo Studio della Neuromorfologia" G.I.S.N. Milan, November 26-27, 2021, European journal of histochemistry a journal of functional cytology ISSN 1121-760Xvolume 65/ supplement 3 2021, Oral presentation.

APPENDICES

Appendix 1

Effects of choline containing phospholipids on the neurovascular unit: A review

Proshanta Roy, Daniele Tomassoni, Giulio Nittari, Enea Traini, Francesco Amenta

Front. Cell. Neurosci.Cellular Neurophysiology, **2022** *,Sep 23;16:988759. doi: 10.3389/fncel.2022.988759*.

Appendix 2

Natural Antioxidants Application on Fat Accumulation: Preclinical Evidence.

Proshanta Roy, Daniele Tomassoni, EneaTraini, Ilenia Martinelli, Francesco Amenta, Seyed Khosrow Tayebati.

Antioxidant, **2021**, *May 27;10(6):858. doi: 10.3390/antiox10060858.*

Appendix 3

Antioxidant properties of alpha-lipoic (thioctic) acid treatment on renal and heart parenchyma in a rat model of hypertension. Ilenia Martinelli, Daniele

Tomassoni, **Proshanta Roy**, Lorenzo Di Cesare Mannelli, Francesco Amenta, Seyed Khosrow Tayebati

Antioxidants, **2021,** *Jun 23;10(7):1006. doi: 10.3390/antiox10071006.*

Appendix 4

Anti Inflammatory and Antioxidant Properties of Tart Cherry Consumption in the Heart of Obese Rats.

Ilenia Martinelli, Daniele Tomassoni, Vincenzo Bellitto, **Proshanta Roy**, Maria Vittoria Micioni Di Bonaventura, Francesco Amenta, Consuelo Amantini, Carlo Cifani 1, and Seyed Khosrow Tayebati.

Biology, **2022**, *Apr 23;11(5):646. doi: 10.3390/biology11050646.*

Appendix 5

Heart alterations in spontaneously hypertensive rats: immunochemical and immunohistochemical assessment of the activity of dextrorotatory form of thioctic acid.

Seyed Khosrow Tayebati, **Proshanta Roy**, Daniele Tomassoni, Ilenia Martinelli, Francesco Amenta,.

74° Congress of the “Società Italiana di Anatomia e Istologia" (SIAI) Bologna 24 25 September 2021 IJAE. Vol. 125, n. 1 (Supplement): 196, 2021.

Appendix 6

Protective effects of the (+)-thioctic acid treatment: possible anti-inflammatory activity on heart of hypertensive rats.

Proshanta Roy, Daniele Tomassoni, Ilenia Martinelli, Vincenzo Bellitto, Giulio Nittari, Francesco Amenta, Seyed Khosrow Tayebati

BMC Complementary Medicine and Therapies 2023.

Appendix 7

Altered Brain Cholinergic and Synaptic Markers in Obese Zucker Rats.

Ilenia Martinelli , Daniele Tomassoni, **Proshanta Roy**, Francesco Amenta, Seyed Khosrow Tayebati

Cells, **2021,** *Sep 24;10(10):2528. doi: 10.3390/cells10102528.*

Appendix 8

Do choline alphoscerate and thioctic acid prevent the cerebrovascular alterations in spontaneously hypertensive rats?

Ilenia Martinelli, **Proshanta Roy**, Seyed Khosrow Tayebati, EneaTraini, Francesco Amenta, Daniele Tomassoni.

74° Congress of the “Società Italiana di Anatomia e Istologia" (SIAI) Bologna 24-25 September 2021. IJAE. Vol. 125, n. 1 (Supplement): 127, 2021.

Appendix 9

Effects of choline-alphoscerate and thiocticacid on the brain of hypertensive rats

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Appendix 10

Choline alphoscerate and thioctic acid activity on brain injury in spontaneously hypertensive rats.

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Appendix 11

Acetylcholine precursors attenuate neuroinflammation in LPS-stimulated BV2cells.

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Effects of choline containing phospholipids on the neurovascular unit: A review

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The roles of choline and of choline-containing phospholipids (CCPLs) on the maintenance and progress of neurovascular unit (NVU) integrity are analyzed. NVU is composed of neurons, glial and vascular cells ensuring the correct homeostasis of the blood-brain barrier (BBB) and indirectly the function of the central nervous system. The CCPLs phosphatidylcholine (lecithin), cytidine 5′-diphosphocholine (CDP-choline), choline alphoscerate or α-glyceryl-phosphorylcholine (α-GPC) contribute to the modulation of the physiology of the NVU cells. A loss of CCPLs contributes to the development of neurodegenerative diseases such as Alzheimer's disease, multiple sclerosis, Parkinson's disease. Our study has characterized the cellular components of the NVU and has reviewed the effect of lecithin, of CDP-choline and α-GPC documented in preclinical studies and in limited clinical trials on these compounds. The interesting results obtained with some CCPLs, in particular with α-GPC, probably would justify reconsideration of the most promising molecules in larger attentively controlled studies. This can also contribute to better define the role of the NVU in the pathophysiology of brain disorders characterized by vascular impairment.

KEYWORDS

neurovascular unit, choline containing phospholipids, phosphatidylcholine, CDP-choline, choline alphoscerate

Introduction

Choline plays a prominent role in the synthesis of different membrane phospholipids (Javaid et al., 2021). Moreover, choline is essential for cholinergic neurons to synthesize the neurotransmitter acetylcholine. The compound *via* choline-containing phospholipids (CCPLs, **Figure 1**) modulates various pathways of intercellular communication and pathophysiological processes through neurovascular coupling

(Attwell et al., 2010), cortical spreading depression (Gursoy-Ozdemir et al., 2004), or induction of oxidative stress (Yemisci et al., 2009). Choline is required for the synthesis of phosphatidylcholine, lyso-phosphatidylcholine, sphingomyelin, and choline plasmalogen, as well as other phospholipids. It has a well-established involvement in neurogenesis and memory development, and its absence may result in neural tube abnormalities (Paoletti et al., 2011). CCPLs such as phosphatidylcholine or lecithin, cytidine 5′-diphosphocholine (CDP-choline) and alpha-glyceryl-phosphorylcholine or choline alphoscerate (α-GPC) are acetylcholine (ACh) precursors that easily traverse the blood brain barrier (BBB). They act through two separate mechanisms of action. The precursors are first used as a substrate for acetylcholine synthesis, which can improve cholinergic neurotransmission (Hurtado et al., 2011). ACh precursors have been shown to prolong the favorable effects of acetylcholinesterase inhibitors on cognitive and behavioral improvements in patients with Alzheimer's disease (AD) and mild to moderate vascular dementia (Adibhatla et al., 2005; Amenta et al., 2020). They may have significant neuroprotective benefits in addition to serving as precursors by maintaining and supplying the membrane structure of neurons (Rao et al., 2000). Previous research has shown that precursor administration to rats 3 weeks after a pilocarpine-induced seizure increased neurogenesis. This suggests that ACh precursors can be used not only as neuroprotective substances but also to aid neurogenesis after ischemic or epileptic damage (Diederich et al., 2012; Lee et al., 2017). CCPLs and the acetylcholinesterase inhibitors were the treatments more largely studied in clinical trials for the AD consistent with the hypothesis that a replacement/enhancement of the cholinergic function may be useful in the treatment of AD patients. Based on the available results, it has been hypothesized that CCPLs could still have a place in the pharmacotherapy of adult-onset dementia disorders (Amenta et al., 2020).

CCPLs participate in the biosynthesis of the cell membrane phospholipids. Phosphatidylcholine is the main form of phosphoglycerides that contains the choline molecule as the head group. It represents the main phospholipid of the outer layer of the cellular and intracellular membranes of mammalian cells, and it accounts for 32.8% of the total glycerophospholipid content of the human brain (Javaid et al., 2021). The synthesis could be mediated by the direct methylation of the ethanolamine residue of phosphatidylethanolamine or *via* the Kennedy pathway. Choline molecules are phosphorylated by choline kinases, which after processing by cytidylyltransferase, generated CDP-choline, which further couples with phosphatidic acid and gives phosphatidylcholine (Javaid et al., 2021). Other CCPLs such as sphingomyelin, are abundant in the myelin sheath and contribute to maintain the integrity of the axonal covering (Javaid et al., 2021).

The neurovascular unit

The neurovascular unit (NVU) (**Figure 2**), which is made up of both neurons, glial and vascular cells is responsible for integrating changes in blood supply to increases or decreases in neuronal activity (Kim and Filosa, 2012). Vascular components of NVU include endothelial cells, pericytes, and vascular smooth muscle cells, whereas glial cells include astrocytes, microglia, and oligodendroglia. Acetylcholine as well as CCPLs play an active role in the regulation of NVU. These molecules display protective effects on neuronal death, microglial activation, BBB disruption and consequent neurological injury (Inazu, 2019). NVU is a relevant structure in the maintenance of brain homeostasis. The concept of NVU was introduced Harder et al. (2002) as a brain structure formed by neurons, interneurons, astrocytes, basal lamina covered with smooth muscular cells and pericytes, endothelial cells and extracellular matrix (Muoio et al., 2014). Each element is intimately and reciprocally interconnected, establishing a comprehensive anatomical and functional system of cerebral blood flow control (Abbott and Friedman, 2012).

In the central nervous system (CNS), astrocytes are the most common glial cells. Astrocytes are essential for molecular transport and the integrity of the BBB and through their end-feet, astrocytes connect with endothelial cells in the NVU (Yamazaki and Kanekiyo, 2017). Neurovascular coupling is a process in which astrocytes link neural activity with blood arteries. They react to neural activity and send out signals to control cerebral blood flow (CBF) (Gordon et al., 2011). Astrocytes are formed in the last stages of neurogenesis, along with neurons and oligodendrocytes. As multifunctional cells, astrocytes are strongly connected with neurons and blood vessels, and communicate with neuronal pre-and post-synaptic terminals to help control synaptic transmission by releasing glutamate, D-serine, and ATP (López-Bayghen and Ortega, 2011; Santello et al., 2012). Astrocytes can be largely coupled into syncytial structures of up to 100 units by gap junctions. Extend end-feet process can modulate CBF or the BBB and high levels of aquaporin-4 water channel proteins enhance perivascular clearance by the newly identified "glymphatic system" (Plog and Nedergaard, 2018).

Microglia cells are derived from the yolk sac and seed in the brain as the first glial cells. During development they grow as highly flexible cells with mobility alongside neurons (Pont-Lezica et al., 2011). Through their interaction in the NVU, active microglia and astrocytes may reach a condition of immunological "optimization". Microglia cells, which are found throughout the brain, differentiate early in embryonic development or as the first response of the CNS to neuroinflammation (Klein and Hunter, 2017).

In the brain, endothelial cells constitute the tubes represented by capillaries. The BBB is formed of continuous endothelial cells with tight junctions, basement membrane, and

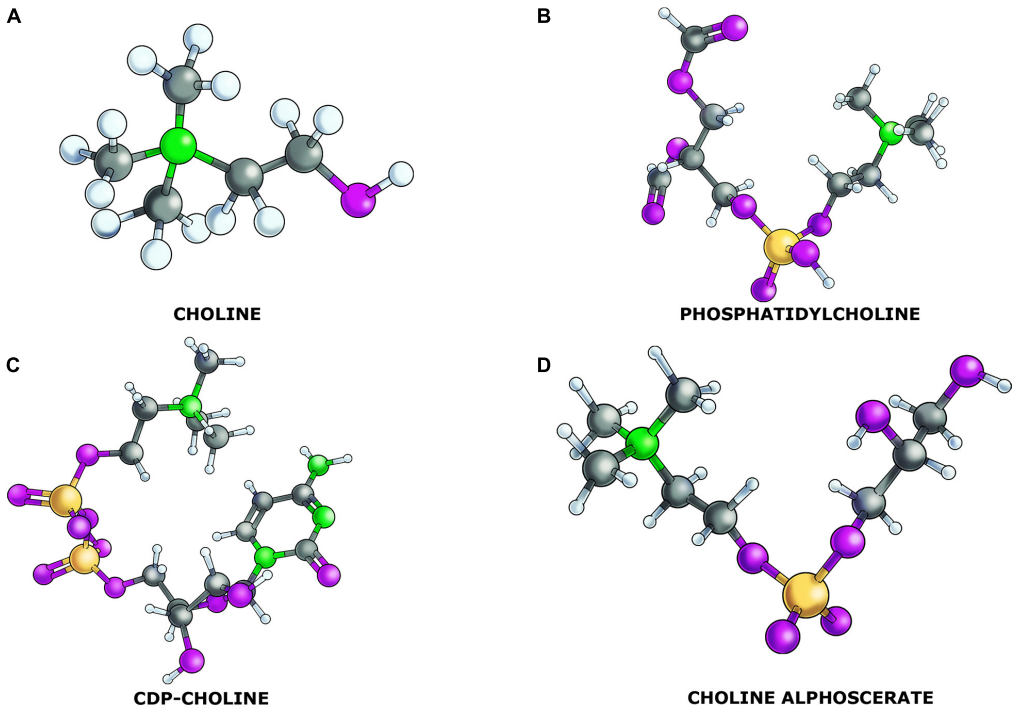

FIGURE 1
Chemical structure of the choline containing phospholipids (CCPLs) and the precursor choline: **(A)** choline; **(B)** phosphatidylcholine; **(C)** CDP-Choline; and **(D)** choline alphoscerate.

astrocyte end-feet in the NVU. BBB controls through chemical and/or anatomic barriers substances entering the brain, and removes dangerous proteins from the brain parenchyma into the bloodstream (Yamazaki and Kanekiyo, 2017). Endothelial cells are joint by tight junctions and adherent junctions, and tight junctions reduce BBB paracellular permeability. All the elements, when combined with neurons, constitute the NVU (Zlokovic, 2008).

The NVU maintains the optimal functioning of the brain microenvironment, contributing to neuronal survival, and information processing by regulating BBB permeability and CBF. Vascular dysfunction has been implicated in various neurodegenerative disorders. The NVU may be damaged, causing BBB malfunction and a drop in CBF, which may concur to the pathophysiology of neurodegenerative diseases. When the NVU is desegregated and CBF drops, the supply of oxygen and nutrients to the brain is reduced as well as the clearance of neurotoxic substances (Sweeney et al., 2018). Claudins, that occlude junctional adhesion molecule, and zonula occludens-1 are all transmembrane proteins involved in the formation of tight junctions (Yamazaki and Kanekiyo, 2017; Bell et al., 2010). The main cadherin that forms the adherent's junction and mediates intercellular adhesion is vascular endothelial cadherin (Zenaro et al., 2017). Tight and adherent junctions are critical in controlling endothelial permeability and help to support normal brain physiological function by limiting the entry of macromolecules, toxins, wastes and perilous blood-borne pathogens (Daneman and Prat, 2015). Tight junctions restrict protein diffusion and seal the paracellular cleft between endothelial cells, whereas adherents' junctions are important for cell-to-cell interaction and cell growth (Tietz and Engelhardt, 2015).

Pericytes cover the abluminal surface of capillaries and adjust capillary diameter to regulate blood flow. BBB permeability, CBF, immunological trafficking, trans endothelial fluid transport, and vascular integrity are all regulated by pericytes (Brown et al., 2019). Pericytes are also involved in the establishment of tight junctions and remove harmful proteins to keep the CNS stable (Daneman et al., 2010; Sagare et al., 2013). Furthermore, for the development, maintenance, and effective functioning of the BBB, interaction between endothelial cells and pericytes is essential (Geranmayeh et al., 2019). **Table 1** summarizes the various component of the NVU.

Choline containing phospholipids

Phosphatidylcholine or lecithin

Neuronal membranes contain different types of lipids present in different amounts: glycerolipids (60%), sterols (20%), glycosphingolipids (15%), and sphingomyelin (5%).

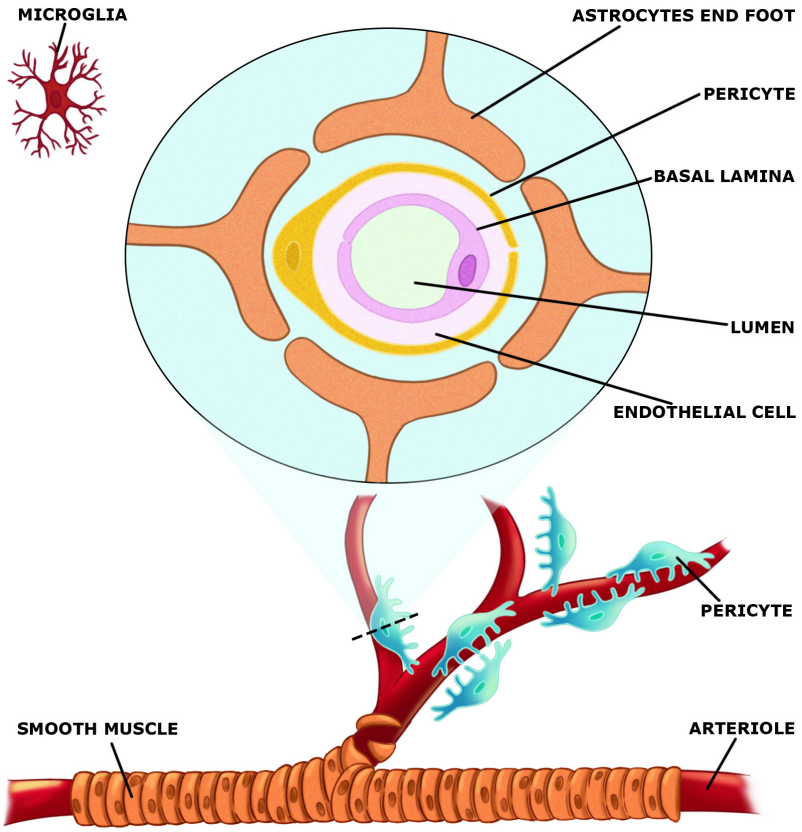

FIGURE 2
Elements of the neurovascular unit (NVU).

Phosphatidylcholine is the most prevalent phospholipid in mammalian membranes, accounting for the 58% of all phospholipids. Other lipids, like sphingolipids and sterols, play key functions during neuronal differentiation (Vance et al., 2000; Schnaar, 2010).

Phosphatidylcholine is a choline-containing, zwitterionic phospholipid (**Figure 1B**). The quaternary amine choline head group of phosphatidylcholine cannot be substituted by primary or secondary amine analogues without adversely affecting cell physiology. Due to its abundance in mammalian cell membranes, depriving cells of phosphatidylcholine by inhibiting synthesis or availability of the precursors results in growth arrest and apoptosis (Ridgway, 2021).

Local synthesis produces half of the phosphatidylcholine that accumulates in distal axons, whereas the other half comes through transport from nerve cell bodies and proximal axons. It is well known that phosphatidylcholine biosynthesis, as a major phospholipid of mammalian membrane, increases during neurogenesis (Carter et al., 2003; Gil et al., 2004). The way through which phosphatidylcholine production is adequately increased to continue growing, as well as the signals that coordinate these processes, are poorly understood. Some evidence demonstrated that phosphatidylcholine or its metabolites could act as a neurotrophic-like factor modulating cell fate and neuronal plasticity (Marcucci et al., 2010). Phosphatidylcholine is the most abundant phospholipid in mammalian plasma and intracellular membranes. Phosphatidylcholine is synthesized from choline through the CDP-choline or Kennedy pathway. In a reaction catalyzed by phosphatidylethanolamine *N*-methyltransferase, it can also be made by directly methylating the ethanolamine residue of phosphatidylethanolamine to form phosphatidylcholine (Vance and Vance, 2004). Phosphatidylcholine-specific phospholipase C activation and phosphocholine cytidylyl-transferase down-regulation inducing accumulation of phosphatidylcholine in membranes, leading to an increase in cell viability (Adibhatla and Hatcher, 2005).

Cytidine 5′-diphosphocholine or citicoline

Cytidine 5′-diphosphocholine, CDP-choline or citicoline, is composed of cytidine and choline linked by a diphosphate bridge (**Figure 1C**). It is an intermediary in the biosynthesis of

TABLE 1 Various components that constitute the neurovascular unit.

NVU components	Classification	Receptors and subtypes	NVU antibodies/Markers
Microglia	Ramified or dormant microglia	Complement type 3 receptor (CR3)	OX-42, MAC-1, CD11b/CD18, OX-6, OX-6, OX-17, and OX-3
	Activated microglia	Major histocompatibility complex class II (MHC II)	CD68, clone ED1, Iba1
	Phagocytic or amoeboid	Major histocompatibility complex class I (MHCI), CD4 receptor	OX42, F4/80, ED1 and ED2, OX-18, OX-6, OX-17, OX-1, and OX-35
	Perivascular microglia	Major histocompatibility complex class II (MHC II)	Iba-1, ED-1, ED-2, OX-17, OX-18 and F4/80, and OX-42
Astrocytes			Glial fibrillary acidic protein (GFAP)
Neurons	Mature neurons	*N*-methyl-D-aspartate (NMDA) 1 glutamate receptors	Neuronal antigen nuclei (NeuN), HuC/D RNA-binding proteins
Pericytes		The tyrosine-kinase receptor PDGFRβ	Platelet-derived growth factor receptor β (PDGFRβ)
			α-Smooth muscle actin (α-SMA)
			Neuron-glial 2 (NG2)
			Desmin (cytoskeleton)
Endothelial cells	Tight junctions proteins	Claudins	Claudin-1, Claudin-3, Claudin-5, and Claudin-12
		Occludins	Zonula occludens (ZO)-1 and ZO-2, ZO-3
		Junctional adhesion molecule (JAM)	VE-cadherin, β-catenin, Caveolin-1, plasma lemma vesicle-associated protein (PLVAP), Platelet-endothelial cell adhesion molecule (PECAM-1), Intercellular adhesion molecule 1 (ICAM-1), Vascular endothelial cell adhesion molecule-1 (VECAM-1)
Basement membrane			Bulins1 (or BM90) and 2; thrombospondins 1 and 2, BM40, Laminin, Collagen IV, Agrin, and Fibronectin
Other blood brain barrier marker		Aquaporins (AQPs)	AQP1, AQP4, and AQP9
		β-Dystroglycan	Dystrophin-glycoprotein complex (DGC), and laminin 2
		Matrix metalloproteinases (MMPs)	Collagenases (MMP-1, MMP 8, MMP-13, MMP-18), gelatinases (MMP-2, MMP-9), stromelysins (MMP-3, MMP-10, MMP-11), matrilysins (MMP-7, MMP-26) and membrane type MMPs (MMP-14, MMP-15, MMP-16, MMP-17, MMP-24, and MMP-25)

cellular membrane phospholipids and is particularly relevant in the production of phosphatidylcholine (Kim J. H. et al., 2015).

From a pharmacological point a view, CDP is classified as a nootropic agent, e.g., a drug used to specifically improve learning or memory, particularly to prevent the cognitive decline associated with dementias (Fioravanti and Yanagi, 2005). Studies in growing sympathetic neurons obtained from rat superior cervical ganglia, showed that when the intracellular concentration of choline is reduced, CDP-choline has beneficial effects in several CNS injury models and pathological conditions. These include cerebral ischemia, traumatic brain injury, hypoxia, AD and Parkinson's diseases. It was proposed that CDP-choline exerted its beneficial effect by attenuating ischemia-induced phospholipase A2.

CDP-choline supplementation promotes brain metabolism by enhancing the synthesis of acetylcholine, and elevates noradrenaline, dopamine, and serotonin levels. CDP-choline restoring neuronal phospholipid affects neuronal membrane excitability and increases neurotransmitter concentrations. The main products of CDP-choline pathways (1,2-diacyl-glycerophosphocholine; sphingomyelin glycerophosphocholine) are incorporated into the membrane phospholipid structure, improving metabolism of mitochondria and phospholipid synthesis (Fagone and Jackowski, 2013).

When administered orally or intravenously, CDP-choline releases its two principal components, cytidine and choline. CDP-choline is virtually entirely absorbed when taken orally, and its bioavailability is the same as when taken intravenously. It has been reported that and citicoline could reverse or prevent neuronal injury (Adibhatla and Hatcher, 2005). Cytidine and choline are absorbed and dispersed throughout the body, crossing the BBB on their way to the CNS, where they are integrated into the phospholipids component of the membrane and microsomes (Secades and Frontera, 1995). In the CNS, citicoline has been demonstrated to increase ACh, noradrenaline, and dopamine levels (Secades and Lorenzo, 2006). In view of its membrane-stabilizing effects, citicoline may affect the BBB integrity (Rao et al., 2000).

CDP-choline boosts brain metabolism and affects the levels of several neurotransmitters by activating structural phospholipids in neuronal membranes. Moreover, CDP-choline has been demonstrated to increase dopamine and noradrenaline levels in the CNS in an experimental setting. CDP-choline has a neuroprotective impact in ischemia and hypoxia improving learning and memory function in animal models of brain aging, owing to these pharmacological properties (Tayebati et al., 2011).

α Glyceryl-phosphorylcholine or choline alphoscerate

α-GPC is semi-synthetic derivative of lecithin. It is a member of the class of CCPLs composed by the choline ester of sn-glycero-3-phosphate (**Figure 1D**). It is also a parasympathomimetic ACh precursor which has been investigated for phosphorylcholine the treatment of AD and other forms of dementia.

α-GPC after administration, is transformed into the metabolically active form of choline, phosphoryl choline that reaches the cholinergic nerve terminals and stimulates ACh synthesis (Amenta et al., 2001). α -GPC improves learning and memory in animal models by increasing acetylcholine levels in the hippocampus (Traini et al., 2013). The cognitive activity of the compound was also demonstrated on symptoms of AD in clinical settings (De Jesus Moreno, 2003). Studies conducted on animals suffering from seizures have suggested that cognitive improvement may depend on increased neuroblast formation, reduced neuronal death, and BBB disruption. Based on these results it has been hypothesized that the compound may contribute to improve cognition in epileptic patients (Lee et al., 2017).

α-GPC is involved in the formation of nerve cell membranes. It is a source of choline that is rapidly absorbed, with the advantage of not carrying the electrical charge of native choline (Parnetti et al., 2001). This probably allows an easier crossing of the BBB. Preclinical studies have shown that α -GPC increases the release of acetylcholine in the rat hippocampus, promotes learning and memory and counters cognitive impairment in the experimental brain aging model of scopolamine-induced memory deficits. Moreover, it counters micro anatomical brain alterations and impairment of cholinergic neurotransmission markers and receptors in old rats (Parnetti et al., 2007).

α-GPC interferes with brain phospholipid metabolism and increases brain choline and ACh synthesis and release. Pharmacodynamic studies on α -GPC during phases of development of the compound were focused primarily on its role in improving brain cholinergic neurotransmission and in interfering with brain phospholipid metabolism. Preclinical studies have demonstrated that α -GPC increases the release of ACh in rat hippocampus, facilitates learning and memory in experimental animals, improves brain transduction mechanisms and decreases the age-dependent structural alterations occurring in the rat brain areas such as frontal cortex and hippocampus. Furthermore, α -GPC contributes to anabolic processes responsible for membrane phospholipid and glycerol-lipid synthesis, positively influencing membrane fluidity. Based on the evidence, the central parasympathomimetic activity of the compound was defined, suggesting its clinical use in patients with cognitive decline (Traini et al., 2013).

Role of choline-containing phospholipids on neurovascular unit elements

Choline-containing phospholipids effects on neurons

Neuroinflammatory response, oxidative stress, protein homeostasis, and apoptotic signaling are involved in the cognitive decline and in the development of neurodegenerative disorders (Teleanu et al., 2022). CDP-choline has been used as a therapeutic agent in combination with levodopa in the treatment of Parkinson's disease (PD). Citicoline reduces the cytotoxic effect of 6-hydroxydopamine (6-OHDA)-treated human dopaminergic SH-SY5Y neuroblastoma cells. Moreover, CDP-choline significantly attenuates substantia nigra dopaminergic cell dropout and tyrosine hydroxylase immunoreactivity in the ipsilateral striatum in rats injected in the striatum with 6-OHDA (Barrachina et al., 2003). Significant increase in dendritic growth and branching of pyramidal neurons from the somatosensory cortex resulted in enlarging the surface area occupied by the neurons were observed in to Long Evans rats supplemented each day with CDP-choline from conception (maternal ingestion) to postnatal day 60 (Rema et al., 2008).

Marked and moderate improvement of cognitive functions was found in patients treated with α-GPC compared to the control one. Deterioration of cognitive functions was seen less often in the treated group than in the control group (Levin et al., 2009).

Treatment with α-GPC for at least 3 weeks was associated with neuroprotective effects and the ability to prevent BBB disruption in an animal model of pilocarpine-induced seizure (Lee et al., 2017). Administration of α-GPC, if immediately after seizure increased neuronal death, starting 3 weeks after administered seizure improved cognitive function through reduced neuronal death and BBB disruption, and increased neurogenesis in the hippocampus (Lee et al., 2018).

Phospholipid metabolites alterations were detected in postmortem AD brains (Nitsch et al., 1991). This

supports the view that phospholipids turnover is elevated in neurodegenerative diseases (Nitsch et al., 1991). A mutual interaction between amyloid formation and membrane phospholipid breakdown has been suggested. The formation of amyloid peptide accelerates membrane breakdown, which is a typical feature of neuronal degeneration in acute and chronic disorders. Moreover, nerve cell loss and phospholipids breakdown may be involved in alterations of central cholinergic neurons. The supplementation of choline or CCPLs, could counter phospholipid breakdown by normalizing phosphatidylcholine, phosphatidyl-ethanolamine, and phosphatidylserine levels (Tayebati et al., 2013).

Based on the evidence that administration of CCPLs, increases ACh levels and modulates the phospholipid breakdown, the administration of CCPLs has been studied as a possible therapeutic approach to treat neurodegenerative disorders (Tayebati et al., 2013). Several studies have clarified the properties of CCPLs, in particular of α-GPC, on the neuronal alterations in animal models of cerebrovascular disease (Tomassoni et al., 2006, 2012; Tayebati et al., 2009, 2015). The beneficial effects of α-GPC may depend by an influence of the compound on brain phospholipids metabolism and/or by its documented activity of increasing free plasma choline levels (Gatti et al., 1992) and brain ACh bioavailability and release (Sigala et al., 1992). α-GPC provides both free choline and phospholipids for synthesizing ACh and could reconstitute the components of the nerves cell membrane. Moreover α-GPC has been found to increase ACh levels and release in rat hippocampus (Sigala et al., 1992; Amenta et al., 2006). These preclinical findings are associated with results of clinical trials in which CCPLs were proposed as potential pharmacotherapies for adult-onset dementia disorders. In particular, α-GPC countered the degeneration occurring in the brain areas involved in learning and memory of AD patients (Amenta et al., 2020).

More recently in a rat model of dual stress, the administration of α-GPC (400 mg/kg) for 7 days, countered the increase of stress hormones, reduced hearing loss, and prevented neuronal injury. This effect has been considered due to the increase of choline acetyltransferase (ChAT) and reduction of brain neuro-inflammation (Jeong Yu et al., 2022). Moreover, in the hippocampus α-GPC enhances brain-derived neurotrophic factor (BDNF) expression and protects the activity of immature cells in the hippocampus (Jeong Yu et al., 2022).

The CCPLs although show mainly a cholinergic profile and affect phospholipids biosynthesis, nervous tissue metabolism and neurotransmitter systems also may have a monoaminergic profile. Dopamine levels in brain areas increase after treatment for 7 days with α-GPC (150 mg/Kg/day) but not with CDP-choline (325 mg/Kg/day) whereas dopamine transporter expression was stimulated in frontal cortex and cerebellum by both CDP and α-GPC. α-GPC also increased serotonin levels in frontal cortex and striatum (Tayebati et al., 2013). CDP-choline in rats does not have effects in the forced swim test, but its primary metabolites showed opposing effects: cytidine has antidepressant-like actions, whereas choline has prodepressant-like actions (Carlezon et al., 2002). The results of this study are consistent with a clinical trial in which depression symptoms in mild to moderate stage AD patients, probably could benefit from stronger cholinergic stimulation induced by combined administration of donepezil and α-GPC (Carotenuto et al., 2022).

Choline-containing phospholipids effects on astrocytes

In the NVU astrocytes play an important role in nutrition, support and protection of neurons and in the neuron-to-neuron signal transduction (Han et al., 2022). Astrocytes wraps the blood vessels with the end feet and mediates the transmission and the movement of molecules between neurons and blood vessels. Projections of astrocytes surround the neurons, can modulate the transmission and asset of synapses, and participate in the synaptic formation and neuronal differentiation (Stogsdill et al., 2017). It has been documented that estrogen released by cultured primary astrocytes, increased the number of newly formed synapses, and enhanced synaptic signal transmission (Fester and Rune, 2015). Cholesterol released by astrocytes induces synaptic formation and affects the number of synapses during the development of neurons (Mauch et al., 2001; Nakayama et al., 2003). Moreover, glial cells can deliver energy and nutrients for neurons, and release citrate, an important substance supplying neuronal energy during hypoglycemia (Meshitsuka and Aremu, 2008). Astrocytes represent a fundamental element in the BBB, a basic component of the NVU and a natural barrier for the brain to maintain the microvascular homeostasis. Astrocytes stretch out to wrap more than 90% of capillary endothelial cells and pericytes, interact with endothelial cells of brain microvasculature and maintain the BBB integrity (Thurgur and Pinteaux, 2018). Astrocytes with microglial cells monitor the brain microenvironment and constitutes the first line of the CNS defense (Li and Barres, 2018). In general, microglia and astrocytes are activated into two states: the neurotoxic phenotype (M1/A1) and the neuroprotection phenotype (M2/A2), corresponding to either the destructive and reparative functions in the NVU, respectively (Liu et al., 2020). Choline transporter-like 1 (CTL1) is expected to have a major role in the synthesis of phospholipids in the plasma membrane since it is functionally expressed in neurons and astrocytes in the CNS (Inazu et al., 2005; Fujita et al., 2006). Choline transporter 1, on the other hand, is expressed in cholinergic neurons and is connected to the production of acetylcholine (Okuda et al., 2000). Although the precise mechanisms are not yet understood, CTL1, which is expressed in astrocytes, is likely involved in astrocyte differentiation and proliferation

(Machová et al., 2009). Membrane lipids like phosphatidic acid are probably transported from astrocytes to neurons either directly or indirectly since astrocytes are involved in the transit of many chemicals and proteins to neurons. It was found that astrocytes produce oleic acid, which was then hypothesized to be taken up by neurons for phospholipid production during neuronal development (Tabernero et al., 2001).

Choline-containing phospholipids modulate transglutaminase activity and expression in primary astrocyte cultures. Transglutaminase is an important Ca^{2+}-dependent protein and a normal element of nervous systems during fetal stages of development. It plays a role in cell signal transduction, differentiation, and apoptosis. In astrocyte cultures supplemented with choline, CDP-choline, or α-GPC at 0.1 or 1 mM concentrations, confocal laser scanning microscopy analysis showed an increase of transglutaminase activity. Comparatively, α-GPC induced the most visible effects enhancing monodansyl-cadaverine fluorescence both in cytosol and in nuclei, supporting a possible active role played by α-GPC during differentiation processes. Western blot analysis showed that in 24 h 1 mM α-GPC and choline-treated astrocytes increased transglutaminase expression, whereas no effect was observed in 24 h 1 mM CDP-choline treated astrocytes (Bramanti et al., 2008a). Moreover, in primary astrocytes cell cultures at 14 and 35 days *in vitro*, CCPLs treatment for 24 h promoted a marked down-regulation of cyclin D1 expression, with reduced cyclin D1 expression in 1 mM α-GPC treated astrocytes. This suggests crucial role of CCPLs independent from ACh, on development and differentiation of astroglial cells *in vitro*, instead of on their maturation, proliferation, and development in culture (Bramanti et al., 2008b).

Another, similar, study in primary astrocytes cell cultures, showed a slight reduction of heme oxygenase-1 (HO-1) expression, a protein that plays a crucial role in oxidative stress processes, cell differentiation and apoptosis, in cell cultures of astrocytes treated with CDP-choline. On the contrary, ACh and choline induced a significant increase of HO-1 expression in 14 day *in vitro* astrocyte cultures (Bramanti et al., 2012). Results concerning p21 expression, a protein that inhibits the cell cycle, showed a significant increment with α-GPC treatment, while CDP-choline treatment caused a higher increase of p21 expression in 14 days *in vitro* astrocyte cultures. These data, suggest that CCPLs modulate HO-1 and p21 expression during astroglial cell differentiation and proliferation in culture and could be considered as a tool to study the induced effects of ischemia and hypoxia diseases (Bramanti et al., 2012). In astrocyte cultures treated with 50 μM (+) lipoic acid or (±)lipoic acid and/or 10 mM α-GPC for 24 h, induced an "upward modulation" in the expression of proliferating and differentiating biomarkers (Grasso et al., 2014).

In spontaneously hypertensive rats (SHR), a preclinical model of cerebrovascular disease associated with hypertension, a treatment for 4 weeks with an oral dose of 100 mg/kg/day of α-GPC decreased astrogliosis and restored expression of aquaporine-4, while galantamine (3 mg/kg/day) treatment countered to a greater extent than α-GPC neuronal alteration induced by hypertension (Tayebati et al., 2009). In the same animal model treatment with α-GPC countered neurons loss and glial reaction particularly in the CA1 subfield and in the dentate gyrus of the hippocampus of while phosphatidylcholine did not modify hypertension-dependent alterations in hippocampal microanatomy (Tomassoni et al., 2006).

In 32-weeks old SHR treated no significant changes in the size of perivascular astrocytes with a-GPC were observed compared to normotensive Wistar-Kyoto rats, while the expression of the BBB marker aquaporin-4 increased in SHR. This enhancement was countered by 4 weeks α-GPC (150 mg/kg/day) treatment. Endothelial markers and vascular adhesion molecules expression were not homogeneously affected by hypertension and α-GPC treatment in intracerebral vessels (Tayebati et al., 2015). This observation could explain data of clinical trials reporting an improvement of cognitive function in patients suffering from cerebrovascular disorders and treated with α-GPC (Amenta et al., 2020).

Choline-containing phospholipids effects on microglia

As brain-resident immune cells, microglia monitor the microenvironment and are ubiquitously distributed throughout the CNS. Microglial cells have been designated as branched, tissue-resident macrophages more abundant in the brain, and represent approximately the 20% of the total glial cells (Wang et al., 2022).

M1–M2 classification, which was applied to peripheral macrophages, divides the microglial activation state into classical activation (M1) or alternative activation (M2) (Dubbelaar et al., 2018). According to the morphology and functions, microglial cells are divided into three categories: M0, M1, and M2. The M0 phenotype, known as the "resting" microglia phenotype, represents microglia that are highly active in their presumed resting state. It is involved in monitoring the presence of pathogens in the local environment and the changes in extracellular concentrations of constitutively expressed neurochemicals (Nimmerjahn et al., 2005). The cells sense the microenvironment by interacting with neurons, blood vessels, ependymal cells, and astrocytes. The M1 phenotype is characterized by the production of pro-inflammatory cytokines (such as TNF-α, IL-6, and IL-1β), chemokines and reactive oxygen species (ROS), leading to an acute immune response. On the contrary, M2 phenotype is characterized by the production of anti-inflammatory cytokines (IL-4 and IL-13), involved in the tissue repair, wound healing, wastes removal, and in the renovation of brain homeostasis (Cherry et al., 2014). The

administration of CCPLs had no impact on the blood levels of the microglial marker ionized calcium-binding adaptor molecule 1 (Iba1), possibly protecting the microglia's crucial functional role in the resolution of local inflammation, the removal of cellular debris, and the provision of protective factors to lessen cell injury in the ischemic brain. Some investigations have shown that serum levels of neuron-specific biomarkers are important and clinically useful indicators of the effectiveness of treating pathologies, and they can be used for further research into the pathophysiology of stroke and the molecular mechanisms underlying nootropic-mediated neuroprotection (Kuryata et al., 2021). ROS and pro-inflammatory cytokines are released by activated microglia and are toxic to nearby neurons, astrocytes, and oligodendrocytes. However, microglia have the capacity to reduce astrocyte reactivity, clear cellular debris, and produce protective substances that can lessen oligodendrocyte injury and/or demyelination as well as local inflammation (Xu et al., 2020). The phagocytosis of active microglia requires the calcium binding protein Iba1, which is specific to macrophages and microglia (Sasaki et al., 2001). Recently, it has been shown that GFAP and Iba1 are two additional biomarkers for neuroinflammation following mild traumatic brain injury (Lafrenaye et al., 2020). CCPLs-based therapeutic strategy to target for demyelination and increasing long-term neurological function (Jayaraj et al., 2019). During systemic inflammation, activated microglial units behave similarly to macrophages and show the potential to phagocytose harmful cellular debris (Lee et al., 2019). Administration of citicoline to the hippocampus significantly decreased microglial activity. In order to more carefully assess the polarization states of microglia, studied at the expression of M1 (IL-6) producers in Iba1-positive microglia. This revealed that citicoline administration may inhibit M1 polarization (Kim J. H. et al., 2018).

Choline-containing phospholipids interact with microglia and exert anti-inflammatory properties in animal models of cerebral disease, while in normal conditions these molecules did not affect the expression of inflammation markers, both inflammation cytokines and endothelial adhesion molecules, in different cerebral areas.

The toll-like receptor (TLR) stimulation enhances choline uptake by macrophages and microglia by the induction of the choline transporter CTL1. Inhibition of CTL1 expression or choline phosphorylation attenuated NLRP3 inflammasome activation and IL-1β and IL-18 production in stimulated macrophages. Correspondingly, choline kinase inhibitors ameliorated acute and chronic models of IL-1β-dependent inflammation (Sanchez-Lopez et al., 2019).

In a rodent model of systemic inflammation induced by i.p. treatment of lipopolysaccharide (LPS), the administration of a special diet enriched with phosphatidylcholine (1% for 5 days before the administration of LPS and thereafter during the 7-day observation period) induced a reduction in the plasma TNF-α and hippocampal NOx changes, prevented the decrease of neurogenesis and the microglial activation (Tokés et al., 2011).

Phosphatidylcholine treatment significantly attenuated docetaxel-induced peripheral neurotoxicity. Phosphatidylcholine decreased oxidative stress in sciatic nerve ameliorated docetaxel-induced neuronal damage and microglial activation in the sciatic nerve and spinal cord (Kim S. T. et al., 2018). The prevention of microglia activation represents a neuroprotective strategy in a rat model of insulin-induced hypoglycemia. In the brain of these rats, the activated microglia represent the M1 polarization and that CDP-choline injections (500 mg/kg, i.p.), may reduce M1 polarization as well as microglial activation following hypoglycemia (Kim J. H. et al., 2018).

In a rodent model of oxaliplatin-induced neuropathy phosphatidylcholine attenuated oxidative stress by increasing antioxidant levels and immunohistochemical evaluation, indicated that phosphatidylcholine administration ameliorated microglial activation, suggesting a therapeutic effect against oxaliplatin-induced peripheral neuropathy due to its antioxidant property (Kim S. T. et al., 2015). Surprisingly, CDP-Choline treatment enhanced seizure-induced neuronal death and microglial activation in the hippocampus of an animal model of pilocarpine-induced epilepsy. CDP-choline administration after seizure-induction increased immunoglobulin leakage *via* BBB disruption indicating that the compound was not able to modulate neuronal death, BBB disruption or microglial activation (Kim S. T. et al., 2015).

α-GPC (0.07 mg/ml) administered in water decreased the deposition of transthyretin (TTR), an amyloidogenic protein, and led to neuroinflammation in senescence-accelerated mouse prone 8 mice. These effects suggest that α-GPC may be a useful compound in anti-aging functional food development (Matsubara et al., 2018).

In spontaneously hypertensive rats, similarly to the reduction of astrogliosis, α-GPC induced a reduction of microglial activation (Tayebati et al., 2015), while in the same animal model vascular adhesion molecules and endothelial markers expression were not homogeneously modified by hypertension or α-GPC treatment (Tayebati et al., 2015).

The nutritional importance of α-GPC in combination with docosahexaenoic acid (DHA) plus triglyceride (TG) was studied. The date showed that the α-GPC in combination with DHA + TG is more neurodevelopmentally effective than DHA + TG or DHA + TG + phosphocholine or DHA + phosphocholine (Tayebati, 2018).

Choline-containing phospholipids effects on endothelial cells and pericytes

Metabolites and neurotoxic substances produced in the brain are cleared through the action of ATP-binding cassette transporters, such as P-glycoprotein, which are found at the luminal membrane of endothelial cells (Okura et al., 2008;

Shimomura et al., 2013). Under physiological circumstances, choline has a positive charge and is a cationic chemical. Therefore, it is anticipated that cationic medicines will competitively limit choline transporter. Indeed, the uptake of several cationic medications (such as clonidine, desipramine, diphenhydramine, quinidine, quinine, and verapamil) hindered choline uptake in human brain microvascular endothelial cells in a concentration-dependent manner (Iwao et al., 2016). Spherical, solitary cells called pericytes are near endothelial cells and they support endothelial cells by anatomical contact.

The effects of CDP-choline on the permeability and expression of tight junction proteins in endothelial cells were tested in an *in vitro* model of human umbilical vein endothelial cells (HUVECs) of hypoxia/aglycemia conditions. In this model, the increased permeability of HUVECs accompanied with down-regulation of zonula occludens-1 (ZO-1) and occluding. Citicoline decreased the permeability with an increased expression of the TJPs, demonstrating that citicoline restores the barrier functions of endothelial cells (Ma et al., 2013). The treatment of CDP-choline (500 mg/kg) in an animal model of cerebral infarct, enhanced cell proliferation, vasculogenesis and presynaptic proteins expression, and reduced astrogliosis levels in the peri-infarct area of the ischemic stroke (Gutiérrez-Fernández et al., 2012a,b). In acute ischemic stroke patients, the increase levels of the in circulating endothelial progenitor cells (EPCs) is associated with a better outcome. A combination of CDP-choline (2,000 mg/day) and recombinant tissue-plasminogen activator (rt-PA) given in acute ischemic stroke and at 6 weeks was able to increase EPC levels and caused protective effects (Sobrino et al., 2011).

The main preclinical observation of possible effects of CCPLs on *in vitro* and *in vivo* models are summarized in **Table 2**.

Clinical activity of choline-containing phospholipids

A natural question after the above description of the role of CCPLs is if and to what extent this class of molecules with a large and documented NVU activity has shown an efficacy in brain disorders characterized by cerebrovascular impairment. CCPLs play an important role in cellular metabolism and are relevant components of various cell membranes (i.e., cellular, mitochondria, endoplasmatic reticulum, Golgi apparatus, peroxisomes, and lysosomes). Choline is a crucial nutrient with a complex role in the body, is necessary for the biosynthesis of the neurotransmitter ACh and is also the major dietary source of methyl groups *via* the synthesis of *S*-adenosylmethionine (Tayebati and Amenta, 2013).

The first approach to restoring deficient cholinergic neurotransmission and alleviating cognitive impairment in dementia disorders was cholinergic precursor-loading therapy. Taking into account the controversial nature of the existence of a direct role of choline on ACh release and given that an increase of brain free choline does not always imply an increase of brain ACh (Tayebati and Amenta, 2013), CCPLs were then more largely investigated. Cholinergic neurons probably have a particular avidity for choline, and it has been hypothesized that when the provision of choline is insufficient, neurons obtain it by hydrolyzing membrane phospholipids. This mechanism, known as auto cannibalism, may render cholinergic neurons more susceptible to injury and may contribute to cholinergic neuron degeneration (Wurtman et al., 1990).

The CCPLs proposed for the treatment of age-related dementia disorders include lecithin, CDP-choline and α-GPC. The main evidence of their activity is reviewed below and summarized in **Table 3**.

Phosphatidylcholine

Phosphatidylcholine is a CCPLs representing the key font of choline. Phosphatidylcholine increases serum choline levels more effectively than orally administered choline (Wurtman et al., 1990). Apparently, phosphatidylcholine may accelerate ACh synthesis in the brain through enhanced availability of choline. Lecithin has been tried out in the treatment of dementia, alone or in combination with an acetylcholinesterase (AChE) inhibitor. Phosphatidylcholine is mainly available as a nutraceutical. The role of this compound in cognitive impairment and dementias has been the focus of a Cochrane review in 2003 (Higgins and Flicker, 2003). No trials reported any clear clinical benefit of phosphatidylcholine for AD or Parkinson's dementia. Only scarce clinical trials have led to data to meta-analyses. The only statistically significant result was in favors of placebo for adverse events, based on one single trial. Relevant results in favor of phosphatidylcholine were gained in a clinical study of subjects with individual problems of cognition (Higgins and Flicker, 2003). More recently the associations of dietary choline intake with the risk of incident dementia and with cognitive performance in middle-aged and older men were evaluated. Total choline and phosphatidylcholine intakes were associated with better performance in cognitive tests and higher phosphatidylcholine intake was associated with lower risk of incident dementia and better cognitive performance in men (Ylilauri et al., 2019).

Cytidine 5′-diphosphocholine

CDP-choline is a CCPLs composed by choline and cytidine, joint together by a diphosphate bridge (**Figure 1C**). Citicoline is the international non-proprietary name of CDP-choline. The functions of CDP-choline include the restoration of the neural membrane through the synthesis of phosphatidylcholine, the

TABLE 2 Effects of choline-containing phospholipids (CCPLs) in different elements of neurovascular unit (NVU) evaluated in different *in vitro* and *in vivo* models.

In vitro or *in vivo* model	Evidence	References
Main results for neurons		
Neuronal cell cultures	Phosphatidylcholine could play a signalling role during neuronal differentiation	Paoletti et al., 2011
Primary cultured neurons treated with Aβ1-42-induced damage	Phosphatidylcholine prevented neuronal death	Ko et al., 2016
Aged mice	Phosphatidylcholine had no effect on hippocampal dendritic spine density compared to control, but improved learning and memory	Muma and Rowell, 1988
Wistar rats with ischemia/reperfusion (I/R)-induced insult	Phosphatidylcholine has a possible neuroprotective effect partly through its antioxidant action	Aabdallah and Eid, 2004
Aging rats	Dietary CDP-choline supplementation can protect impairment in hippocampal-dependent long-term memory.	Teather and Wurtman, 2003
Long Evans rats	CDP-choline significant increases in neurite length, branch points and total area occupied by the neurons were observed	Rema et al., 2008
Human dopaminergic SH-SY5Y neuroblastoma cells	CDP-choline reduces the cytotoxic effect of 6-hydroxydopamine (6-OHDA)	Barrachina et al., 2003
Rats	α-GPC increase acetylcholine level, facilitates learning and memory, improves brain transduction mechanisms	Traini et al., 2013
Rats	α-GPC increase dopamine levels, whereas dopamine transporter expression was stimulated by both CDP and α-GPC in brain areas	Tayebati et al., 2013
Spontaneously hypertensive rats (SHR) as a model of cerebrovascular disease	α-GPC prevent neuronal alterations	Tayebati et al., 2009, 2015; Tomassoni et al., 2006, 2012
Pilocarpine-induced seizure in rat	α-GPC, starting 3 weeks after seizure improved cognitive	Lee et al., 2018
Rat model of dual stress	α-GPC countered the increase of stress hormones, reduced hearing loss, and prevented neuronal injury	Jeong Yu et al., 2022
Main results for astrocytes		
Primary astrocyte cultures	Active role played by CCPLs., particularly a-GPC throughout differentiation processes	Bramanti et al., 2008a,b, 2012; Grasso et al., 2014
Spontaneously hypertensive rats (SHR) as a model of cerebrovascular disease	α-GPC reduced astrogliosis and the expression of aquaporin-4	Tayebati et al., 2009, 2015; Tomassoni et al., 2006
Rat with middle cerebral artery occlusion as a model of stroke	CDP-choline reduced glial fibrillary acidic protein levels in the peri-infarct area of the ischemic stroke	Gutiérrez-Fernández et al., 2012a,b
Main results for microglia		
Rat model of on oxaliplatin-induced neuropathy	CDP-choline administration ameliorated microglial activation	Kim S. T. et al., 2015
pilocarpine-induced epilepsy in rat	CDP-Choline treatment enhanced seizure-induced neuronal death and microglial activation	Kim J. H. et al., 2015
Rat with docetaxel-induced peripheral neurotoxicity	CDP choline significantly decreased microglial activation and M1 polarization in rat hippocampus	Kim S. T. et al., 2018
Senescence-accelerated mouse prone 8 (SAMP-8)	α-GPC protect the brain by reducing TTR deposition and preventing neuroinflammation.	Matsubara et al., 2018
Spontaneously hypertensive rats (SHR) as a model of cerebrovascular disease	α-GPC induce a reduction of microglial activation	Tayebati et al., 2015
Main results for endothelial cells and pericytes		
Human umbilical vein endothelial cells (HUVECs) under hypoxia/aglycemia conditions	CDP-choline decreased the permeability with an increased expression of the tight junction proteins	Ma et al., 2013
Rat with middle cerebral artery occlusion as a model of stroke	CDP-choline increased cell proliferation, and vasculogenesis	Gutiérrez-Fernández et al., 2012a,b
Spontaneously hypertensive rats (SHR) as a model of cerebrovascular disease	α-GPC affect endothelial markers and vascular adhesion molecules expression ICAM-1, VCAM-1, and PECAM-1	Tayebati et al., 2015

TABLE 3 Evidence of the effects of choline-containing phospholipids (CCPLs) in clinical studies.

Disease	Types of study and patients	Evidence	References
		Phosphatidylcholine	
Dementia and cognitive decline	265 with Alzheimer's disease, 21 with Parkinsonian dementia and 90 with subjective memory problems in randomized trials	Evidence doesn't support the use of lecithin in the treatment of patients with dementia	Higgins and Flicker, 2003
	2,497 in a population study	Higher phosphatidylcholine intake was associated with lower risk of incident dementia and better cognitive performance in men	Ylilauri et al., 2019
		CDP-choline	
Acute ischemic stroke	4,281 patients in randomized controlled trials	Little to no difference compared controls regarding all-cause mortality, disability or dependence in daily activities, severe adverse events, functional recovery and neurological function	Martí-Carvajal et al., 2020
Chronic cerebral disorder in elderly and dementia	1,307 in clinical trials	Evidence that CDP-choline has a positive effect on memory and behaviour	Fioravanti and Yanagi, 2005
	736 in observational studies	Reduction of cognitive dysfunction Reduction of disturbances of visual/spatial Positive effects on the emotional Decreasing the level of depression	Mashin et al., 2017
	174 Outpatients in retrospective case-control study (CDP-Choline + Rivastigmine)	Effectiveness of combined administration versus the Rivastigmine alone, mainly in slowing disease progression and consequently in disease management, of Alzheimer's disease (AD) and in mixed dementia (MD)	Castagna et al., 2016
	170 Retrospective multi-centric case-control study	After 12 months, triple therapy with citicoline, memantine, and AChEI was more effective than memantine and AChEI without citicoline in maintaining the MMSE total score	Castagna et al., 2021a
	104 in retrospective case-control study	Absence of a statistically significant difference between case and control groups for the MMSE total scores	Castagna et al., 2021b
		Choline alphoscerate	
Acute cerebrovascular disease	4,315 in 11 trials on dementia disorders of neurodegenerative or vascular origin and in cerebrovascular disease	Choline alphoscerate provided some modest symptomatic relief primarily on memory and attention	Parnetti et al., 2007
AD associated with cerebrovascular injury	113 Participants to the randomized, placebo-controlled, double-blind ASCOMALVA trial	Findings suggest that the combination of choline alphoscerate with a ChE-I may prolong/increase the effectiveness of cholinergic therapies in AD with concomitant ischemic cerebrovascular injury	Amenta et al., 2014
	56 Participants to the randomized, placebo-controlled, double-blind ASCOMALVA trial	Addition of choline alphoscerate to standard treatment with the cholinesterase inhibitor donepezil counters to some extent the loss in volume occurring in some brain areas of AD patients	Traini et al., 2020
Mild cognitive impairment	50 Patients	Psychometric measures showed a significant improvement	Gavrilova et al., 2018
Ischemic and hemorrhagic stroke	277 Patients in 6 trials	Regress in neurological deficit, better recovery in cognitive functions and functional status	Traini et al., 2013
chronic brain ischemia and moderate cognitive impairment.	25 Patients, 16 women, and 9 men	significant positive effect on patient's condition including cognitive function	Pizova, 2014
Parkinson's disease with cognitive disorders	40 Patients (main group) 20 patients (control group)	Marked and moderate improvement of cognitive functions was found in patients of the main group compared to the control one	Levin et al., 2009
vascular cognitive impairment (VCI) due to cerebral small vessel disease (SVD).	62 Patients, pilot, single-center (university hospital), double-blinded, randomized clinical trial (Choline Alphoscerate and Nimodipine)	Combined choline alphoscerate-nimodipine treatment showed no significant effect	Salvadori et al., 2021

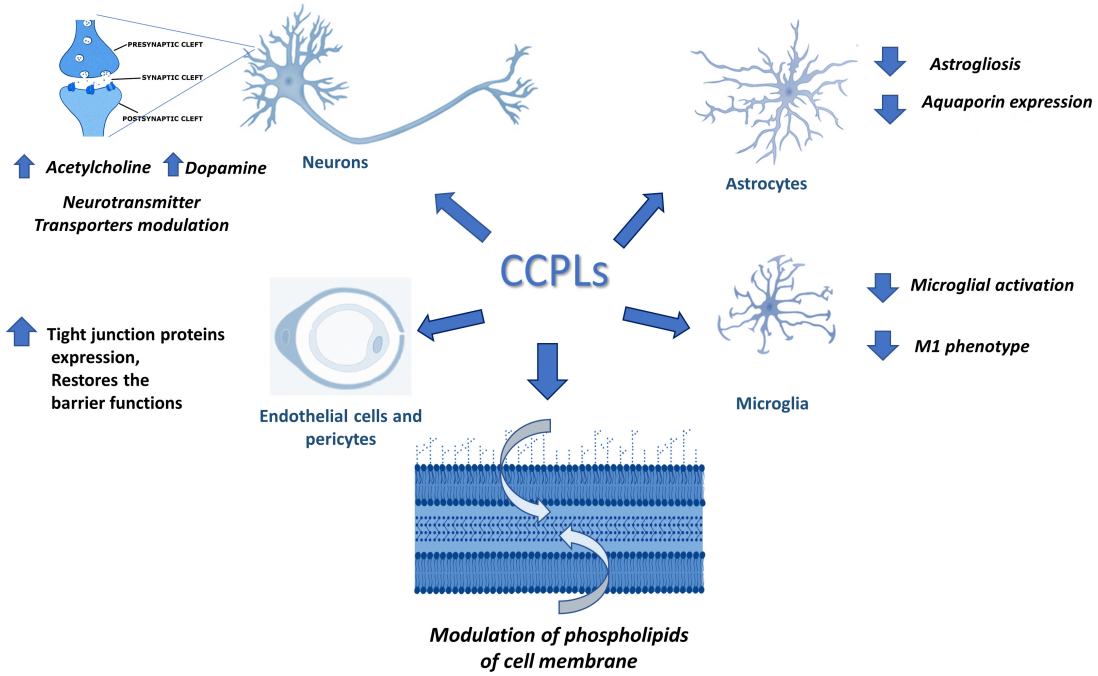

FIGURE 3
Possible activity of choline containing phospholipids (CCPLs) on different elements of Neurovascular Unit or in cell membrane phospholipids metabolism.

reduction of stored fat responsible for the cognitive decline, and the increase of ACh levels. In post-stroke patients a neuroprotective effect of CDP-choline, contributing to an improvement in attention tasks, executive function, temporal orientation, along with an ability, although in an experimental setting, to improve the neural repair has been reported.

Several studies have demonstrated positive effects of the compound on cognition, but other studies failed to confirm the results. In view of these discrepancies, additional clinical studies are necessary to confirm the potential benefits of citicoline in the treatment of adult-onset dementia disorders (Amenta et al., 2020).

Choline alphoscerate

α-GPC has been on the pharmaceutical market since 1987, however, its popularity has declined since the introduction of cholinesterase inhibitors. The last 10 years have seen a renewed attention on this compound, with preclinical studies, clinical investigations and review articles published in the literature (Traini et al., 2013).

The majority of clinical studies available on the effect of α-GPC on cognitive function in neurodegenerative and cerebrovascular disorders were detailed in two review articles as showed in Parnetti et al. (2007) and Traini et al. (2013). Administration of α-GPC improved cognitive functions, as well as affective and somatic symptoms (fatigue, vertigo). The effects of α-GPC were greater than those of placebo and of the same extent or superior to those of reference compounds (Amenta et al., 2001). Studied in rat models showed that the association of α-GPC with AChE inhibitors enhance the effects of both drugs on cholinergic neurotransmission (Amenta et al., 2006). Based on this evidence an independent clinical trial: "Effect of association between a cholinesterase inhibitor and α-GPC on cognitive deficits in AD associated with cerebrovascular impairment" (ASCOMALVA) was performed in Italy. In agreement with literature data, in patients selected to the reference treatment group (donepezil + placebo), a slight time-dependent worsening of Mini Mental State Examination (MMSE) and the Alzheimer's Disease Assessment Scale–Cognitive Subscale (ADAS-cog) scores was found. In the active treatment group, the administration of donepezil+ α-GPC countered the decline of MMSE and ADAS-cog scores. The effect of the association on psychometric tests was statistically significant after 12 months of treatment (Amenta et al., 2014). The combination of donepezil plus α-GPC was more effective than donepezil alone in countering symptoms of apathy in AD. This suggests that the availability in the brain of a higher amount of acetylcholine may affect apathy in AD subjects with spared executive functions (Rea et al., 2015).

A supplementary contribution of the ASCOMALVA trial was the study of the influence of the combine treatment of α-GPC and donepezil on brain atrophy in AD. Cerebral

atrophy is a common feature of neurodegenerative disorders. This pathology includes a loss of gyri and sulci in the temporal lobe and parietal lobe, and in parts of the frontal cortex and of the cingulate gyrus. Patients enrolled in the ASCOMALVA trial underwent yearly Magnetic Resonance Imaging (MRI) analysis for diagnostic purposes. In 56 patients who achieved 3 years of therapy, brain MRI were examined by voxel morphometry techniques. At the end of 3 years of treatment, in patients enrolled in the active group of donepezil plus α-GPC, a reduction of the volume loss of the grey matter (with a concomitant increase of the volume of the ventriculi and cerebrospinal fluid space) was observed, compared to the reference group (donepezil alone). Frontal and temporal lobes, hippocampus, amygdala and basal ganglia represented the areas in which brain atrophy was more sensitive to combination treatment. No significant differences were noticeable in other areas, between the two groups. Morphological data were confirmed by neuropsychological assessment performed alongside the trial (Traini et al., 2020).

In conclusion, a cholinergic precursor-loading strategy with α-GPC in combination with donepezil counters to some extent the atrophy occurring in some brain areas of AD patients. In these patients, the parallel observation of an improvement in cognitive and functional tests suggests that morphological changes observed could have functional relevance (Traini et al., 2020).

Conclusion

This manuscript has reviewed the role of CCPLs on the function of the different components of the NVU. CCPLs promote metabolism in different elements of the NVU by increasing the synthesis of neurotransmitters, or by incorporation into the membrane phospholipid structure, improving synthesis of phospholipids and mitochondrial metabolism, as shown in **Figure 3**.

A comparative analysis on the results obtained with animal models and in clinical trials revealed a discrepancy between quite positive results observed in preclinical studies and the limited positive outcomes reported in clinical trials. The value of animal research in drug development is increasingly questionable primarily in case of typical human diseases such as neurodegenerative disorders. For instance, a relevant lack of success in AD drug development over the past two decades was observed (Kim et al., 2022), whereas preclinical studies reported quite encouraging results. Several factors can contribute to these inconsistencies (Mehta et al., 2017). One is the inadequacy in the selection of animal models not always done with a clear and specific translational rationale. Another reason could be the lack of experiments conducted according to the best practices and mimicking the course of a human disease. We should also consider that the use of animal models such as the rodents with a short lifespan compared with the long time necessary for the development of symptoms of a typical neurodegenerative disorder represents a potential obstacle for the translatability of animal data to humans. On the other hand, treatment starting in advanced stages of a disease is difficult to bring positive results. Hence, the use of suitable animal models and the treatment of diseases for enough time at a not too advanced stage may represent a reasonable approach to give a true value of evidence coming from animal studies.

Despite the large evidence about the biochemistry of these molecules and the role in modulating the activity of the NVU, clinical studies on the activity of CCPLs in pathologies have suggested a dysfunction of the NVU (adult-onset dementia disorders of vascular origin) are limited. Phosphatidylcholine was the first cholinergic precursor molecule used, but it did not demonstrate clear clinical benefits. The same is not true for other phospholipids, CDP-choline and α-GPC, involved in choline biosynthetic pathways. For these an uncertain improvement of cognitive dysfunction in neurodegenerative and vascular dementia is documented. Positive results obtained with selected cholinergic precursors cannot be generalized due to the small numbers of patients studied in appropriate clinical trials. However, they probably would justify reconsideration of the most promising molecules in larger carefully controlled studies. This can also contribute to better define the role of the NVU in the pathophysiology of brain disorders characterized by vascular impairment.

Author contributions

PR, DT, ET, and GN conducted critical analysis of the literature on the topic of manuscript. DT and FA designed the review. PR, DT, and ET wrote the draft of the manuscript. PR, DT, ET, GN, and FA revised the ultimate version of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Aabdallah, D. M., and Eid, N. I. (2004). Possible neuroprotective effects of lecithin and alpha-tocopherol alone or in combination against ischemia/reperfusion insult in rat brain. *J. Biochem. Mol. Toxicol.* 18, 273–278. doi: 10.1002/jbt.20037

Abbott, N. J., and Friedman, A. (2012). Overview and introduction: The blood-brain barrier in health and disease. *Epilepsia* 53(Suppl. 6), 1–6. doi: 10.1111/j.1528-1167.2012.03696.x

Adibhatla, R. M., and Hatcher, J. F. (2005). Cytidine 5′-diphosphocholine (CDP-choline) in stroke and other CNS disorders. *Neurochem. Res.* 30, 15–23. doi: 10.1007/s11064-004-9681-8

Adibhatla, R. M., Hatcher, J. F., and Tureyen, K. (2005). CDP-choline liposomes provide significant reduction in infarction over free CDP-choline in stroke. *Brain Res.* 1058, 193–197. doi: 10.1016/j.brainres.2005.07.067

Amenta, F., Battineni, G., Traini, E., and Pallotta, G. (2020). "Choline-containing phospholipids and treatment of adult-onset dementia disorders," in *The neuroscience of dementia. Martin victor preedy*, 1st Edn, ed. R. Colin (London: Academic Press), 477–493.

Amenta, F., Carotenuto, A., Fasanaro, A. M., Rea, R., and Traini, E. (2014). The ASCOMALVA (association between the cholinesterase inhibitor donepezil and the cholinergic precursor choline alphoscerate in Alzheimer's disease) trial: Interim results after two years of treatment. *J. Alzheimer's Dis.* 42(Suppl. 3), S281–S288. doi: 10.3233/JAD-140150

Amenta, F., Parnetti, L., Gallai, V., and Wallin, A. (2001). Treatment of cognitive dysfunction associated with Alzheimer's disease with cholinergic precursors. Ineffective treatments or inappropriate approaches? *Mech. Ageing Dev.* 122, 2025–2040. doi: 10.1016/s0047-6374(01)00310-4

Amenta, F., Tayebati, S. K., Vitali, D., and Di Tullio, M. A. (2006). Association with the cholinergic precursor choline alphoscerate and the cholinesterase inhibitor rivastigmine: An approach for enhancing cholinergic neurotransmission. *Mech. Ageing Dev.* 127, 173–179. doi: 10.1016/j.mad.2005.09.017

Attwell, D., Buchan, A. M., Charpak, S., Lauritzen, M., Macvicar, B. A., and Newman, E. A. (2010). Glial and neuronal control of brain blood flow. *Nature* 468, 232–243. doi: 10.1038/nature09613

Barrachina, M., Domínguez, I., Ambrosio, S., Secades, J., Lozano, R., and Ferrer, I. (2003). Neuroprotective effect of citicoline in 6-hydroxydopamine-lesioned rats and in 6-hydroxydopamine-treated SH-SY5Y human neuroblastoma cells. *J. Neurol. Sci.* 215, 105–110. doi: 10.1016/s0022-510x(03)00204-1

Bell, R. D., Winkler, E. A., Sagare, A. P., Singh, I., LaRue, B., Deane, R., et al. (2010). Pericytes control key neurovascular functions and neuronal phenotype in the adult brain and during brain aging. *Neuron* 68, 409–427. doi: 10.1016/j.neuron.2010.09.043

Bramanti, V., Bronzi, D., Tomassoni, D., Li Volti, G., Cannavò, G., Raciti, G., et al. (2008a). Effect of choline-containing phospholipids on transglutaminase activity in primary astroglial cell cultures. *Clin. Exp. Hypertens.* 30, 798–807. doi: 10.1080/10641960802563576

Bramanti, V., Campisi, A., Tomassoni, D., Li Volti, G., Caccamo, D., Cannavò, G., et al. (2008b). Effect of acetylcholine precursors on proliferation and differentiation of astroglial cells in primary cultures. *Neurochem. Res.* 33, 2601–2608. doi: 10.1007/s11064-008-9829-z

Bramanti, V., Tomassoni, D., Grasso, S., Bronzi, D., Napoli, M., Campisi, A., et al. (2012). Cholinergic precursors modulate the expression of heme oxigenase-1, p21 during astroglial cell proliferation and differentiation in culture. *Neurochem. Res.* 37, 2795–2804. doi: 10.1007/s11064-012-0873-3

Brown, L. S., Foster, C. G., Courtney, J. M., King, N. E., Howells, D. W., and Sutherland, B. A. (2019). Pericytes and neurovascular function in the healthy and diseased brain. *Front. Cell Neurosci.* 13:282. doi: 10.3389/fncel.2019.00282

Carlezon, W. A., Pliakas, A. M., Parow, A. M., Detke, M. J., Cohen, B. M., and Renshaw, P. F. (2002). Antidepressant-like effects of cytidine in the forced swim test in rats. *Biol. Psychiatry* 51, 882–889. doi: 10.1016/s0006-3223(01)01344-0

Carotenuto, A., Fasanaro, A. M., Manzo, V., Amenta, F., and Traini, E. (2022). Association between the cholinesterase inhibitor donepezil and the cholinergic precursor choline alphoscerate in the treatment of depression in patients with Alzheimer's disease. *J. Alzheimers Dis. Rep.* 6, 235–243. doi: 10.3233/ADR-200269

Carter, J. M., Waite, K. A., Campenot, R. B., Vance, J. E., and Vance, D. E. (2003). Enhanced expression and activation of CTP: Phosphocholinecytidylyltransferase beta2 during neurite outgrowth. *J. Biol. Chem.* 278, 44988–44994. doi: 10.1074/jbc.M307336200

Castagna, A., Cotroneo, A. M., Ruotolo, G., and Gareri, P. (2016). The CITIRIVAD study: CITIcoline plus RIVAstigmine in elderly patients affected with dementia study. *Clin. Drug Investig.* 36, 1059–1065. doi: 10.1007/s40261-016-0454-3

Castagna, A., Fabbo, A., Manzo, C., Lacava, R., Ruberto, C., and Ruotolo, G. (2021a). A retrospective study on the benefits of combined citicoline, memantine, and acetylcholinesterase inhibitor treatments in older patients affected with Alzheimer's disease. *J. Alzheimers Dis.* 79, 1509–1515. doi: 10.3233/JAD-201211

Castagna, A., Manzo, C., Fabbo, A., Lacava, R., Ruberto, C., and Ruotolo, G. (2021b). The CITIMERIVA study: CITIcoline plus MEmantina plus RIVAstigmine in older patients affected with Alzheimer's disease [published correction appears in Clin Drug Investig. 2021 41(4):413]. *Clin. Drug Investig.* 41, 177–182. doi: 10.1007/s40261-020-00996-2

Cherry, J. D., Olschowka, J. A., and Banion, M. O. (2014). Neuroinflammation and M2 microglia: The good, the bad, and the inflamed. *J. Neuroinflammation* 11:98. doi: 10.1186/1742-2094-11-98

Daneman, R., and Prat, A. (2015). The blood-brain barrier. *Cold Spring Harb. Perspect. Biol.* 7:a020412. doi: 10.1101/cshperspect.a020412

Daneman, R., Zhou, L., Kebede, A. A., and Barres, B. A. (2010). Pericytes are required for blood-brain barrier integrity during embryogenesis. *Nature* 468, 562–566. doi: 10.1038/nature09513

De Jesus Moreno, M. (2003). Cognitive improvement in mild to moderate Alzheimer's dementia after treatment with the acetylcholine precursor choline alfoscerate: A multicenter, double-blind, randomized, placebo-controlled trial. *Clin. Ther.* 25, 178–193. doi: 10.1016/s0149-2918(03)90023-3

Diederich, K., Frauenknecht, K., Minnerup, J., Schneider, B. K., Schmidt, A., Altach, E., et al. (2012). Citicoline enhances neuroregenerative processes after experimental stroke in rats. *Stroke* 43, 1931–1940. doi: 10.1161/STROKEAHA.112.654806

Dubbelaar, M. L., Kracht, L., Eggen, B. J. L., and Boddeke, W. G. M. (2018). The kaleidoscope of microglial phenotypes. *Front. Immunol.* 9:1753. doi: 10.3389/fimmu.2018.01753

Fagone, P., and Jackowski, S. (2013). Phosphatidylcholine and the CDP-choline cycle. *Biochim. Biophys. Acta* 1831, 523–532. doi: 10.1016/j.bbalip.2012.09.009

Fester, L., and Rune, G. M. (2015). Sexual neurosteroids and synaptic plasticity in the hippocampus. *Brain Res.* 1621, 162–169. doi: 10.1016/j.brainres.2014.10.033

Fioravanti, M., and Yanagi, M. (2005). Cytidinediphosphocholine (CDP-choline) for cognitive and behavioural disturbances associated with chronic cerebral disorders in the elderly. *Cochrane Database Syst. Rev.* 2:CD000269. doi: 10.1002/14651858.CD000269.pub3

Fujita, T., Shimada, A., Okada, N., and Yamamoto, A. (2006). Functional characterization of Na+-independent choline transport in primary cultures of neurons from mouse cerebral cortex. *Neurosci. Lett.* 393, 216–221. doi: 10.1016/j.neulet.2005.09.069

Gatti, G., Barzaghi, N., Acuto, G., Abbiati, G., Fossati, T., and Perucca, E. (1992). A comparative study of free plasma choline levels following intramuscular administration of-alpha-glycerylphosphorylcholine and citicoline in normal volunteers. *Int. J. Clin. Pharmacol. Ther. Toxicol.* 30, 331–335.

Gavrilova, S. I., Kolykhalov, I. V., Ponomareva, E. V., Fedorova, Y. B., and Selezneva, N. D. (2018). Klinicheskaia éffektivnost' i bezopasnost' primeneniia kholina al'fostserata v lechenii dodementnykh kognitivnykh narusheniĭ v

pozdnem vozraste [Clinical efficacy and safety of choline alfoscerate in the treatment of late-onset cognitive impairment]. *Zh. Nevrol. Psikhiatr. Im. Korsakova* 118, 45–53. doi: 10.17116/jnevro20181185145

Geranmayeh, M. H., Rahbarghazi, R., and Farhoudi, M. (2019). Targeting pericytes for neurovascular regeneration. *Cell Commun. Signal.* 17:26. doi: 10.1186/s12964-019-0340-8

Gil, G. A., Bussolino, D. F., Portal, M. M., Alfonso-Pecchio, A., Renner, M. L., Borioli, G. A., et al. (2004). c-Fos activated phospholipid synthesis is required for neurite elongation in differentiating PC12 cells. *Mol. Biol. Cell* 15, 1881–1894. doi: 10.1091/mbc.e03-09-0705

Gordon, G. R., Howarth, C., and Macvicar, B. A. (2011). Bidirectional control of arteriole diameter by astrocytes. *Exp. Physiol.* 96, 393–399. doi: 10.1113/expphysiol.2010.053132

Grasso, S., Bramanti, V., Tomassoni, D., Bronzi, D., Malfa, G., Traini, E., et al. (2014). Effect of lipoic acid and α-glyceryl-phosphoryl-choline on astroglial cell proliferation and differentiation in primary culture. *J Neurosci. Res.* 92, 86–94. doi: 10.1002/jnr.23289

Gursoy-Ozdemir, Y., Qiu, J., Matsuoka, N., Bolay, H., Bermpohl, D., Jin, H., et al. (2004). Cortical spreading depression activates and upregulates MMP-9. *J. Clin. Invest.* 113, 1447–1455. doi: 10.1172/JCI21227

Gutiérrez-Fernández, M., Leciñana, M. A., Rodríguez-Frutos, B., Ramos-Cejudo, J., Roda, J. M., and Díez-Tejedor, E. (2012a). CDP-choline at high doses is as effective as i.v.thrombolysis in experimental animal stroke. *Neurol. Res.* 34, 649–656. doi: 10.1179/1743132812Y.0000000058

Gutiérrez-Fernández, M., Rodríguez-Frutos, B., Fuentes, B., Vallejo-Cremades, M. T., Alvarez-Grech, J., Expósito-Alcaide, M., et al. (2012b). CDP-choline treatment induces brain plasticity markers expression in experimental animal stroke. *Neurochem. Int.* 60, 310–317. doi: 10.1016/j.neuint.2011.12.015

Han, G., Song, L., Ding, Z., Wang, Q., Yan, Y., Huang, J., et al. (2022). The important double-edged role of astrocytes in neurovascular unit after ischemic stroke. *Front. Aging Neurosci.* 14:833431. doi: 10.3389/fnagi.2022.833431

Harder, D. R., Zhang, C., and Gebremedhin, D. (2002). Astrocytes function in matching blood flow to metabolic activity. *News Physiol. Sci.* 17, 27–31. doi: 10.1152/physiologyonline.2002.17.1.27

Higgins, J. P., and Flicker, L. (2003). Lecithin for dementia and cognitive impairment. *Cochrane Database Syst. Rev.* CD001015. doi: 10.1002/14651858.CD001015

Hurtado, O., Lizasoain, I., and Moro, M. A. (2011). Neuroprotection and recovery: Recent data at the bench on citicoline. *Stroke* 2(Suppl.), S33–S35. 110.597435 doi: 10.1161/STROKEAHA

Inazu, M. (2019). Functional expression of choline transporters in the blood-brain barrier. *Nutrients* 11:2265. doi: 10.3390/nu11102265

Inazu, M., Takeda, H., and Matsumiya, T. (2005). Molecular and functional characterization of an Na+-independent choline transporter in rat astrocytes. *J. Neurochem.* 94, 1427–1437. doi: 10.1111/j.1471-4159.2005.03299.x

Iwao, B., Yara, M., Hara, N., Kawai, Y., Yamanaka, T., Nishihara, H., et al. (2016). Functional expression of choline transporter like-protein 1 (CTL1) and CTL2 in human brain microvascular endothelial cells. *Neurochem. Int.* 93, 40–50. doi: 10.1016/j.neuint.2015.12.011

Javaid, S., Farooq, T., Rehman, Z., Afzal, A., Ashraf, W., Rasool, M., et al. (2021). Dynamics of choline-containing phospholipids in traumatic brain injury and associated comorbidities. *Int. J. Mol. Sci.* 22:11313. doi: 10.3390/ijms222111313

Jayaraj, R., Azimullah, S., Beiram, R., Jalal, F. Y., and Rosenberg, G. A. (2019). Neuroinflammation: Friend and foe for ischemic stroke. *J. Neuroinflammation* 16:142. doi: 10.1186/s12974-019-1516-2

Jeong Yu, H., Lin Kim, Y., Jung Kim, M., Mee Park, J., Young Park, S., Nae Park, S., et al. (2022). The effect of choline alphoscerate on non spatial memory and neuronal differentiation in a rat model of dual stress. *Brain Res.* 1786:147900. doi: 10.1016/j.brainres.2022.147900

Kim, C. K., Lee, Y. R., Ong, L., Gold, M., Kalali, A., and Sarkar, J. (2022). Alzheimer's disease: Key insights from two decades of clinical trial failures. *J. Alzheimers Dis.* 87, 83–100. doi: 10.3233/JAD-215699

Kim, J. H., Choi, B. Y., Kho, A. R., Lee, S. H., Jeong, J. H., Hong, D. K., et al. (2018). Acetylcholine precursor, citicoline (cytidine 5'-diphosphocholine), reduces hypoglycaemia-induced neuronal death in rats. *J. Neuroendocrinol.* 30. doi: 10.1111/jne.12567

Kim, J. H., Lee, D. W., Choi, B. Y., Sohn, M., Lee, S. H., Choi, H. C., et al. (2015). Cytidine 5′-diphosphocholine (CDP-choline) adversely effects on pilocarpine seizure-induced hippocampal neuronal death. *Brain Res.* 1595, 156–165. doi: 10.1016/j.brainres.2014.11.01

Kim, K. J., and Filosa, J. A. (2012). Advanced *in vitro* approach to study neurovascular coupling mechanisms in the brain microcirculation. *J. Physiol.* 590, 1757–1770. doi: 10.1113/jphysiol.2011.222778

Kim, S. T., Chung, Y. H., Lee, H. S., Chung, S. J., Lee, J. H., Sohn, U. D., et al. (2015). Protective effects of phosphatidylcholine on oxaliplatin-induced neuropathy in rats. *Life Sci.* 130, 81–87. doi: 10.1016/j.lfs.2015.03.013

Kim, S. T., Kyung, E. J., Suh, J. S., Lee, H. S., Lee, J. H., Chae, S. I., et al. (2018). Phosphatidylcholine attenuated docetaxel-induced peripheral neurotoxicity in rats. *Drug Chem. Toxicol.* 41, 476–485. doi: 10.1080/01480545.2017.1390580

Klein, R. S., and Hunter, C. A. (2017). Protective and pathological immunity during central nervous system infections. *Immunity* 46, 891–909. doi: 10.1016/j.immuni.2017.06.012

Ko, M., Hattori, T., Abdullah, M., Gong, J. S., Yamane, T., and Michikawa, M. (2016). Phosphatidylcholine protects neurons from toxic effects of amyloid β-protein in culture. *Brain Res.* 1642, 376–383. doi: 10.1016/j.brainres.2016.04.035

Kuryata, O. V., Kushnir, Y. S., Nedzvetsky, V. S., Korsa, V. V., and Tykhomyrov, A. A. (2021). Serum levels of the biomarkers associated with astrocytosis, neurodegeneration, and demyelination: Neurological benefits of citicoline treatment of patients with ischemic stroke and atrial fibrillation. *Neurophysiology* 53, 2–12. doi: 10.1007/s11062-021-09907-3

Lafrenaye, A. D., Mondello, S., Wang, K. K., Yang, Z., Povlishock, J. T., Gorse, K., et al. (2020). Circulating GFAP and Iba-1 levels are associated with pathophysiological sequelae in the thalamus in a pig model of mild TBI. *Sci. Rep.* 10:13369. doi: 10.1038/s41598-020-70266-w

Lee, M., Choi, B. Y., and Suh, S. W. (2018). Unexpected effects of acetylcholine precursors on pilocarpine seizure- induced neuronal death. *Curr. Neuropharmacol.* 16, 51–58. doi: 10.2174/1570159X15666170518150053

Lee, J., Hamanaka, G., Lo, E. H., and Arai, K. (2019). Heterogeneity of microglia and their differential roles in white matter pathology. *CNS Neurosci. Ther.* 25, 1290–1298. doi: 10.1111/cns.13266

Lee, S. H., Choi, B. Y., Kim, J. H., Kho, A. R., Sohn, M., Song, H. K., et al. (2017). Late treatment with choline alfoscerate (l-alpha glycer-ylphosphorylcholine, alpha-GPC) increase hippocampal neurogenesis and provides protection against seizure induced neuronal death and cognitive impairment. *Brain Res.* 1654(Pt A), 66–76. doi: 10.1016/j.brainres.2016.10.011

Levin, O. S., Batukaeva, L. A., Anikina, M. A., and Iunishchenko, N. A. (2009). Efficacy and tolerability of choline alphoscerate (cereton) in patients with Parkinson's disease with cognitive disorders. *Zh. Nevrol. Psikhiatr. Im. S S Korsakova* 109, 42–46.

Li, Q., and Barres, B. A. (2018). Microglia and macrophages in brain homeostasis and disease. *Nat. Rev. Immunol.* 18, 225–242. doi: 10.1038/nri.2017.125

Liu, L. R., Liu, J. C., Bao, J. S., Bai, Q. Q., and Wang, G. Q. (2020). Interaction of microglia and astrocytes in the neurovascular unit. *Front. Immunol.* 11:1024. doi: 10.3389/fimmu.2020.01024

López-Bayghen, E., and Ortega, A. (2011). Glial glutamate transporters: New actors in brain signaling. *IUBMB Life* 63, 816–823. doi: 10.1002/iub.536

Ma, X., Zhang, H., Pan, Q., Zhao, Y., Chen, J., Zhao, B., et al. (2013). Hypoxia/Aglycemia-induced endothelial barrier dysfunction and tight junction protein downregulation can be ameliorated by citicoline. *PLoS One* 8:e82604. doi: 10.1371/journal.pone.0082604

Machová, E., O'Regan, S., Newcombe, J., Meunier, F. M., Prentice, J., Dove, R., et al. (2009). Detection of choline transporter-like 1 protein CTL1 in neuroblastoma x glioma cells and in the CNS, and its role in choline uptake. *J. Neurochem.* 110:1297–1309. doi: 10.1111/j.1471-4159.2009.06218.x

Marcucci, H., Paoletti, L., Jackowski, S., and Banchio, C. (2010). Phosphatidylcholine biosynthesis during neuronal differentiation and its role in cell fate determination. *J. Biol. Chem.* 285, 25382–25393. doi: 10.1074/jbc.M110.139477

Martí-Carvajal, A. J., Valli, C., Martí-Amarista, C. E., Solà, I., Martí-Fàbregas, J., and Bonfill Cosp, X. (2020). Citicoline for treating people with acute ischemic stroke. *Cochrane Database Syst. Rev.* 8:CD013066. doi: 10.1002/14651858.CD013066.pub2

Mashin, V. V., Belova, L. A., Bakhtogarimov, I. R., Bergelson, T. M., and Sharafutdinova, R. R. (2017). Mnogotsentrovaia nabliudatel'naia programma po otsenke éffektivnosti preparata rekognan (tsitikolin) v korrektsii kognitivnykh narusheniĭ u patsientov s khronicheskoĭ tserebrovaskuliarnoĭ patologieĭ [Multicenter observational program for evaluation of the effectiveness of the recognan (citicoline) in the correction of cognitive impairment in patients with chronic cerebrovascular pathology]. *Zh. Nevrol. Psikhiatr. Im. S S Korsakova* 117, 39–43. doi: 10.17116/jnevro2017117818139-43

Matsubara, K., Okuda, M., Shibata, S., Miyaki, S., Ohkubo, T., Izu, H., et al. (2018). The delaying effect of alpha-glycerophosphocholine on senescence, transthyretin deposition, and osteoarthritis in senescence-accelerated mouse prone 8 mice. *Biosci. Biotechnol. Biochem.* 82, 647–653. doi: 10.1080/09168451.2017.1403883

Mauch, D. H., Nägler, K., Schumacher, S., Göritz, C., Müller, E. C., Otto, A., et al. (2001). CNS synaptogenesis promoted by glia-derived cholesterol. *Science* 294, 1354–1357. doi: 10.1126/science.294.5545.1354

Mehta, D., Jackson, R., Paul, G., Shi, J., and Sabbagh, M. (2017). Why do trials for Alzheimer's disease drugs keep failing? A discontinued drug perspective for 2010-2015. *Expert Opin. Investig. Drugs* 26, 735–739. doi: 10.1080/13543784.2017.13238

Meshitsuka, S., and Aremu, D. A. (2008). (13)C heteronuclear NMR studies of the interaction of cultured neurons and astrocytes and aluminum blockade of the preferential release of citrate from astrocytes. *J. Biol. Inorgan. Chem.* 13, 241–247. doi: 10.1007/s00775-007-0317-8

Muma, N. A., and Rowell, P. P. (1988). Effects of chronic choline and lecithin on mouse hippocampal dendritic spine density. *Exp. Aging Res.* 14, 137–141. doi: 10.1080/03610738808259738

Muoio, V., Persson, P. B., and Sendeski, M. M. (2014). The neurovascular unit - concept review. *Acta Physiol (Oxf).* 210, 790–798. doi: 10.1111/apha.1225

Nakayama, T., Momoki-Soga, T., and Inoue, N. (2003). Astrocyte-derived factors instruct differentiation of embryonic stem cells into neurons. *Neurosci. Res.* 46, 241–249. doi: 10.1016/s0168-0102(03)00063-4

Nimmerjahn, A., Kirchhoff, F., and Helmchen, F. (2005). Resting microglial cells are highly dynamic surveillants of brain parenchyma *in vivo*. *Science* 308, 1314–1318. doi: 10.1126/science.1110647

Nitsch, R., Pittas, A., Blusztajn, J. K., Slack, B. E., Growdon, J. H., and Wurtman, R. J. (1991). Alterations of phospholipid metabolites in postmortem brain from patients with Alzheimer's disease. *Ann. N. Y. Acad. Sci.* 640, 110–113.

Okuda, T., Haga, T., Kanai, Y., Endou, H., Ishihara, T., and Katsura, I. (2000). Identification and characterization of the high-affinity choline transporter. *Nat. Neurosci.* 3, 120–125. doi: 10.1038/72059

Okura, T., Hattori, A., Takano, Y., Sato, T., Hammarlund-Udenaes, M., Terasaki, T., et al. (2008). Involvement of the pyrilamine transporter, a putative organic cation transporter, in blood-brain barrier transport of oxycodone. *Drug Metab. Dispos.* 36, 2005–2013. doi: 10.1124/dmd.108.022087

Paoletti, L., Elena, C., Domizi, P., and Banchio, C. (2011). Role of phosphatidylcholine during neuronal differentiation. *IUBMB Life* 63, 714–720. doi: 10.1002/iub.521

Parnetti, L., Mignini, F., Tomassoni, D., Traini, E., and Amenta, F. (2007). Cholinergic precursors in the treatment of cognitive impairment of vascular origin: Ineffective approaches or need for re-evaluation? *J. Neurol. Sci.* 257, 264–269. doi: 10.1016/j.jns.2007.01.043

Parnetti, L., Amenta, F., and Gallai, V. (2001). Choline alphoscerate in cognitive decline and in acute cerebrovascular disease: An analysis of published clinical data. *Mech. Ageing Dev.* 122, 2041–2055. doi: 10.1016/s0047-6374(01)00312-8

Pizova, N. V. (2014). The use of cereton in patients with chronic brain ischemia and moderate cognitive impairment. *Zh. Nevrol. Psikhiatr. Im. S S Korsakova* 114, 78–83. doi: 10.17116/jnevro201411412178-83

Plog, B. A., and Nedergaard, M. (2018). The glymphatic system in central nervous system health and disease: Past, present, and future. *Annu. Rev. Pathol.* 13, 379–394. doi: 10.1146/annurev-pathol-051217-111018

Pont-Lezica, L., Béchade, C., Belarif-Cantaut, Y., Pascual, O., and Bessis, A. (2011). Physiological roles of microglia during development. *J. Neurochem.* 119, 901–908. doi: 10.1111/j.1471-4159.2011.07504.x

Rao, A. M., Hatcher, J. F., and Dempsey, R. J. (2000). Lipid alterations in transient forebrain ischemia: Possible new mechanisms of CDP-choline neuroprotection. *J. Neurochem.* 75, 2528–2535. doi: 10.1046/j.1471-4159.2000.0752528.x

Rea, R., Carotenuto, A., Traini, E., Fasanaro, A. M., Manzo, V., and Amenta, F. (2015). Apathy treatment in Alzheimer's disease: Interim results of the ASCOMALVA trial. *J. Alzheimers Dis.* 48, 377–383. doi: 10.3233/JAD-141983

Rema, V., Bali, K. K., Ramachandra, R., Chugh, M., Darokhan, Z., and Chaudhary, R. (2008). Cytidine-5-diphosphocholine supplement in early life induces stable increase in dendritic complexity of neurons in the somatosensory cortex of adult rats. *Neuroscience* 155, 556–564. doi: 10.1016/j.neuroscience.2008.04

Ridgway, D. N. (2021). "Phospholipid synthesis in mammalian cells," in *Biochemistry of lipids, lipoproteins and membranes*, 7th Edn, Vol. 7, eds N. Ridgway and R. McLeod 227–258.

Sagare, A. P., Bell, R. D., Zhao, Z., Ma, Q., Winkler, E. A., Ramanathan, A., et al. (2013). Pericyte loss influences Alzheimer-like neurodegeneration in mice. *Nat. Commun.* 4:2932. doi: 10.1038/ncomms3932

Salvadori, E., Poggesi, A., Donnini, I., Rinnoci, V., Chiti, G., Squitieri, M., et al. (2021). Efficacy and safety of the association of nimodipine and choline alphoscerate in the treatment of cognitive impairment in patients with cerebral small vessel disease. The CONIVaD trial. *Drugs Aging* 38, 481–491. doi: 10.1007/s40266-021-00852-8

Sanchez-Lopez, E., Zhong, Z., Stubelius, A., Sweeney, S. R., Booshehri, L. M., Antonucci, L., et al. (2019). Choline uptake and metabolism modulate macrophage IL-1β and IL-18 production. *Cell Metab.* 29, 1350–1362.e7. doi: 10.1016/j.cmet.2019.03.011

Santello, M., Calì, C., and Bezzi, P. (2012). Glio transmission and the tripartite synapse. *Adv. Exp. Med. Biol.* 970, 307–331. doi: 10.1007/978-3-7091-0932-8_14

Sasaki, Y., Ohsawa, K., Kanazawa, H., Kohsaka, S., and Imai, Y. (2001). Iba1 is an actin-cross-linking protein in macrophages/microglia. *Biochem. Biophys. Res. Commun.* 286, 292–297. doi: 10.1006/bbrc.2001.5388

Schnaar, R. L. (2010). Brain gangliosides in axon-myelin stability and axon regeneration. *FEBS Lett.* 584, 1741–1747. doi: 10.1016/j.febslet.2009.10.011

Secades, J. J., and Frontera, G. (1995). CDP-choline: Pharmacological and clinical review. *Methods Find. Exp. Clin. Pharmacol.* 17 Suppl B, 1–54.

Secades, J. J., and Lorenzo, J. L. (2006). Citicoline: Pharmacological and clinical review, 2006 update. *Methods Find. Exp. Clin. Pharmacol.* 28(Suppl. B), 1–56.

Shimomura, K., Okura, T., Kato, S., Couraud, P. O., Schermann, J. M., Terasaki, T., et al. (2013). Functional expression of a proton-coupled organic cation (H+/OC) antiporter in human brain capillary endothelial cell line hCMEC/D3, a human blood-brain barrier model. *Fluids Barriers CNS* 10:8. doi: 10.1186/2045-8118-10-8

Sigala, S., Imperato, A., Rizzonelli, P., Casolini, P., Missale, C., and Spano, P. (1992). L-alpha-glycerylphosphorylcholine antagonizes scopolamine-induced amnesia and enhances hippocampal cholinergic transmission in the rat. *Eur. J. Pharmacol.* 211, 351–358. doi: 10.1016/0014-2999(92)90392-h

Sobrino, T., Rodríguez-González, R., Blanco, M., Brea, D., Pérez-Mato, M., Rodríguez-Yáñez, M., et al. (2011). CDP-choline treatment increases circulating endothelial progenitor cells in acute ischemic stroke. *Neurol. Res.* 33, 572–577. doi: 10.1179/016164110X12807570510176

Stogsdill, J. A., Ramirez, J., Liu, D., Kim, Y. H., Baldwin, K. T., Enustun, E., et al. (2017). Astrocytic neuroligins control astrocyte morphogenesis and synaptogenesis. *Nature* 551, 192–197. doi: 10.1038/nature24638

Sweeney, M. D., Kisler, K., Montagne, A., Toga, A. W., and Zlokovic, B. V. (2018). The role of brain vasculature in neurodegenerative disorders. *Nat. Neurosci.* 21, 1318–1331. doi: 10.1038/s41593-018-0234

Tabernero, A., Lavado, E. M., Granda, B., Velasco, A., and Medina, J. M. (2001). Neuronal differentiation is triggered by oleic acid synthesized and released by astrocytes. *J. Neurochem.* 79, 606–616. doi: 10.1046/j.1471-4159.2001.00598.x

Tayebati, S. K., Di Tullio, M. A., Tomassoni, D., and Amenta, F. (2009). Neuroprotective effect of treatment with galantamine and choline alphoscerate on brain microanatomy in spontaneously hypertensive rats. *J. Neurol. Sci.* 283, 187–194. doi: 10.1016/j.jns.2009.02.349

Tayebati, S. K., Tomassoni, D., Di Stefano, A., Sozio, P., Cerasa, L. S., and Amenta, F. (2011). Effect of choline-containing phospholipids on brain cholinergic transporters in the rat. *J. Neurol. Sci.* 302, 49–57. doi: 10.1016/j.jns.2010.11.028

Tayebati, S. K., and Amenta, F. (2013). Choline-containing phospholipids: Relevance to brain functional pathways. *Clin. Chem. Lab. Med.* 51, 513–521. doi: 10.1515/cclm-2012-0559

Tayebati, S. K., Tomassoni, D., Nwankwo, I. E., Di Stefano, A., Sozio, P., Cerasa, L. S., et al. (2013). Modulation of monoaminergic transporters by choline-containing phospholipids in rat brain. *CNS Neurol. Disord. Drug Targets* 12, 94–103. doi: 10.2174/18715273113120100015

Tayebati, S. K., Amenta, F., and Tomassoni, D. (2015). Cerebrovascular and blood-brain barrier morphology in spontaneously hypertensive rats: Effect of treatment with choline alphoscerate. *CNS Neurol. Disord. Drug Targets* 14, 421–429. doi: 10.2174/1871527314666150225140855

Tayebati, S. K. (2018). Phospholipid and lipid derivatives as potential neuroprotective compounds. *Molecules* 23:2257. doi: 10.3390/molecules23092257

Teather, L. A., and Wurtman, R. J. (2003). Dietary cytidine (5')-diphosphocholine supplementation protects against development of memory deficits in aging rats. *Prog. Neuro Psychopharmacol. Biol. Psychiatry* 27, 711–717. doi: 10.1016/S0278-5846(03)00086-1

Teleanu, D. M., Niculescu, A. G., Lungu, I. I., Radu, C. I., Vladâcenco, O., Roza, E., et al. (2022). An overview of oxidative stress, neuroinflammation, and neurodegenerative diseases. *Int. J.Mol. Sci.* 23:5938. doi: 10.3390/ijms23115938

Thurgur, H., and Pinteaux, E. (2018). Microglia in the neurovascular unit: Blood-brain barrier-microglia interactions after central nervous system disorders. *Neuroscience* 405, 55–67. doi: 10.1016/j.neuroscience.2018.06.046

Tietz, S., and Engelhardt, B. (2015). Brain barriers: Crosstalk between complex tight junctions and adherens junctions. *J. Cell Biol.* 209, 493–506. doi: 10.1083/jcb.201412147

Tokés, T., Eros, G., Bebes, A., Hartmann, P., Várszegi, S., Varga, G., et al. (2011). Protective effects of a phosphatidylcholine-enriched diet in lipopolysaccharide-induced experimental neuroinflammation in the rat. *Shock* 36, 458–465. doi: 10.1097/SHK.0b013e31822f36b0

Tomassoni, D., Avola, R., Mignini, F., Parnetti, L., and Amenta, F. (2006). Effect of treatment with choline alphoscerate on hippocampus microanatomy and glial reaction in spontaneously hypertensive rats. *Brain Res.* 1120, 183–190. doi: 10.1016/j.brainres.2006.08.068

Tomassoni, D., Catalani, A., Cinque, C., Di Tullio, M. A., Tayebati, S. K., Cadoni, A., et al. (2012). Effects of cholinergic enhancing drugs on cholinergic transporters in the brain and peripheral blood lymphocytes of spontaneously hypertensive rats. *Curr. Alzheimer Res.* 9, 120–127. doi: 10.2174/156720512799015118

Traini, E., Bramanti, V., and Amenta, F. (2013). Choline alphoscerate (alpha-glyceryl-phosphoryl-choline) an old choline- containing phospholipid with a still interesting profile as cognition enhancing agent. *Curr. Alzheimer. Res. Dec.* 10, 1070–1079. doi: 10.2174/15672050113106660173

Traini, E., Carotenuto, A., Fasanaro, A. M., and Amenta, F. (2020). Volume analysis of brain cognitive areas in Alzheimer's disease: Interim 3-year results from the ASCOMALVA trial. *J. Alzheimers Dis.* 76, 317–329. doi: 10.3233/JAD-190623

Vance, J. E., Campenot, R. B., and Vance, D. E. (2000). The synthesis and transport of lipids for axonal growth and nerve regeneration. *Biochim. Biophys. Acta* 1486, 84–96. doi: 10.1016/s1388-1981(00)00050-0

Vance, J. E., and Vance, D. E. (2004). Phospholipid biosynthesis in mammalian cells. *Biochem. Cell Biol.* 82, 113–128. doi: 10.1139/o03-073

Wang, M., Pan, W., Xu, Y., Zhang, J., Wan, J., and Jiang, H. (2022). Microglia-mediated neuroinflammation: A potential target for the treatment of cardiovascular diseases. *J. Inflamm. Res.* 15, 3083–3094. doi: 10.2147/JIR.S350109

Wurtman, R. J., Blusztajn, J. K., Ulus, I. H., Coviella, I. L., Buyukuysal, R. L., Growdon, J. H., et al. (1990). Choline metabolism in cholinergic neurons: Implications for the pathogenesis of neurodegenerative diseases. *Adv. Neurol.* 51, 117–125.

Xu, S., Lu, J., Shao, A., Zhang, J. H., and Zhang, J. (2020). Glial cells: Role of the immune response in ischemic stroke. *Front. Immunol.* 11:294. doi: 10.3389/fimmu.2020.00294

Yamazaki, Y., and Kanekiyo, T. (2017). Blood-brain barrier dysfunction and the pathogenesis of Alzheimer's disease. *Int. J. Mol. Sci.* 18:1965. doi: 10.3390/ijms18091965

Yemisci, M., Gursoy-Ozdemir, Y., Vural, A., Can, A., Topalkara, K., and Dalkara, T. (2009). Pericyte contraction induced by oxidative-nitrative stress impairs capillary reflow despite successful opening of an occluded cerebral artery. *Nat. Med.* 15, 1031–1037. doi: 10.1038/nm.2022

Ylilauri, M. P. T., Voutilainen, S., Lönnroos, E., Virtanen, H. E. K., Tuomainen, T. P., Salonen, J. T., et al. (2019). Associations of dietary choline intake with risk of incident dementia and with cognitive performance: The Kuopio ischaemic heart disease risk factor study. *Am. J. Clin. Nutr.* 110, 1416–1423. doi: 10.1093/ajcn/nqz148

Zenaro, E., Piacentino, G., and Constantin, G. (2017). The blood-brain barrier in Alzheimer's disease. *Neurobiol. Dis.* 107, 41–56.

Zlokovic, B. V. (2008). The blood-brain barrier in health and chronic neurodegenerative disorders. *Neuron* 57, 178–201. doi: 10.1016/j.neuron.2008.01.003

antioxidants

MDPI

Review

Natural Antioxidant Application on Fat Accumulation: Preclinical Evidence

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Abstract: Obesity represents one of the most important challenges in the contemporary world that must be overcome. Different pathological consequences of these physical conditions have been studied for more than 30 years. The most nagging effects were found early in the cardiovascular system. However, later, its negative impact was also investigated in several other organs. Damage at cellular structures due to overexpression of reactive oxygen species together with mechanisms that cause under-production of antioxidants leads to the development of obesity-related complications. In this view, the negative results of oxidant molecules due to obesity were studied in various districts of the body. In the last ten years, scientific literature has reported reasonable evidence regarding natural and synthetic compounds' supplementation, which showed benefits in reducing oxidative stress and inflammatory processes in animal models of obesity. This article attempts to clarify the role of oxidative stress due to obesity and the opposing role of antioxidants to counter it, reported in preclinical studies. This analysis aims to clear-up different mechanisms that lead to the build-up of pro-oxidants during obesity and how various molecules of different origins hinder this phenomenon, behaving as antioxidants.

Keywords: antioxidant; obesity; inflammation; preclinical studies

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1. Introduction

In the last years, several studies tried to spot the link between antioxidant compounds and oxidative stress due to obesity. Obesity is one of the public health problems because it may lead to metabolic and cardiovascular disorders. Obesity is a chronic and multifactorial disease characterized by an increase in the white adipose tissue (WAT) that results mainly in fat accumulation localized in the abdominal region [1]. Obesity is considered a medical challenge because it is associated with chronic disease development. Actually, high body mass index (BMI) or the increase of waist circumference is also correlated with the development of cardiovascular risk factors such as hypertension, dyslipidemia, insulin resistance, and diabetes mellitus [2–5]. In a meta-analysis, including 2.88 million individuals, all obesity grades were associated with a significant increase in mortality rate [6].

Oxidative stress plays an essential role in the development of co-morbidities in obese condition [7]. Obesity promotes oxidative stress through different biochemical mechanisms [7], as summarized in Figure 1. Moreover, evidence accumulated over the past two decades has pointed to significant connections between inflammation and oxidative stress, where every process contributes to fuel the other one, thereby establishing a vicious cycle able to perpetuate and propagate the inflammatory response. This correlation is mediated by the downregulation of nuclear factor kappa-light-chain-enhancer of activated B cells

(NF-κB) signaling, the activation of the NLR family pyrin domain containing 3 (NLRP3) inflammasome, and Toll-like receptors (TLR). On the TLR, the extracellular domain connects through a transmembrane domain to the cytoplasmic Toll/interleukin-1 receptor (TIR) domain, which interacts with adaptor molecules. Consequently, the expression of inflammatory mediators is upregulated, comprising notably pro-oxidant enzymes such as NADPH oxidase (NOX) and inducible nitric oxide synthase (iNOS) and producing high levels of reactive oxygen species (ROS). TLR engagement also facilitates the generation of ROS within mitochondria and promotes activation of NOX [8].

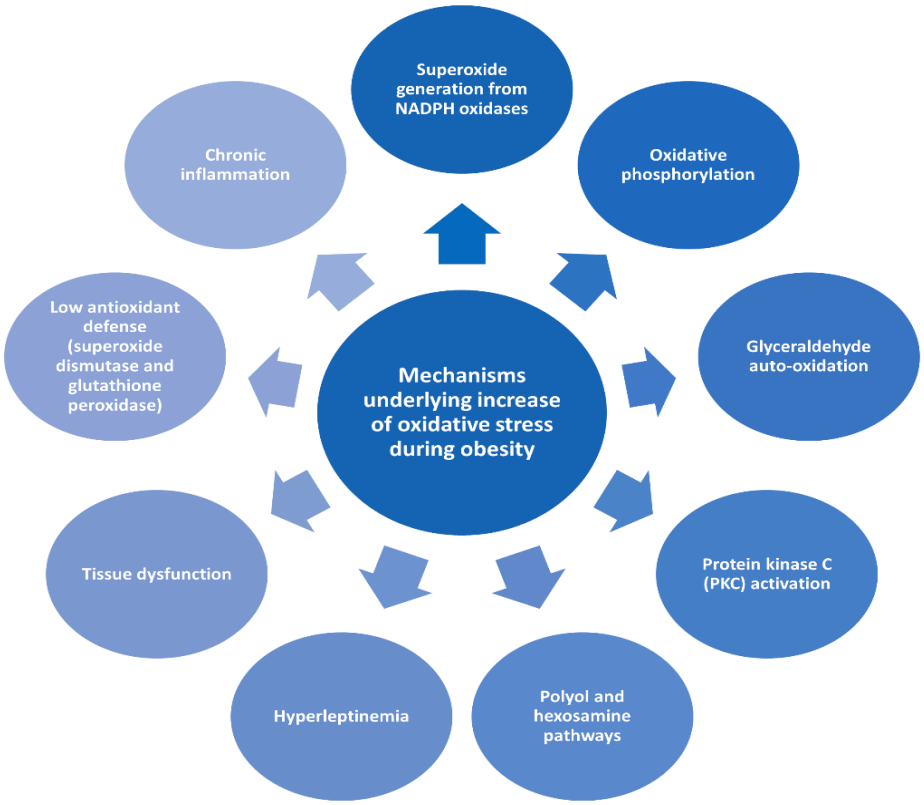

Figure 1. Mechanisms underlying increase of oxidative stress during obesity.

The role of plant-derived antioxidants in food and human health has been widely investigated. The positive effect of many food products and beverages such as fruits, vegetables, coffee, tea, and cacao on human health is due to their antioxidant activity [9]. Antioxidants' supplementation is not necessary in a balanced diet. Indeed, the Mediterranean diet, characterized more by intake of plant-based foods and fish and less consumption of meat and dairy products, represents the gold standard in preventive medicine, thanks to the synergy of many foods with antioxidant and anti-inflammatory effects [10]. As reviewed in [11], clinical trials failed to demonstrate beneficial effects of synthetic antioxidants' supplementation in preventing diseases. There is also some concern that inappropriate use of dietary supplements may induce "antioxidative stress" and thus they may be harmful. Before prescribing antioxidants, an accurate determination of an individual's oxidative stress levels is required [11].

Antioxidants are compounds that decrease the levels of ROS, which can modulate mechanisms of the homeostasis of glucose, lipids, and amino acids and suppress inflammation [12–14].

The development of functional foods for health benefit is important to the determination of bioaccessibility and bioavailability [15]. Having various compounds in bioactive food molecules that result in a complex chemical structure by adding the lipophilic and hydrophilic molecules may differ the absorption mechanisms. There is also a significant

difference in absorption of polyphenols groups, even within the same subclass of compounds [16]. Phenolic compounds are cited as a secondary metabolite in plants. At low concentration, phenolic compounds may act as an antioxidant. On the contrary, at high concentrations, these compounds may interact with proteins, carbohydrates, and minerals [17]. Flavonoids are polyphenolic antioxidant compounds that are typically both ROS scavengers and metal chelators that potentially produce double protection. They are generally accepted as heavily advocated antioxidants for the interesting polymer-based delivery and its short-time circulation ability, which correlate their antioxidant activity with the degree of polymerization [18].

Sugar molecules have an effective role in the absorption of phenolic compounds. If the phenolic compounds contain a sugar molecule, such as galactose or xylose, glucose, they will be absorbed through the small intestine by the cytosolic β-glucosidase/lactase phlorizin hydrolase [19]. If the sugar molecules are not present, the hydrophilic cannot be absorbed in the upper gastrointestinal part. (−)-epicatechin (EC) and (−)-epigallocatechin (EGC) are acylated flavonoids. They can be directly absorbed without deconjugation and hydrolysis [20]. The aglycone is a phase II substrate for metabolism, typically glucuronidation, sulfation, and methylation, carried out by UDP-glucuronosyltransferases, sulfotransferases, and catechol-*O*-methyltransferases (COMT) [21]. During the phase II of metabolism, metabolites are rapidly reached in the liver via the portal vein.

In vitro methods result in the changes in bioactive compounds' bioaccessibility with the variations of food matrix and food processing. Several studies have demonstrated that the bioavailability and bioactivity of some functional components receive support from microbiota. Increasing the antioxidant bioactivity using probiotics and prebiotic strains is an innovative manner, which alternatively increases the involvement of natural and metabolic components [22]. The rearrangement of the structure of polyphenols (by the addition or removal of hydroxyl and methoxy groups) at the colonic level might affect metabolism on microbiota [23].

Increasing attention has been given to the different strategies and possible therapies targeting differentiation of adipogenesis, glucose intake and transport, attenuation of inflammation, and changes within the immune response. Long-term and low-level inflammation usually present in obese subjects, and alterations presented within the metabolism, could lead to changed immunity. Understanding the mechanisms of action of antioxidants in human health conservation and disease prevention could promote interest in new drug discovery. Besides, it may also clear the potential of diets in the prevention of obesity and several diseases linked to it. This review attempts to clarify the role of natural antioxidant compounds in oxidative stress and inflammation due to obesity, focusing on preclinical studies.

2. Natural Antioxidant Compounds

2.1. Flavonoids

More and more people are coming to discover the powerful healing properties of antioxidants. One of them that is getting the most attention is anthocyanin.

Anthocyanin represents one of the largest groups of phenolic pigments with antioxidant properties. The molecules are found in red wine, some cereals, root vegetables, and red and purple fruits. Anthocyanins are potentially integrated into food and in medical products by pharmaceutical industries due to their potential health benefits [24]. They are backed up by new research that shows their beneficial effects. Anthocyanin has a high antioxidant potential as measured by laboratory tests and contains several compounds that fight free radicals [25,26]. They have been shown to cut down on inflammation of different sorts, including decreased muscle soreness, greater strength following exercise, and decreased inflammatory markers [27–29]. Anthocyanin has beneficial metabolic effects, such as decreasing fat, sugar, and insulin levels in the blood [30].

It showed useful effects on sleep, encouraging positive effects against some cancers such as reducing tumor burden in the gut and slowing tumor growth, and it might promote

brain health [31–33]. Anthocyanins are antioxidants that eliminate ROS. In particular, among them, delphinidin represents the most active scavenger against superoxide anions. In cell lines, a different study reports that anthocyanin has protective effects against oxidative stress [34]. In vivo studies demonstrated the antioxidant functions of anthocyanins. Cyanidin-3-glucoside improved oxidative stress-induced hepatic ischemia-reperfusion in rats, and cyanidin, delphinidin, and malvidin induced upregulation of antioxidant response element (ARE) pathways. Moreover, anthocyanins are effective against cytotoxicity, lipidic peroxidation, and as protectors of DNA. Besides, the capacity of the anthocyanins for stabilizing triple-helical complexes of DNA by forming complexes of anthocyanins-DNA is well-established [24]. Anthocyanins have cellular antioxidant mechanisms comparable to or greater than other micronutrients, such as vitamin E. Indirectly, anthocyanins show anti-inflammatory effects: cyanidin-3-glucoside, delphinidin-3-glucoside, and petunidin-3-glucoside inhibited NF-κB activities, while other cyanidins inhibited cyclooxygenase enzyme activities [34].

Basically, anthocyanin is a subfamily of flavonoids (Figure 2), which are abundant in fruits, seeds, and plant leaves [35]. Six major compounds are derived from the basic structure of 2-phenylchromenylium (flavylium B-ring). They are cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin, depending on their flavylium B-ring substitutions. Sugars such as glucose, arabinose, and galactose can be attached to the basic structure of anthocyanins [36,37] and could modulate the changes in the antioxidant activity. [38]. For example, in cyanidin, glycosylation in position 3 of the flavylium B-ring with glucose or rhamnose increases the antioxidant activity, but with galactose, it declines, as shown by Oxygen Radical Absorbance Capacity (ORAC) assays [24].

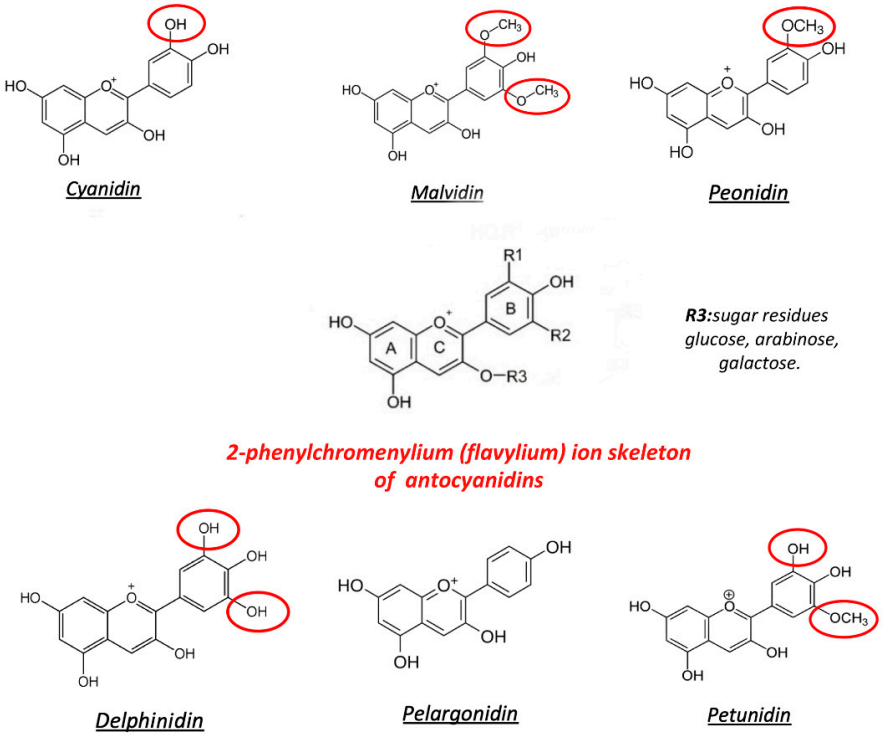

Figure 2. Structure of the most common anthocyanins. Red circles represent the substitutions of the flavylium group, in the positions R1 and R2.

The scientific literature on anthocyanin concerns small changes in the instance of the amount and position of oxygen–hydrogen (hydroxyl) units on the caffeic acid structures, which act like cannons on a ship ready to aim free radicals. Besides, when the smoke cleared, these compounds turned out to be nearly as effective as other commercial antioxidants, namely BHT (butylated hydroxytoluene) and TBHQ (tert-butylhydroquinone).

Furthermore, the anthocyanin compounds may work in an additional way, that is, they may chelate or build-up metals (e.g., iron) in the body, which produce free radicals such as hydroxyls that cause oxidative stress and organ damage [39].

Evidence continues to mount for the anti-inflammatory and antioxidant effects of anthocyanin. Different researchers checked out how cherries blocked lipid peroxidation of the plasma membrane. All of the compounds, including the base structure of cyanidin (without sugar attached), and, therefore, the three major sugar-added compounds, blocked this peroxidation. Even certain glycoside products of cyanidin appear to hold onto iron and other heavy metals, which may cause peroxidation. Overall, cyanidin actually performed better than aspirin at inhibiting cyclooxygenase (COX) [40].

Anthocyanins have the power to eliminate ROS. In vitro, a large number of studies have proven that anthocyanins, in particular cyanidin-3-glucoside, have an ORAC [41]. Delphinidin is the most active scavenger against superoxide anion [42]. Moreover, various studies suggest that it has a protective effect against oxidative stress in different cell lines [43–45]. In vivo, the antioxidant effect of anthocyanin is positive. The upregulation of the ARE pathways is induced by cyanidin, delphinidin, and malvidin antioxidant enzymes. [46].

Studies highlighted that the consumption of anthocyanins reduced body weight and insulin resistance, resulting in restored glucose tolerance. Mitogen-activated protein kinase (MAPK) pathways induce cyanidin-3-glucoside, delphinidin-3-glucoside, and petunidin-3-glucoside to inhibit NF-κB [47–49], and also the activities of cyclooxygenase enzyme were inhibited by cyanidins [50]. These biological activities of anthocyanins are closely associated with the kind and progression of diseases [24].

Red cabbage microgreen, blueberry, blackcurrant, mulberry, cherry, black elderberry, black soybean, chokeberry, and jaboticaba's peel contain a variety of anthocyanins, including cyanidins, delphinidins, malvidins, pelargonidins, peonidins, and petunidins, that showed a different positive effect on an animal model of obesity. Red cabbage microgreen decreased weight gain, low-density lipoprotein (LDL) levels, triacylglycerol, and cholesterol levels in high-fat diet-fed mice. Inflammatory cytokines such as C-reactive protein (CRP) and tumor necrosis factor alpha (TNF-α) also significantly decreased in mice. In high-fat diet (HFD) mice, the supplementation with blueberry has shown a reduction in body weight and blood glucose levels as well as TNF-α and interleukin 6 (IL-6) levels. The whole blueberry improved high-fat diet-induced insulin resistance and decreased TNF-α, IL-6, monocyte chemoattractant protein-1 (MCP-1), $CD11c^+$ also known as integrin alpha X, and iNOS. Again, in HFD mice, blueberry juice reduced the weight gain, the plasma level of insulin and leptin, the cholesterol, and triacylglycerol in the liver, reducing inflammatory markers such as TNF-α, IL-6, iNOS, and NF-kB in WAT. A similar effect was highlighted in HFD mice supplemented with anthocyanin-rich fruit such as blackcurrant, mulberry, cherry, black elderberry, black soybean, and freeze-dried jaboticaba peel. Chokeberry extracts led to improve metabolic disturbance and inflammation in rats with a fructose-rich diet, while tart cherry reduced metabolic and inflammatory markers in Zucker fatty rats [34].

Recently, the supplementation of tart cherry juice and seeds rich in anthocyanins, even if it did not reduce the weight gain, was able to reduce the neuroinflammatory process, liver steatosis, inflammation, and adipose gene transcription in visceral adipose tissue in rats under diet-induced obesity (DIO) [51–54]. Several analyses find that some compounds showed higher antioxidant activities than other compounds, particularly cyanidin derivatives such as kaempferol, quercetin, and melatonin. Each of these compounds could contribute to the antioxidant effects attributed to crude extracts of tart cherry fruit. However, some minimal level of particular constituents is needed for these molecules to exhibit their antioxidant effects most efficiently. Concentrations of antioxidant compounds in cherry may be influenced by many factors, including environmental conditions, degree of ripeness, cultivar, cultivation site, processing, and storage of the fruit [50].

The anthocyanins and other flavonoids, similarly to melatonin, found in tart cherry fruits are reported to have various phytotherapeutic activities that are supported by their modes of action at different target sites [55].

Quercetin has been shown to inhibit COX and lipoxygenase activities. These enzymes are involved in the release of arachidonic acid, the initiator of a general inflammatory response. Quercetin also exerts a preferential cytotoxic effect on dividing colon carcinoma HT29 and CACO-2 cells and induces apoptosis in human leukemia HL60 cells, following inhibition of growth. Possible mechanisms of action could include increased expression of wild-type p53, reduction of Ki RAS levels, or p21 upregulation. Besides, animal studies indicate that the incidence of carcinogen-induced mammary tumors and lung tumors was decreased by dietary administration of quercetin [56]. The flavonols, quercetin, and catechin synergistically act to inhibit platelet aggregation and adhesion to collagen, reducing atherosclerotic lesions [57]. The antioxidant effectiveness of anthocyanins and other polyphenols, in vitro, is essential because of the spontaneity of the chemical structure in which a hydrogen atom from an aromatic hydroxyl group is donated to a free radical. Besides, their ability to chelate transition metal ions, involved in radical forming processes such as Fenton reactions and the induction of endogenous antioxidants, could also contribute to the antioxidant efficacy of these compounds. Consequently, numerous studies have shown that many phenolic compounds found in fruits and vegetables, including berries, inhibit the oxidation of LDL and DNA in vitro [58].

Membrane lipid viscosity or protein movement is probably affected by the flavonoids. However, the combined bioactive mixture is potentiated when the interaction leads to improved solubility, absorption, safety, stability, or bioavailability of the active principles. Such synergistic interactions are also explained by enhanced uptake, absorption, metabolism, and reduced excretion (pharmacokinetics), or by enhanced effectiveness (binding to receptor molecules like bioactive proteins that enhance protein–protein or protein–ligand interactions) at the target sites of action (pharmacodynamics). This idea has been adopted by pharmacologists to explore combinations of several metabolites in multi-target therapy. The importance of affecting multiple targets may be beneficial when handling complex diseases, such as cancer, chronic inflammation, chronic infection, and plenty of others [59].

2.2. Resveratrol

Resveratrol (trans-3, 4′, 5-trihydroxystilbene, RSV, Figure 3) is a small polyphenol that has been largely studied for decades in a wide spectrum of therapeutic research areas [60,61]. The natural occurrence of RSV in a large variety of plant species, specifically, mulberries, peanuts, and grapes, has further fostered public opinion and claims around the possibility of using RSV within the fields of natural medicine and dietary supplementations [62].

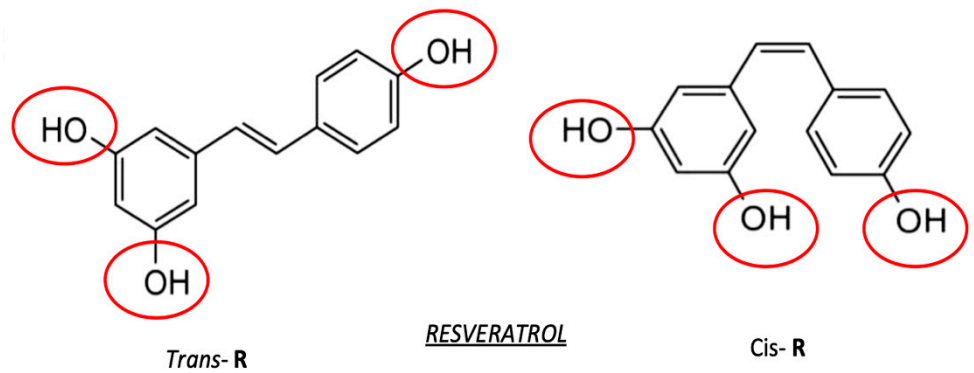

Figure 3. Configuration of *trans* (left part) and *cis* (right part) forms of RSV. Red circles represent the reactive site of the molecule.

In obese subjects, RSV exhibited a vascular protective effect, mimicking calorie restriction [63–65]. RSV also has an antioxidant effect, protected from deregulated glucose

tolerance that leads to a beneficial effect on hypertriglyceridemia and improves memory performance (maintenance of brain health) [63].

Supplementation with RSV of rodents protected animals against HFD-induced body weight gain and obesity. RSV also increased the energy expenditure, which was partly mediated by stimulating intracellular mitochondrial functions (fatty acid oxidation) in adipose tissue, by the fatty acid synthesis suppression, and by producing brown-like adipocyte formation in WAT [66–72]. The in vitro anti-inflammatory effect of RSV was also confirmed in animal models. In mice, RSV reduced HFD-induced inflammation of WAT by downregulating pro-inflammatory cytokines TNF-α, interferon alpha and beta (IFN-α and IFN-β), and IL-6 [60,73]. Moreover, RSV prevented the production of regulatory T cells and reduced adipose tissue macrophage infiltration in HFD-induced obese mice [74]. In Zucker rats, the levels of IL-6 and the activity of NF-κB were suppressed by RSV reducing macrophage infiltration in adipose tissue [60]. Amusingly, Jimenez-Gomez et al. showed that RSV reported similar effects on a high-fat-treated adult rhesus monkey model as effects on HFD-induced obese rodent models. RSV decreased the mRNA levels, suppressed the activation of pro-inflammatory cytokines IL-6, TNF-α, IL-1β, and NF-κB, and adiponectin in the visceral adipose tissue of the HFD-treated monkey model [75]. The antioxidant effect of RSV was also proven in other animal models. Lv and co-workers reported that RSV reduced oxidative stress, which is associated with diet induced partly by the reduction of sirtuin 1 (SIRT1) and manganese superoxide dismutase (MnSOD) levels [76].

Obesity is induced by providing a high-calorie diet (e.g., excessive amount of dietary fat or sugar). Common markers for obesity include weight and fasting serum levels of glucose and insulin. The effect of RSV on body weight is controversial, but it is demonstrated that there is a significant difference in glucose tolerance between standard diet and fat/sugar-enriched diet controls [62]. It was also demonstrated that RSV regimens improved glucose tolerance or lowered fasting glucose level and decreased serum insulin, in mice or swine models, compared with obese controls [77].

Numerous studies have reported the anti-inflammatory properties of RSV in several inflammation models, including arthritis, asthma, encephalomyelitis, atherosclerosis, and intestinal inflammatory diseases, among others [78–81]. Nevertheless, recently, the effect of RSV on the inflammatory process, which takes place in adipocytes under several metabolic conditions such as obesity, was studied.

The vast majority of these recent studies have been performed in in vitro conditions, by using cultured adipocytes or macrophages [67,82,83], or adipose tissue explants [84]. These data demonstrate that RSV may prevent inflammatory processes in adipocytes, but it is essential to remember that important limitations exist when extrapolating these data to the in vivo situation.

Various studies have shown the in vivo anti-inflammatory effects of several polyphenols, such as quercetin [85] or polyphenol extracts [86–88]. There are some other possible effectors which are additionally relevant targets proposed for RSV (Figure 4). Particularly, investigations on classic and novel possible targets relevant for the metabolic effects of RSV, and, more specifically, the direct action of RSV, have been described [89–92].

Despite that RSV has interesting properties and potential applications, there are some limits for its use in aqueous formulations, due to its poor solubility, stability, and bioavailability. To overcome these limitations, various solutions are being studied, including the development of new formulations using nanoparticles or nanoemulsions.

2.3. Thioctic Acid

Thioctic acid (TIO), also called alpha-lipoic acid, is a naturally occurring short-chain fatty acid that contains a thiol bond. It is an essential cofactor for energy production in the mitochondria [93]. TIO also has a powerful antioxidant effect, and it is a free radical scavenger. TIO is marketed in several places as an over-the-counter nutritional antioxidant supplement, alone or together with other antioxidants.

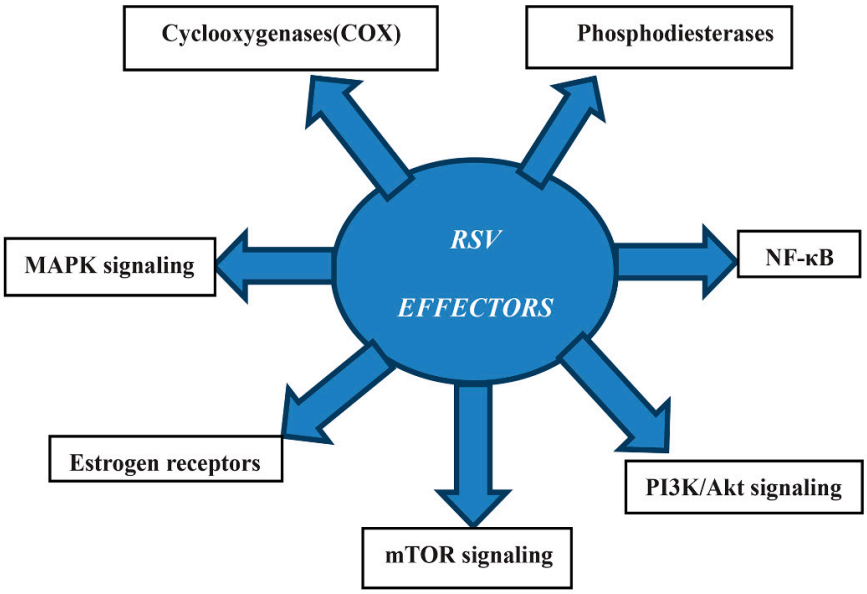

Figure 4. Mechanism of action of RSV effectors. Abbreviations: nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT), mechanistic target of rapamycin (mTOR), mitogen-activated protein kinase (MAPK).

TIO is a dithiol eight-carbon molecule (chemical formula: $C_8H_{14}O_2S_2$) [94]. It presents a chiral center inducing its isomerization into two optical enantiomers, (+)-TIO and (−)-TIO (Figure 5) [95]. The (+)-TIO is synthesized de novo in mammalian mitochondria by the enzyme lipoic acid synthase (LASY), from octanoic acid and cysteine [96]. Thanks to its amide linkage to a lysine residue, (+)-TIO acts as a cofactor for some critical mitochondrial enzymes, such as pyruvate dehydrogenase (PDH), branched-chain α-keto-acid dehydrogenase (KDH), and α-ketoglutarate dehydrogenase (KGDH) [97].

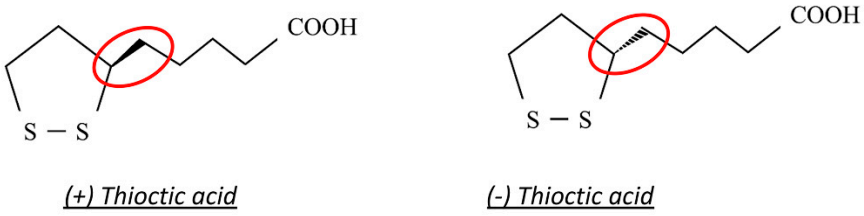

Figure 5. Chemical structure of (+)-thioctic acid and (−)-thioctic acid. Red circles represent the chiral center of the molecule.

TIO has been widely studied since the 1950s when its antioxidant properties were first discovered [98]. It has been pointed out that TIO is effective in relieving some symptoms associated with certain diseases like diabetes, age-related cardiovascular and neuromuscular defects, antipsychotic drug-related weight gain, and metabolic obesity [99–102]. Its potential effects on different types of diseases have drawn attention since the results from studies were promising, namely in the field of neurodegenerative conditions [103]. Additionally, the number of clinical trials increased to deepen knowledge on other TIO therapeutic properties and found hopeful effects. TIO is absorbed from a wide variety of animals and vegetables, and once it is inside the cell, it starts to convert its reduced form, dihydrolipoic acid [104]. It appears that TIO or its reduced form dihydrolipoic acid (DHLA) possesses many biochemical functions, acting as biological antioxidants, as metal chelators, able to regenerate endogenous antioxidants, and as a modulator of the signaling transduction of several pathways [105]. Briefly, it also has a protective mechanism that protects membranes by interacting with vitamin C and glutathione (GSH), which may, in turn, recycle vitamin E.

Moreover, dihydrolipoate may exert prooxidant actions through the reduction of iron. The application of TIO is beneficial in several oxidative stress models like ischemia-reperfusion injury, diabetes (both α-lipoic acid and dihydrolipoic acid exhibit hydrophobic binding to proteins which might prevent glycation reactions, such as albumin), neurodegeneration, cataract formation, and radiation injury [93].

In medicine, TIO has been shown to scale back symptoms of diabetic polyneuropathy, and several clinical trials established some efficacy and an excellent safety profile in this patient population [106]. Previous studies suggested that alpha-lipoic acid has anti-obesity properties [107]. In animal studies, it has been shown that TIO supplementation excites to reduce body weight and fat mass by decreasing food intake and enhancing energy expenditure, possibly by suppressing hypothalamic AMP-activated protein kinase (AMPK) activity [108]. However, studies in humans with TIO supplementation are limited, and therefore the results have been inconsistent. Some clinical trials suggest that TIO supplementation may help overweight or obese individuals [109,110], while other studies have observed no effects of TIO on weight [111,112]. Nevertheless, TIO appears to provide a large range of beneficial effects on obesity-related conditions such as insulin resistance, metabolic syndrome, and type 2 diabetes, including their complications such as vascular damage [106,113].

TIO has many clinically valuable properties [114]. It works as an enzymatic cofactor [93], and it is involved in glucose metabolism, lipid metabolism, and control gene transcription. TIO also restores the intrinsic antioxidant systems and supports their production or cell accessibility [94,95]. It efficiently removes heavy metals from the bloodstream. TIO is also responsible for oxidative stress [115]. The most identifiable characteristic of TIO over other antioxidant substances is that it reacts both as lipid and as water-soluble compounds [113]. TIO also has some other functions because it is involved in mitochondria-producing energy, acting as a cofactor for various enzymes that deal with metabolism [113].

Besides, TIO plays a key role in glucose metabolism. A racemic form of TIO was applied for the treatment of diabetic polyneuropathy-associated pain and paresthesia [116,117]. TIO also has a pivotal function in energy transduction through mitochondria [118,119]. Two reduced or oxidized thiol groups are present within TIO. It inactivates free radicals, and the reduced form interacts with ROS.

TIO is synthesized de novo at small amounts within the body from cysteine and fatty acids, and thus it is necessary to supplement it from exogenous sources [120]. TIO improves glycemic control [118], alleviates diabetes mellitus (DM) complications [121,122], and even symptoms of peripheral neuropathy, and at the same time, it effectively lessens the heavy metals toxicity [123].

TIO can enhance body weight and fat mass loss through its ability to suppress the hypothalamic region. AMPK and decreasing dietary energy intake reduce lipoprotein lipase activity, increase energy expenditure, lipolysis, insulin sensitivity, and inhibit lipogenesis [97,99,124–126]. Furthermore, clinical trials have demonstrated that TIO is safe, and no serious adverse effects have been reported [127,128]. Discrepancies in findings might be related to different study designs, such as characteristics of study samples, dosage, and duration of the studies.

2.4. Curcumin

Curcumin is a bioactive polyphenol derived from spice turmeric. Curcumin plays several biological functions, such as antioxidative, anti-inflammatory, and anti-angiogenesis in different organs, including adipose tissue (Figure 6).

Curcumin contains approximately 70% carbohydrates, 13% moisture, 6% protein, 6% essential oils, 5% fat, 3% mineral (potassium, calcium, phosphorus, iron, and sodium), 3–5% curcuminoids, and tiny amounts of vitamins [129,130]. Curcumin was isolated as diferuloylmethane, or 1,6-heptadiene-3,5-dione-1,7-bis (4-hydroxy-3-methoxyphenyl)-(1E,6E) [131].

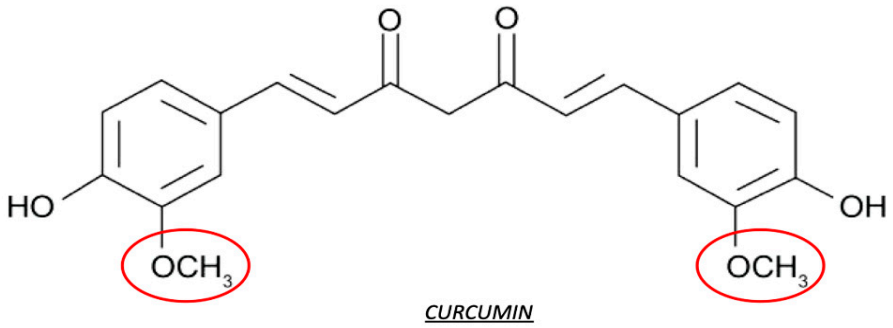

Figure 6. Configuration of curcumin. Red circles represent the reactive site of the molecule.

Curcumin, and other dietary polyphenols, due to their potential mechanisms of metabolic actions, may be useful for the therapy of obesity. Nowadays, the controversy concerning the therapeutic potential of curcumin indicates challenges and interest in this research field. Here, we try to introduce some investigations on antioxidant properties of curcumin that exhibit its application to metabolic diseases, in particular to obesity and diabetes. The beneficial effect of curcumin has been reported by some preclinical and clinical investigations which showed that curcumin and other dietary polyphenols reduce body weight, ameliorate insulin sensitivity, and prevent diabetes development both in rodent models and in prediabetic subjects [132]. We also found improvements in insulin actions by significantly decreased serum triglyceride levels after curcumin treatment and reducing the inflammatory cytokines IL-1β and interleukin 4 (IL-4) in the serum levels of obese individuals [133,134]. Recent studies suggest that there are some links to changes in gut microbiota because of the metabolic effects of curcumin and polyphenols. In this way, research into the supplementation of curcumin continues to provide novel insights into metabolic regulation and its effectiveness in reducing the oxidative stress burden in obese individuals.

Curcumin may have a relevant effect on adipogenesis. Curcumin treatment suppressed the expression of adipogenic genes peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT-enhancer-binding proteins α (C/EBPα) in primary human adipocytes and murine 3T3-L1 adipocytes [135]. Due to its antiadipogenic effects, curcumin suppresses the preadipocyte's maturation, preventing their differentiation by inactivation of MAPK, including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38, that inhibit 3T3-L1 adipocyte differentiation [136]. The inactivation effect by curcumin may also suppress the PPARγ expression in a dose-dependent manner in human adipocytes. Besides, curcumin also showed anti-inflammatory effects. In 3T3-L1 adipocytes, the secretion of a proinflammatory cytokine and the MCP-1 were inhibited by the pretreatment of curcumin [137]. Curcumin showed beneficial effects on energy metabolism and the reduction of body weight. Some evidence suggests that in rats, two weeks of high dietary curcumin supplementation reduced epididymal adipose tissue and increased fatty acid β-oxidation, indicating the increase of energy expenditure after curcumin treatment [138]. In rodent models of HFD that induced insulin resistance, many supplementations of compounds targeted to inhibit the pathogenic factors led to improvements in insulin sensitivity and resistance to weight gain. Among these compounds, curcumin has shown some beneficial effects. Nevertheless, the observation of decreased adipogenesis and/or fat mass accumulation in HFD animals, and the inhibition of adipocyte proliferation and differentiation by curcumin supplementation, suggest an enhanced catabolism in adipose tissue [139,140]. Moreover, another study demonstrated that curcumin promoted browning of WAT in diet-induced obese mice, which indicates increased energy expenditure as another mechanism linking curcumin supplementation to reduced fat mass deposition [141]. Several in vivo studies in rodents demonstrate anti-inflammatory effects of curcumin both in an HFD animal model of obesity and in genetic obesity (ob/ob mice) [132]. Moreover, curcumin, similarly to other polyphenols, could activate the nuclear factor erythroid 2–related fac-

tor 2/Kelch-like ECH-associated protein 1 (Nrf2-Keap1) antioxidant response due to the presence of a reactive Michael acceptor that reacts with cysteine residues on Keap1 [142]. This sulfhydryl reactivity may explain the other actions of curcumin but, at the same time, it has an apparent lack of specificity, particularly in cell culture experiments [132]. Curcumin also showed anti-inflammatory functions. In HFD-induced obesity and genetic obesity (ob/ob mice) models, curcumin reduced adipose tissue inflammation by reducing macrophage infiltration into adipose tissue and by increasing adiponectin production (Figure 7) [143,144].

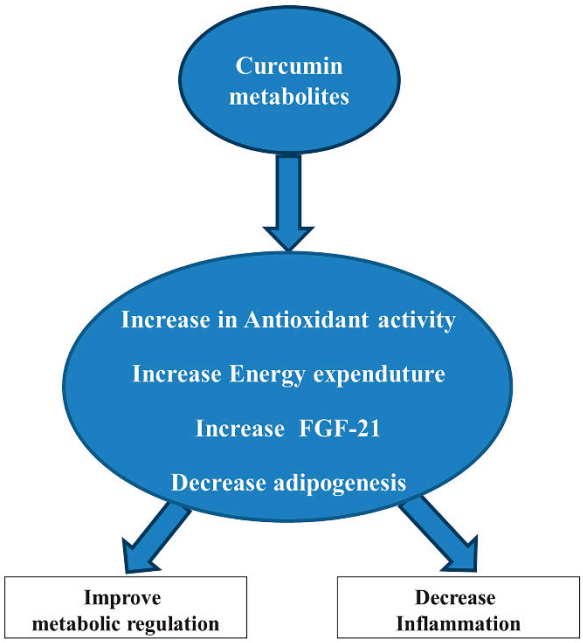

Figure 7. Possible antioxidant effects of curcumin metabolites. Abbreviation: Fibroblast growth factor 21 (FGF-21).

Furthermore, other investigations suggested that high doses of curcumin can prompt gastrointestinal upset, skin inflammation [145], and advanced liver toxicity in humans [146]. Major pathogenetic factors which link obesity to metabolic diseases involve increasing levels of circulating free fatty acids, endoplasmic reticulum (ER) stress, altered levels of adipokines, decreased adiponectin, and infiltrating macrophage-derived cytokines, such as increased MCP-1, TNF-α, and IL-6 [147–154].

2.5. Caffeine and Catechin

Green tea is one of the most widely consumed beverages. Nowadays, its medicinal properties have been widely explored. The green tea plant named *Camellia sinensis* is a member of the Theaceae family, and green tea is produced from its leaves. *Camellia sinensis* thrive mainly in tropical and subtropical climates. The tea plant is cultured from seed which needs 7 to 10 years to become ready for harvesting. Green tea is a non-alcoholic beverage. Two different groups of tea have been found, particularly one is *Camellia sinensis. var. assamica*, used in India, and the other one is *Camellia sinensis var. sinensis,* used in Japan and China [155].

Green tea contains several bioactive components, including free polyphenols, amino acids, and caffeine. Chemically, caffeine is a methylxanthine (1,3,7-trimethylxanthine). Many plants contain caffeine in their seeds, fruits, and leaves. Besides coffee and tea, these plants include cacao beans, yerba matte leaves, and guarana berries [156]. Preclinical studies reported that caffeine had antioxidant properties, for example in brain and in liver. Chronic coffee and caffeine intake diminishes the lipid peroxidation and protects membranes from damage caused by ROS, and amplifies the activity of antioxidant enzymes

(GSH and SOD) [157–159]. Caffeine also provided anti-fibrogenic and anti-inflammatory effects (decreased serum levels of cytokines TNF-α, IL-1β, and IL-6) that were associated with recovery of hepatic histological and functional alterations from thioacetamide-induced hepatotoxicity [159]. Experimental studies in animals reported a lower incidence of obesity, metabolic syndrome, and type 2 diabetes in regular coffee drinkers, of 3–4 cups per day [160,161]. Indeed, coffee as well as caffeine reduced lipogenesis, regulated lipid uptake and transport, increased fatty acid β-oxidation, increased lipolysis, and reduced lipid digestion, as reviewed in [161].

Basically, catechins are the major class of polyphenols that have high biological activity. Generally, catechin and its derivatives are expected in the green tea infusion, including epicatechin, epicatechin-3-gallate, epigallocatechin, and epigallocatechin-3-gallate (EGCG) [162]. Green tea is extracted from boiling fresh leaves at high temperatures. This procedure leaves the polyphenol content intact by inactivating the oxidizing enzymes. Flavanols or catechins are the most commonly found polyphenols in green tea.

There are several investigations about green tea polyphenols that have demonstrated significant antioxidant, anti-inflammatory, antibacterial, antiviral, and anti-angiogenic properties in numerous human, animal, and in vitro studies [163,164]. Green tea also has some beneficial effects against cancer, obesity, diabetes, and cardiovascular diseases [165].

Their antioxidant perspective is directly involved in the combination of aromatic rings and hydroxyl groups that make up their structure. It may be a result of the binding and neutralization of free radicals by the hydroxyl groups. Moreover, this kind of polyphenols augment the activity of hepatic detoxification enzymes, which promote the detoxification of xenobiotic compounds. These are also able of chelating metal ions such as iron that can generate ROS [166,167]. Green tea polyphenols have some adverse effects. They inhibit the production of arachidonic acid, metabolites such as pro-inflammatory prostaglandins and leukotrienes, and as a result, they decrease the inflammatory response.

Green tea has the ability to decrease the denaturation of proteins and increase the production of anti-inflammatory cytokines [168,169]. Besides, they can control the number of free radicals by binding to ROS, upregulating basal levels of antioxidant enzymes, and increasing the activity of these antioxidant enzymes [170–172].

We mentioned before that there are mainly five catechins in green tea. They are (+)-catechin (C), (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG), and (−)-epigallocatechin-3-gallate (EGCG) (Figure 8). Among these four, the most abundant catechin is EGCG (∼60%), and the next most abundant is EGC (∼20%), then ECG (∼14%) and EC (∼6%). EGCG is the most studied catechin, which is related to health benefits, although EGC and ECG have been studied as well. There is a variation in the number of catechins in any particular green tea beverage. However, standardized extracts are available for use as supplements [173,174].

Several studies on humans and animals have demonstrated the ability of EGCG's to block the inflammatory response due to ultraviolet A and B radiation and significant inhibition of the migration of neutrophils, which occurs during the inflammatory process [175,176]. The thermogenic properties of green tea indicate a synergistic interaction between the caffeine content and catechin polyphenol that may be a result of prolonged stimulation of thermogenesis.

Thermogenesis is a bioenergetic process, which is associated with adiposity, obesity, insulin sensitivity, blood glucose concentration, and its related disorders [155]. The brown adipocytes have two thermogenic cells, which are highly expressed in uncoupling protein 1 (UCP1). In terms of thermogenesis, UCP1 is activated and evaporates energy as heat instead of ATP synthesis [177,178]. Several studies have shown that green tea and its components can induce fat oxidation. Catechins in green tea increase energy expenditure (EE) by inhibiting COMT, which degrades catecholamines, such as norepinephrine [179]. The inhibition results in the stimulation of catecholamines and the increase of EE.

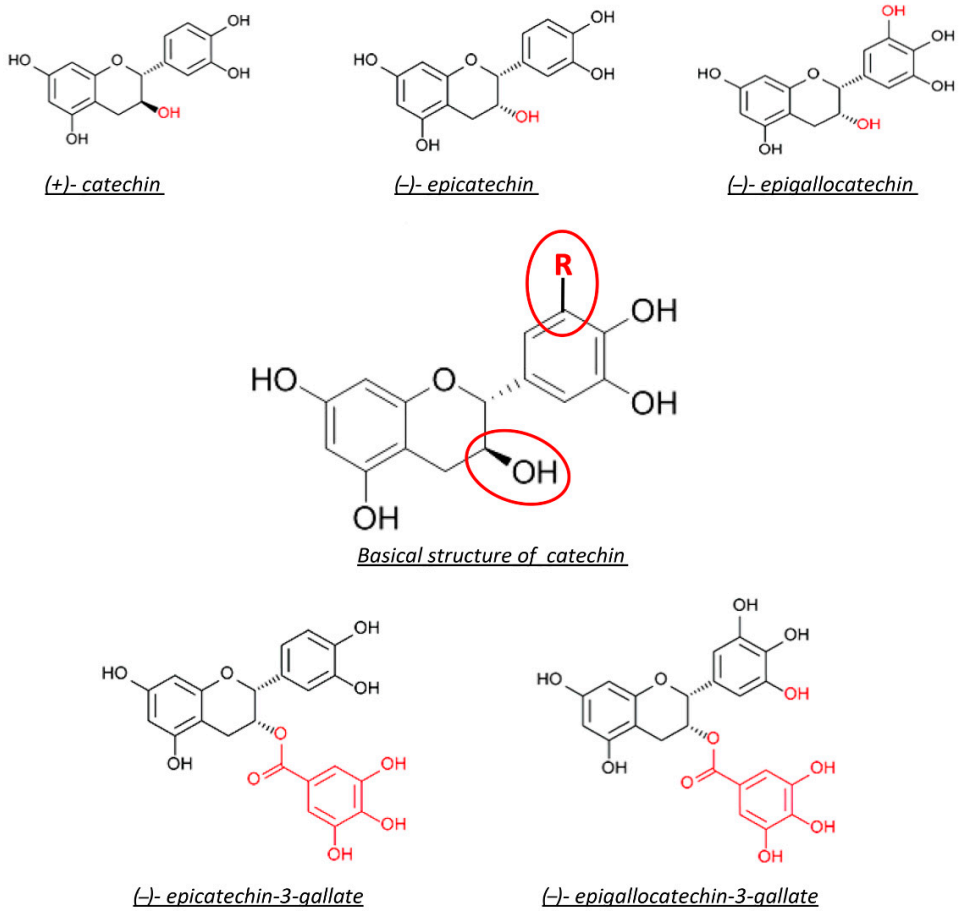

Figure 8. Chemical structure of green tea catechin. Red circles represent the reactive site of the molecule.

EGCG's catechins increased, directly or indirectly, total plasma antioxidant activity. Besides, they inhibited LDL oxidation and decreased high-density lipoprotein (HDL) cholesterol [180,181]. A randomized trial revealed that in postmenopausal women, total blood cholesterol was significantly reduced after the use of green tea extract for four weeks, compared to the control group [182].

Some studies are presenting that green tea is used for the prevention and treatment of obesity and type 2 diabetes. Green tea catechins decreased serum glucose levels [183], and especially EGCG could mimic the action of insulin and enhance insulin resistance [184,185], and catechins also reduced the absorption of triglycerides, cholesterol, and also regulated the glucose and lipid metabolism in a HFD mouse model [185–187]. The bioactive components of green tea had reduced miRNA levels of lipogenic, adipogenic, and fatty acid uptake genes. In green tea, EGCG is a bioactive molecule that reduces the expression of FAS and glycerol-3-phosphatase acyltransferase, acetyl coenzyme A carboxylase-1, and sterol-coenzyme A desaturase 1 (SCD1) mRNA [188,189].

3. Discussion

Although several drugs are approved for the treatment of obese patients, many of them were withdrawn due to severe adverse events such as heart and psychiatric disorders [190]. Recent studies demonstrated that consumption of bioactive components of food such as phenolic compounds is positively associated with reducing the risk of obesity and associated chronic diseases. [191,192]. Thus, creating new dietary treatments based on various bioactive components in food has been emerging as a new possible intervention against obesity [193].

As summarized in Table 1, different antioxidant compounds could have possible anti-obesogenic and anti-inflammatory effects.

Table 1. Effects of dietary antioxidants on inflammation and obesity (in vivo and in vitro studies).

Antioxidant	Sources	Bioactive Dose of Antioxidant	Moderator	Metabolic Marker	Inflamatory Marker	Reference
Anthocyanine	Tart cherry powder	Cyanidin(3-sophoroside), cyanidin (3-glucosylrutinoside), cyanidin- glucose(3-glucoside) insulin, cyanidincholesterol (3-rutinoside), triglyceride (TG) peonidin (3-glucoside).	Zucker rats	Reduced glycemia and insulinemia as well as improved insulin resistance.	Decreased plasma levels of IL-6 and TNF-α.	[194]
	Whole blueberry powder	Delphinidins, cyanidins, peonidins, malvidins.	C57BL/6 mice	Reduced levels of fasting glucose improved insulin tolerance test (ITT)	Reduced TNF-α expression in adipose tissue.	[195]
	Blueberry juice	Cyanidi(3-galactoside) cyanidi (3-arabinoside delphinidi(3-glucoside), delphinidinadiponectinpetunidin(3 arabinoside), malvidin (3-galactoside), malvidin(3-glucoside). Dose: 4.09 mg/mL	Mice	Reduced body weight, decreased the level of TG, leptin, and cholesterol, percentage of WAT.	Reduced TNF-α and IL-6 expression.	[196]
	Purple sweet potato	Cyanidnin(3-caffeylferulysophoroside-5-glucoside), peonidin(3caffeylferulysophoroside-5-glucoside). Dose: 4.28 to 12.84 μg/mL	Murine 3T3-L1 adipocytes	Decreased leptin and adipogenic factors.	Decreased COX-2, MCP-1, IL-6.	[197]
Resveratrol	Red wine, acai, blueberry, cranberry, pomegranate, Japanese knotwood, Ziziphus.	Resveratrol-4′-*O*-glucuronide, resveratrol-3-*O*-glucuronide and resveratrol-3-*O*-sulfate. Dose: 15 mg/kg body weight/day (gavage).	Male Zucker rats	Decreased TG content, increased epinephrine-stimulated glycerol release, increaseD hormone-sensitive lipase (HSL) mRNA.	Reduced IL-6, TNF-α, IL-1β, and NF-κB.	[60,198]
			Obese Zucker (fa/fa) rats			
		1, 10, 25 μM resveratrol, resveratrol-4′-*O*-glucuronide, resveratrol-3-*O* glucuronide and resveratrol-3-*O* sulfate.	Murine 3T3-L1 adipocytes	Increased SIRT1 mRNA, increased TG content, increased peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) mRNA, increased adipose triglyceride lipase (ATGL) mRNA, increased HSL mRNA.	Reduced IL-6 and TNF-α.	[199]
Lipoic acid	Red meat, spinach, broccoli, tomatoes, peas, Brussels sprouts.	Oxoaciddehydrogenase, pyruvatedehydrogenase complex, 2-oxoglutarate dehydrogenase complex.	HFD-induced obesity	Improved glycemic control and lipid profile, decreased weight.	Reduced IL-6 and TNF-α.	[200]

Table 1. *Cont.*

Antioxidant	Sources	Bioactive Dose of Antioxidant	Moderator	Metabolic Marker	Inflamatory Marker	Reference
Curcumine	Rhizome, or rootstalk of the turmeric plant.	1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione.	Primary human adipocytes and murine 3T3-L1 adipocytes	Suppressed the expression of adipogenic genes, PPARγ, and C/EBP α.	Reduced (MCP-1, a proinflammatory cytokine.	[137]
		1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione	HFD-induced obesity and in genetic obesity (ob/ob mice).	Reduced body weight and energy metabolism, reduced epididymal adipose tissue, increased fatty acid β-oxidation.	Increased adiponectin production and reduced inflammation.	[144]
Catechin	*Camellia sinensis* leaves and buds, green tea.	(−)-EGCG	Obese Zucker (fa/fa) rats	Reduced deleterious effects, including hepatic injury.	Decreased TNF-α, IL-1β, COX-2, and matrix metallopeptidase 9 (MMP-9).	[165]
Caffeine	Coffee and tea.	1,3,7-trimethylxanthine. Doses: 20 and 40 mg/kg per day; 37.5 mg/kg per day; 3–4 cups of coffee per day.	Male rats	Reduced lipogenesis, regulated lipid uptake and transport, increased fatty acid β-oxidation, increased lipolysis and reduced lipid digestion. Decreased lipid peroxidation and increased antioxidant enzyme activities.	Decreased serum levels of inflammatory cytokines TNF-α, IL-1β, and IL-6.	[157–161]

Other compounds demonstrated positive effects in animal models of obesity. Some studies have suggested that ursolic acid may reduce fat storage by enhancing lipolysis in adipocytes and inhibiting pancreatic lipases [201], decreasing the synthase of fatty acid activity [202], and acting as a PPARα agonist to regulate hepatic lipid metabolism [203]. Furthermore, ursolic acid may increase energy reserves in muscles by enhancing glycogen storage and lean muscle mass through increased sensitivity to insulin and insulin-like growth factor 1 (IGF-1) [204].

Vitamins C and E are antioxidants and also act as cofactors in many enzymatic reactions. To prevent the oxidation of membrane lipids, vitamin E acts as a peroxyl scavenger [205]. Vitamin E is present in low amounts, and it recycles oxidized form to the reduced form, and it is coupled with vitamin C, which is present in the body at a greater concentration. Vitamin C is a water-soluble vitamin naturally present in some foods that represent an essential dietary component for animals and humans [206,207].

Zinc acts on several antioxidant enzymes as a cofactor, such as copper-zinc (Cu-Zn) SOD [208]. There have been supplementations of Zn tested to view the effects on oxidative stress and type 2 diabetes. Zn supplementation showed an improved glycemic control and lipid profile in patients with higher postprandial glucose levels or higher fasting blood glucose [209].

Omega-3 polyunsaturated fatty acids (Omega-3 PUFAs) have been shown to contribute to human obesity [210]. Omega-3 fatty acids are dietary essentials of PUFAs [211]. The vegetal form of Omega-3 fatty acids is a short-chain fatty acid α-linolenic acid obtained from plant oil, including leafy vegetables, walnuts, soybean oil, canola oil, and flaxseed oil [212]. The marine forms of Omega-3 fatty acids are the long-chain fatty acids, such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). They are obtained from seafood, fish, and algae [213]. Obesity has been connected with low levels of Omega-3 PUFAs [214]. In fact, the supplementation of Omega-3 PUFAs may help to reduce the incidence of obesity and its comorbidities [215].

Coenzyme Q10 is involved in energy production via the mitochondrial electron transport chain [216]. Some literature reviews suggested that coenzyme Q10 has anti-inflammatory effects in vitro, but coenzyme Q10 supplementation seems to have only an antihypertensive effect, and there have no benefits to reduce the body weight, fat mass, or glycemia [217].

4. Conclusions

Obesity is a medical condition consisting of abnormal deposition of adipose tissue, with a negative effect on health status. An imbalance between caloric intake and energy expenditure results in fat accumulation due to excessive lipogenesis in adipose tissues. Many preclinical and clinical studies suggested a possible convergence of an inflammatory state, which results in chronic inflammation and oxidative stress that is localized within adipose tissue. Oxidative stress and inflammation play a crucial role in developing obesity-related metabolic complications. No specific therapeutical policies are available to counteract these complications. The evidence of the possible protective properties of natural antioxidant compounds, despite no specific effects shown on weight gain, could represent an important strategy to prevent metabolic alterations in adipose tissue. More specific clinical trials are necessary to confirm the role of these compounds on human health.

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References

1. Rosen, E.D.; Spiegelman, B.M. What we talk about when we talk about fat. *Cell* **2014**, *156*, 20–44. [CrossRef]
2. Poirier, P.; Giles, T.D.; Bray, G.A.; Hong, Y.; Stern, J.S.; Pi-Sunyer, F.X.; Eckel, R.H. Obesity and cardiovascular disease: Pathophysiology, evaluation, and effect of weight loss: An update of the 1997 American Heart Association Scientific Statement on obesity and heart disease from the Obesity Committee of the Council on Nutrition, Physical. *Circulation* **2006**, *113*, 898–918. [CrossRef] [PubMed]
3. Wormser, D.; Kaptoge, S.; Di Angelantonio, E.; Wood, A.M.; Pennells, L.; Thompson, A.; Sarwar, N.; Kizer, J.R.; Lawlor, D.A.; Nordestgaard, B.G.; et al. Separate and combined associations of body-mass index and abdominal adiposity with cardiovascular disease: Collaborative analysis of 58 prospective studies. *Lancet* **2011**, *377*, 1085–1095.
4. Nimptsch, K.; Konigorski, S.; Pischon, T. Diagnosis of obesity and use of obesity biomarkers in science and clinical medicine. *Metabolism* **2019**, *92*, 61–70. [CrossRef]
5. Hoyas, I.; Leon-Sanz, M. Nutritional challenges in metabolic syndrome. *J. Clin. Med.* **2019**, *8*, 1301. [CrossRef]
6. Flegal, K.M.; Kit, B.K.; Orpana, H.; Graubard, B.I. Association of all-cause mortality with overweight and obesity using standard body mass index categories: A systematic review and meta-analysis. *JAMA* **2013**, *309*, 71–82. [CrossRef]
7. Manna, P.; Jain, S.K. Obesity, oxidative stress, adipose tissue dysfunction, and the associated health risks: Causes and therapeutic strategies. *Metab. Syndr. Relat. Disord.* **2015**, *13*, 423–444. [CrossRef] [PubMed]
8. Lugrin, J.; Rosenblatt-Velin, N.; Parapanov, R.; Liaudet, L. The role of oxidative stress during inflammatory processes. *Biol. Chem.* **2014**, *395*, 203–230. [CrossRef]
9. Gülçin, I. Antioxidant activity of food constituents: An overview. *Arch. Toxicol.* **2012**, *86*, 345–391. [CrossRef] [PubMed]
10. Abenavoli, L.; Boccuto, L.; Federico, A.; Dallio, M.; Loguercio, C.; Di Renzo, L.; De Lorenzo, A. Diet and non-alcoholic fatty liver disease: The mediterranean way. *Int. J. Environ. Res. Public Health* **2019**, *16*, 3011. [CrossRef]
11. Poljsak, B.; Šuput, D.; Milisav, I. Achieving the balance between ROS and antioxidants: When to use the synthetic antioxidants. *Oxid. Med. Cell Longev.* **2013**, *2013*, 956792. [CrossRef]
12. Hussain, T.; Tan, B.; Yin, Y.; Blachier, F.; Tossou, M.C.; Rahu, N. Oxidative stress and inflammation: What polyphenols can do for Us? *Oxid. Med. Cell Longev.* **2016**, *2016*, 7432797. [CrossRef]
13. Kattoor, A.J.; Pothineni, N.V.K.; Palagiri, D.; Mehta, J.L. Oxidative Stress in Atherosclerosis. *Curr. Atheroscler. Rep.* **2017**, *19*, 42. [CrossRef]
14. Unuofin, J.O.; Lebelo, S.L. Antioxidant effects and mechanisms of medicinal plants and their bioactive compounds for the prevention and treatment of type 2 diabetes: An updated review. *Oxid. Med. Cell Longev.* **2020**, *2020*, 1356893. [CrossRef] [PubMed]
15. Sensoy, I. A review on the relationship between food structure, processing, and bioavailability. *Crit. Rev. Food Sci. Nutr.* **2014**, *54*, 902–909. [CrossRef] [PubMed]
16. Rein, M.J.; Renouf, M.; Cruz-Hernandez, C.; Actis-Goretta, L.; Thakkar, S.K.; da Silva Pinto, M. Bioavailability of bioactive food compounds: A challenging journey to bioefficacy. *Br. J. Clin. Pharmacol.* **2013**, *75*, 588–602. [CrossRef]
17. Karakaya, S. Bioavailability of phenolic compounds. *Crit. Rev. Food Sci. Nutr.* **2004**, *44*, 453–464. [CrossRef]
18. Lith, R.V.; Ameer, G.A. Antioxidant polymers as biomaterial. *Oxidative Stress Biomater.* **2016**, *2*, 251–296.
19. Hollman, P.C.H.; Arts, I.C.W. Flavonols, flavones, and flavanols—Nature, occurrence, and dietary burden. *J. Sci. Food Agric.* **2000**, *80*, 1081–1093. [CrossRef]
20. Scalbert, A.; Williamson, G. Dietary intake and bioavailability of polyphenols. *J. Nutr.* **2000**, *130*, 2073S–2085S. [CrossRef]
21. Zhang, L.; Zuo, Z.; Lin, G. Intestinal and hepatic glucuronidation of flavonoids. *Mol. Pharm.* **2007**, *4*, 833–845. [CrossRef]
22. Vamanu, E.; Gatea, F. Correlations between Microbiota Bioactivity and Bioavailability of Functional Compounds: A Mini-Review. *Biomedicines* **2020**, *20*, 39. [CrossRef]
23. Angelino, D.; Berhow, M.; Ninfali, P.; Jeffery, E.H. Caecal absorption of vitexin-2-O-xyloside and its aglycone apigenin, in the rat. *Food Funct.* **2013**, *4*, 1339–1345. [CrossRef]
24. Martín, J.; Kuskoski, E.M.; Navas, M.J.; Asuero, A.G. Antioxidant capacity of anthocyanin pigments. In *Flavonids: From Biosynthesis to Human Health*; IntechOpen: Rijeka, Croatia, 2017.
25. Kirakosyan, A.; Seymour, E.M.; UrcuyoLlanes, D.E.; Kaufman, P.B.; Bolling, S.F. Chemical profile and antioxidant capacities of tart cherry products. Chemical profile and antioxidant capacities of tart cherry products. *Food Chem.* **2009**, *30*, 20–25. [CrossRef]
26. Wojdyło, A.; Nowicka, P.; Laskowski, P.; Oszmiański, J. Evaluation of sour cherry (*Prunus cerasus* L.) fruits for their polyphenol content, antioxidant properties, and nutritional components. *J. Agric. Food Chem.* **2014**, *62*, 12332–12345. [CrossRef]
27. Tall, J.M.; Seeram, N.P.; Zhao, C.; Nair, M.G.; Meyer, R.A.; Raja, S.N. Tart cherry anthocyanins suppress inflammation-induced pain behavior in rat. *Behav. Brain Res.* **2004**, *153*, 181–188. [CrossRef] [PubMed]
28. Kuehl, K.S.; Perrier, E.T.; Elliot, D.L.; Chesnutt, J.C. Efficacy of tart cherry juice in reducing muscle pain during running: A randomized controlled trial. *J. Int. Soc. Sports Nutr.* **2010**, *7*, 17. [CrossRef] [PubMed]
29. Smeriglio, A.; Barreca, D.; Bellocco, E.; Trombetta, D. Chemistry, pharmacology and health benefits of anthocyanins. *Phytother. Res.* **2016**, *30*, 1265–1286. [CrossRef] [PubMed]
30. Tsuda, T. Recent progress in anti-obesity and anti-diabetes effect of berries. *Antioxidants* **2016**, *5*, 13. [CrossRef] [PubMed]
31. Kang, S.Y.; Seeram, N.P.; Nair, M.G.; Bourquin, L.D. Tart cherry anthocyanins inhibit tumor development in Apc (Min) mice and reduce proliferation of human colon cancer cells. *Cancer Lett.* **2003**, *194*, 13–19. [CrossRef]

32. Traustadóttir, T.; Davies, S.S.; Stock, A.A.; Su, Y.; Heward, C.B.; Roberts, L.J., 2nd; Harman, S.M. Tart cherry juice decreases oxidative stress in healthy older men and women. *J. Nutr.* **2009**, *139*, 1896–1900. [CrossRef]
33. Howatson, G.B.; Tallent, P.G.J.; Middleton, B.; McHugh, M.P.; Ellis, J. Effect of tart cherry juice (*Prunus cerasus*) on melatonin levels and enhanced sleep quality. *Eur. J. Nutr.* **2012**, *51*, 909–916. [CrossRef]
34. Lee, Y.M.; Yoon, Y.; Yoon, H.; Park, H.M.; Song, S.; Yeum, K.J. Dietary Anthocyanins against Obesity and Inflammation. *Nutrients* **2017**, *9*, 1089. [CrossRef] [PubMed]
35. Mazza, G.; Miniati, E. *Anthocyanins in Fruits, Vegetables and Grains*, 1st ed.; CRC Press: Boca Raton, FL, USA, 1993; p. 384.
36. Pascual-Teresa De, S.; Sanchez-Ballesta, M.T. Anthocyanins: From plant to health. *Phytochem. Rev.* **2008**, *7*, 281–299. [CrossRef]
37. He, J.; Giusti, M.M. Anthocyanins: Natural colorants with health-promoting properties. *Annu. Rev. Food Sci. Technol.* **2010**, *1*, 163–187. [CrossRef]
38. Kähkonen, M.P.; Heinonen, M. Antioxidant activity of anthocyanins and their aglycons. *J. Agric. Food Chem.* **2003**, *51*, 628–633. [CrossRef]
39. Wang, H.; Nair, M.G.; Strasburg, G.M.; Chang, Y.C.; Booren, A.M.; Gray, J.I.; DeWitt, D.L. Antioxidant and antiinflammatory activities of anthocyanins and their aglycon, cyanidin, from tart cherries. *J. Nat. Prod.* **1999**, *62*, 294–296. [CrossRef]
40. Saric, A.; Sobocanec, S.; Balog, T.; Kusic, B.; Sverko, V.; Dragovic-Uzelec, V.; Levaj, B.; Cosic, Z.; Safranko, Z.M.; Marotti, T. Improved antioxidant and anti-inflammatory potential in mice consuming sour cherry juice (*Prunus Cerasus* cv. Maraska). *Plant Foods Hum. Nutr.* **2009**, *64*, 231–237. [CrossRef] [PubMed]
41. Wang, H.; Cao, G.; Prior, R.L. Oxygen radical absorbing capacity of anthocyanins. *J. Agric. Food Chem.* **1997**, *45*, 304–309. [CrossRef]
42. Tsuda, T.; Shiga, K.; Ohshima, K.; Kawakishi, S.; Osawa, T. Inhibition of lipid peroxidation and the active oxygen radical scavenging effect of anthocyanin pigments isolated from *Phaseolus vulgaris* L. *Biochem. Pharmacol.* **1996**, *52*, 1033–1039. [CrossRef]
43. Tedesco, I.; Luigi Russo, G.; Nazzaro, F.; Russo, M.; Palumbo, R. Antioxidant effect of red wine anthocyanins in normal and catalase-inactive human erythrocytes. *J. Nutr. Biochem.* **2001**, *12*, 505–511. [CrossRef]
44. Heo, H.J.; Lee, C.Y. Strawberry and its anthocyanins reduce oxidative stress-induced apoptosis in pc12 cells. *J. Agric. Food Chem.* **2005**, *53*, 1984–1989. [CrossRef] [PubMed]
45. Isaak, C.K.; Petkau, J.C.; Blewett, H.; Karmin, O.; Siow, Y.L. Lingonberry anthocyanins protect cardiac cells from oxidative-stress-induced apoptosis. *Can. J. Physiol. Pharmacol.* **2017**, *95*, 904–910. [CrossRef]
46. Thoppil, R.J.; Bhatia, D.; Barnes, K.F.; Haznagy-Radnai, E.; Hohmann, J.; Darvesh, A.S.; Bishayee, A. Black currant anthocyanins abrogate oxidative stress through nrf2-mediated antioxidant mechanisms in a rat model of hepatocellular carcinoma. *Curr. Cancer Drug Targets* **2012**, *12*, 44–1257.
47. Afaq, F.; Saleem, M.; Krueger, C.G.; Reed, J.D.; Mukhtar, H. Anthocyanin- and hydrolyzable tannin-rich pomegranate fruit extract modulates mapk and nf-kappab pathways and inhibits skin tumorigenesis in cd-1 mice. *Int. J. Cancer* **2005**, *113*, 423–433. [CrossRef]
48. Jeong, J.W.; Lee, W.S.; Shin, S.C.; Kim, G.Y.; Choi, B.T.; Choi, Y.H. Anthocyanins down regulate lipopolysaccharide-induced inflammatory responses in bv2 microglial cells by suppressing the nf-kappab and akt/mapks signaling pathways. *Int. J. Mol. Sci.* **2013**, *14*, 1502–1515. [CrossRef] [PubMed]
49. Vendrame, S.; Klimis-Zacas, D. Anti-inflammatory effect of anthocyanins via modulation of nuclear factor-kappab and mitogen-activated protein kinase signaling cascades. *Nutr. Rev.* **2015**, *73*, 348–358. [CrossRef]
50. Wang, H.; Nair, M.G.; Strasburg, G.M.; Booren, A.M.; Gray, J.I. Novel antioxidant compounds from tart cherries (*Prunus cerasus*). *J. Nat. Prod.* **1999**, *62*, 86–88. [CrossRef]
51. Micioni Di Bonaventura, M.V.; Martinelli, I.; Moruzzi, M.; Micioni Di Bonaventura, E.; Giusepponi, M.E.; Polidori, C.; Lupidi, G.; Tayebati, S.K.; Amenta, F.; Cifani, C.; et al. Brain alterations in high fat diet induced obesity: Effects of tart cherry seeds and juice. *Nutrients* **2020**, *12*, 623. [CrossRef]
52. Martinelli, I.; Micioni Di Bonaventura, M.V.; Moruzzi, M.; Amantini, C.; Maggi, F.; Gabrielli, M.G.; Fruganti, A.; Marchegiani, A.; Dini, F.; Marini, C.; et al. Effects of *Prunus cerasus* L. seeds and juice on liver steatosis in an animal model of diet-induced obesity. *Nutrients* **2020**, *12*, 1308. [CrossRef]
53. Cocci, P.; Moruzzi, M.; Martinelli, I.; Maggi, F.; Micioni Di Bonaventura, M.V.; Cifani, C.; Mosconi, G.; Tayebati, S.K.; Damiano, S.; Lupidi, G.; et al. Tart cherry (*Prunus cerasus* L.) dietary supplement modulates visceral adipose tissue CB1 mRNA levels along with other adipogenesis-related genes in rat models of diet-induced obesity. *Eur. J. Nutr.* **2021**, in press. [CrossRef] [PubMed]
54. Moruzzi, M.; Kloting, N.; Blüher, M.; Martinelli, I.; Tayebati, S.K.; Gabrielli, M.G.; Roy, P.; Micioni Di Bonaventura, M.V.; Cifani, C.; Lupidi, G.; et al. Tart cherry juice and seeds affect pro-inflammatory markers in visceral adipose tissue of high-fat diet obese rats. *Molecules* **2021**, *26*, 1403. [CrossRef]
55. Blando, F.; Gerardi, C.; Nicoletti, I. Sour Cherry (*Prunus cerasus* L.) Anthocyanins as ingredients for functional foods. *J. Biomed. Biotechnol.* **2004**, *2004*, 253–258. [CrossRef] [PubMed]
56. Tumbas, V.T.; Canadanović-Brunet, J.M.; Cetojević-Simin, D.D.; Cetković, G.S.; Ethilas, S.M.; Gille, L. Effect of rosehip (*Rosa canina* L.) phytochemicals on stable free radicals and human cancer cells. *J. Sci. Food Agric.* **2012**, *92*, 1273–1281. [CrossRef] [PubMed]
57. Pignatelli, P.; Pulcinelli, F.M.; Celestini, A.; Lenti, L.; Ghiselli, A.; Gazzaniga, P.P.; Violi, F. The flavonoids quercetin and catechin synergistically inhibit platelet function by antagonizing the intracellular production of hydrogen peroxide. *Am. J. Clin. Nutr.* **2000**, *1150*, 5. [CrossRef]

58. Wu, T.; Guo, X.; Zhang, M.; Yang, L.; Liu, R.; Yin, J. Anthocyanins in black rice, soybean and purple corn increase fecal butyric acid and prevent liver inflammation in high fat diet-induced obese mic. *Food Funct.* **2017**, *8*, 3178–3186. [CrossRef] [PubMed]
59. Kirakosyan, A.; Seymour, E.M.; Noon, K.R.; Llanes, D.E.U.; Kaufman, P.B.; Warber, S.L.; Bolling, S.F. Interactions of antioxidants isolated from tart cherry (*Prunus cerasus*) fruits. *Food Chem.* **2010**, *122*, 78–83. [CrossRef]
60. Zorita, S.G.; Quintela, A.F.; Lasa, A.; Hijona, E.; Bujanda, L.; Portillo, M. Effects of resveratrol on obesity-related inflammation markers in adipose tissue of genetically obese rats. *Nutrition* **2013**, *29*, 1374–1380. [CrossRef]
61. Sameer, S.K.; Carles, C. The molecular targets of resveratrol. *Biochim. Biophys. Acta* **2015**, *1852*, 1114–1123.
62. Park, E.J.; Pezzuto, J.M. The pharmacology of resveratrol in animals and humans. *Biochim. Biophys. Acta* **2015**, *1852*, 1071–1113. [CrossRef]
63. Wong, R.H.; Howe, P.R.; Buckley, J.D.; Coates, A.M.; Kunz, I.; Berry, N.M. Acute resveratrol supplementation improves flow-mediated dilatation in overweight/obese individuals with mildly elevated blood pressure. *Nutr. Metab. Cardiovasc.* **2011**, *21*, 851–856. [CrossRef]
64. Timmers, S.; Konings, E.; Bilet, L.; Houtkooper, R.H.; Weijer, T.V.D.; Goossens, G.H.; Hoeks, J.; Krieken, S.V.D.; Kersten, D.; Ryu, S.; et al. Calorie restriction-like effects of 30 days of resveratrol supplementation on energy metabolism and metabolic profile in obese humans. *Cell Metab.* **2011**, *14*, 612–622. [CrossRef]
65. Wong, R.H.; Berry, N.M.; Coates, A.M.; Buckley, J.D.; Bryan, J.; Kunz, I.; Howe, P.R. Chronic resveratrol consumption improves brachial flow-mediated dilatation in healthy obese adults. *J. Hypertens.* **2013**, *31*, 819–1827. [CrossRef] [PubMed]
66. Lagouge, M.; Argmann, C.; Gerhart-Hines, Z.; Meziane, H.; Lerin, C.; Daussin, F.; Messadeq, N.; Milne, J.; Lambert, P.; Elliott, P.; et al. Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. *Cells* **2006**, *127*, 1109–1122. [CrossRef] [PubMed]
67. Kim, S.; Jin, Y.; Choi, Y.; Park, T. Resveratrol exerts antiobesity effects via mechanisms involving down-regulation of adipogenic and inflammatory processes in mice. *Biochem. Pharmacol.* **2011**, *81*, 1343–1351. [CrossRef]
68. Cho, S.J.; Jung, U.J.; Choi, M.S. Differential effects of low-dose resveratrol on adiposity and hepatic steatosis in diet-induced obese mice. *Br. J. Nutr.* **2012**, *108*, 2166–2175. [CrossRef] [PubMed]
69. Alberdi, G.; Rodriguez, V.M.; Miranda, J.; Macarulla, M.T.; Churruca, I.; Portillo, M.P. Thermogenesis is involved in the body-fat lowering effects of resveratrol in rats. *Food Chem.* **2013**, *141*, 1530–1535. [CrossRef]
70. Wang, S.; Liang, X.; Yang, Q.; Fu, X.; Rogers, C.J.; Zhu, M.; Rodgers, B.D.; Jiang, Q.; Dodson, M.V.; Du, M. Resveratrol induces brown-like adipocyte formation in white fat through activation of AMP-activated protein kinase (AMPK) alpha1. *Int. J. Obes.* **2015**, *39*, 967–976. [CrossRef]
71. Ku, C.R.; Cho, Y.H.; Hong, Z.Y.; Lee, H.; Lee, S.J.; Hong, S.S.; Lee, E.J. The effects of high fat diet and resveratrol on mitochondrial activity of brown adipocytes. *Endocrinol. Metab.* **2016**, *31*, 328–335. [CrossRef]
72. Arias, N.; Pico, C.; Macarulla, M.T.; Oliver, P.; Miranda, J.; Palou, A.; Portillo, M.P. A combination of resveratrol and quercetin induces browning in white adipose tissue of rats fed an obesogenic diet. *Obesity* **2017**, *25*, 111–121. [CrossRef] [PubMed]
73. Wang, B.; Sun, J.; Li, X.; Zhou, Q.; Bai, J.; Shi, Y.; Le, G. Resveratrol prevents suppression of regulatory T-cell production, oxidative stress, and inflammation of mice prone or resistant to high-fat diet-induced obesity. *Nutr. Res.* **2013**, *33*, 971–981. [CrossRef]
74. Jeon, B.T.; Jeong, E.A.; Shin, H.J.; Lee, Y.; Lee, D.H.; Kim, H.J.; Kang, S.S.; Cho, G.J.; Choi, W.S.; Roh, G.S. Resveratrol attenuates obesity-associated peripheral and central inflammation and improves memory deficit in mice fed a high-fat diet. *Diabetes* **2012**, *61*, 1444–1454. [CrossRef] [PubMed]
75. Jimenez-Gomez, Y.; Mattison, J.A.; Pearson, K.J.; Martin-Montalvo, A.; Palacios, H.H.; Sossong, A.M.; Ward, T.M.; Younts, C.M.; Lewis, K.; Allard, J.S.; et al. Resveratrol improves adipose insulin signaling and reduces the inflammatory response in adipose tissue of rhesus monkeys on high-fat, high-sugar diet. *Cell Metab.* **2013**, *18*, 533–545. [CrossRef] [PubMed]
76. Lv, Z.M.; Wang, Q.; Chen, Y.H.; Wang, S.H.; Huang, D.Q. Resveratrol attenuates inflammation and oxidative stress in epididymal white adipose tissue: Implications for its involvement in improving steroidogenesis in diet induced obese mice. *Mol. Reprod. Dev.* **2015**, *82*, 321–328. [CrossRef]
77. Wang, C.Y.; Liao, J.K. A mouse model of diet-induced obesity and insulin resistance. *Methods Mol. Biol.* **2012**, *821*, 421–433.
78. Kowalski, J.; Samojedni, A.; Paul, M.; Pietsz, G.; Wilczok, T. Effect of apigenin, kaempferol and resveratrol on the expression of interleukin-1 beta and tumor necrosis factor-alpha genes in J774.2 macrophages. *Pharmacol. Rep.* **2005**, *390*, 4.
79. Ma, Z.H.; Ma, Q.Y.; Wang, L.C.; Sha, H.C.; Wu, S.L.; Zhang, M. Effect of resveratrol NF-kappaB activity in rat peritoneal macrophages. *Am. J. Chin. Med.* **2006**, *34*, 623–630. [CrossRef]
80. Das, S.; Das, D.K. Anti-inflammatory responses of resveratrol. *Inflamm. Allergy Drug Targets* **2007**, *6*, 168–173. [CrossRef]
81. Gonzalez, R.; Ballester, I.L.; Opez-Posadas, R.; Suarez, M.D.; Zarzuelo, A.; Martınez Augustin, O.; Sanchez de Madina, F. Effects of flavonoids and other polyphenols on inflammation. *Crit. Rev. Food Sci. Nutr.* **2011**, *51*, 331–362. [CrossRef]
82. Chuang, C.C.; Martinez, K.; Xie, G.; Kennedy, A.; Bumrungper, A.; Overman, A.; Jia, W.; McIntosh, M.K. Quercetin is equally or more effective than resveratrol in attenuating tumor necrosis factor-a–mediated inflammation and insulin resistance in primary human adipocytes. *Am. J. Clin. Nutr.* **2010**, *92*, 1512–1521. [CrossRef] [PubMed]
83. Mercader, J.; Palou, A.; Bonet, M.L. Resveratrol enhances fatty acid oxidation capacity and reduces resistin and Retinol-Binding Protein 4 expression in white adipocytes. *J. Nutr. Biochem.* **2011**, *22*, 828–834. [CrossRef] [PubMed]
84. Olholm, J.; Paulsen, S.K.; Cullberg, K.B.; Richelsen, B.; Peersen, S.B. Anti-inflammatory effect of resveratrol on adipokine expression and secretion inhuman adipose tissue explants. *Int. J. Obes.* **2010**, *34*, 1546–1553. [CrossRef] [PubMed]

85. Chuang, C.C.; McIntosh, M.K. Potential mechanisms by which polyphenol-rich grapes prevent obesity-mediated inflammation and metabolic diseases. *Annu. Rev. Nutr.* **2011**, *31*, 155–176. [CrossRef]
86. Terra, X.; Montagut, G.; Bustos, M.; Llopiz, N.; Ardevol, A.; Blade, C.; Fernández-Larrea, J.; Pujadas, G.; Salvadó, J.; Arola, L.; et al. Grape-seed procyanidins prevent low-grade inflammation by modulating cytokine expression in rats fed a high-fat diet. *J. Nutr. Biochem.* **2009**, *20*, 210–218. [CrossRef]
87. Hogan, S.; Canning, C.; Sun, S.; Sun, X.; Kadouh, H.; Zhou, K. Dietary supplementation of grape skin extract improves glycemia and inflammation indiet-induced obese mice fed a Western high fat diet. *J. Agric. Food Chem.* **2011**, *59*, 3035–3041. [CrossRef] [PubMed]
88. Terra, X.; Pallares, V.; Ardevol, A.; Blad, C.F.; Andez-Larrea, J.; Pujadas, G.; Salvado, J.; Arola, L.; Blay, M. Modulatory effect of grape-seed procyanidins on local and systemic inflammation in diet-induced obesity rats. *J. Nutr. Biochem.* **2011**, *22*, 380–387. [CrossRef] [PubMed]
89. Cucciolla, V.; Borriello, A.; Oliva, A.; Galletti, P.; Zappia, V.; Della, R.F. Resveratrol: From basic science to the clinic. *Cell Cycle* **2007**, *6*, 2495–2510. [CrossRef] [PubMed]
90. Shankar, S.; Singh, G.; Srivastava, R.K. Chemoprevention by resveratrol: Molecular mechanisms and therapeutic potential. *Front. Biosci.* **2007**, *12*, 4839–4854. [CrossRef] [PubMed]
91. Athar, M.; Back, J.H.; Kopelovich, L.; Bickers, D.R.; Kim, A.L. Multiple molecular targets of resveratrol: Anti-carcinogenic mechanisms. *Arch. Biochem. Biophys.* **2009**, *486*, 95–102. [CrossRef] [PubMed]
92. Pervaiz, S.; Holme, A.L. Resveratrol: Its biologic targets and functional activity. *Antioxid. Redox Signal.* **2009**, *11*, 2851–2897. [CrossRef]
93. Packer, L.; Cadenas, E. Lipoic acid: Energy metabolism and redox regulation of transcription and cell signaling. *J. Clin. Biochem. Nutr.* **2011**, *48*, 26–32. [CrossRef] [PubMed]
94. Goraca, A.; Huk-Kolega, H.; Piechota, A.; Kleniewska, P.; Ciejka, E.; Skibska, B. Lipoic acid biological activity and therapeutic potential. *Pharmacol. Rep.* **2011**, *63*, 849–858. [CrossRef]
95. Shay, K.P.; Moreau, R.F.; Smith, E.J.; Smith, A.R.; Hagen, T.M. Alpha-lipoic acid as a dietary supplement: Molecular mechanisms and therapeutic potential. *Biochim. Biophys. Acta* **2009**, *1790*, 1149–1160. [CrossRef]
96. Szeląg, M.; Mikulski, D.; Molski, M. Quantum-chemical investigation of the structure and the antioxidant properties of α-lipoic acid and its metabolites. *J. Mol. Model.* **2012**, *18*, 2907–2916. [CrossRef] [PubMed]
97. Gomes, M.B.; Negrato, C.A. Alpha-lipoic acid as a pleiotropic compound with potential therapeutic use in diabetes and other chronic diseases. *Diabetol. Metab. Syndr.* **2014**, *6*, 80. [CrossRef]
98. Reed, L.J. From lipoic acid to multi-enzyme complexes. *Protein Sci.* **1998**, *7*, 220–224. [CrossRef]
99. Liu, J.; Head, E.; Gharib, A.M.; Yuan, W.; Ingersoll, R.T.; Hagen, T.M.; Cotman, C.W.; Ames, B.N. Memory loss in old rats is associated with brain mitochondrial decay and RNA/DNA oxidation: Partial reversal by feeding acetyl-L-carnitine and/or R-α-lipoic acid. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 2356–2361. [CrossRef]
100. Suh, J.H.; Zhu, B.Z.; de Szoeke, E.; Frei, B.; Hagen, T.M. Dihydrolipoic acid lowers the redox activity of transition metal ions but does not remove them from the active site of enzymes. *Redox Rep.* **2004**, *9*, 57–61. [CrossRef] [PubMed]
101. Kim, N.W.; Song, Y.M.; Kim, E.; Cho, H.S.; Cheon, K.A.; Kim, S.J.; Park, J.Y. Adjunctive α-lipoic acid reduces weight gain compared with placebo at 12 weeks in schizophrenic patients treated with atypical antipsychotics: A double-blind randomized placebo-controlled study. *Int. Clin. Psychopharmacol.* **2016**, *31*, 265–274. [CrossRef]
102. Sun, H.; Yao, W.; Tang, Y.; Zhuang, W.; Wu, D.; Huang, S.; Sheng, H. Urinary exosomes as a novel biomarker for evaluation of α-lipoic acid's protective effect in early diabetic nephropathy. *J. Clin. Lab. Anal.* **2017**, *31*, 22129. [CrossRef] [PubMed]
103. De Sousa, C.N.S.; da Silva Leite, C.M.G.; da Silva Medeiros, I.; Vasconcelos, L.C.; Cabral, L.M.; Patrocínio, C.F.V.; Patrocínio, M.L.V.; Mouaffak, F.; Kebir, O.; Macedo, D.; et al. Alpha-lipoic acid in the treatment of psychiatric and neurological disorders: A systematic review. *Metab. Brain Dis.* **2019**, *34*, 39–52. [CrossRef]
104. Cakatay, U.; Telci, A.; Salman, S.; Satman, L.; Sivas, A. Oxidative protein damage in type I diabetic patients with and without complications. *Endocr. Res.* **2000**, *26*, 65–79. [CrossRef]
105. Rochette, L.; Ghibu, S.; Richard, C.; Zeller, M.; Cottin, Y.; Vergely, C. Direct and indirect antioxidant properties of α-lipoic acid and therapeutic potential. *Mol. Nutr. Food Res.* **2013**, *57*, 114–125. [CrossRef] [PubMed]
106. Ziegler, D.; Nowak, H.; Kempler, P.; Vargha, P.; Low, P.A. Treatment of symptomatic diabetic polyneuropathy with the antioxidant alpha-lipoic acid: A meta-analysis. *Diabet. Med.* **2004**, *21*, 114–121. [CrossRef] [PubMed]
107. Huerta, A.E.; Navas-Carretero, S.; Prieto-Hontoria, P.L.; Martinez, J.A.; Moreno-Aliaga, M.J. Effects of alpha-lipoic acid and eicosapentaenoic acid in overweight and obese women during weight loss. *Obesity* **2015**, *23*, 313–321. [CrossRef]
108. Prieto-Hontoria, P.L.; Perez-Matute, P.; Fernandez-Galilea, M.; Martinez, J.A.; Moreno-Aliaga, M.J. Effects of lipoic acid on AMPK and adiponectin in adipose tissue of low- and high-fat-fed rats. *Eur. J. Nutr.* **2013**, *52*, 779–787. [CrossRef]
109. Carbonelli, M.G.; Renzo, L.D.; Bigioni, M.D.; Daniele, N.; Lorenzo, A.D.; Fusco, M.A. Alpha-lipoic acid supplementation: A tool for obesity therapy? *Curr. Pharm. Des.* **2010**, *16*, 840–846. [CrossRef] [PubMed]
110. Koh, E.H.; Lee, W.J.; Lee, S.A.; Kim, E.H.; Cho, E.H.; Jeong, E.; Kim, D.W.; Kim, M.-S.; Park, J.-Y.; Park, K.-G.; et al. Effects of alpha-lipoic Acid on body weight in obese subjects. *Am. J. Med.* **2011**, *124*, 1–8. [CrossRef]
111. Ansar, H.; Mazloom, Z.; Kazemi, F.; Hejazi, N. Effect of alpha-lipoic acid on blood glucose, insulin resistance and glutathione peroxidase of type 2 diabetic patients. *Saudi Med. J.* **2011**, *32*, 584–588. [PubMed]

112. McNeilly, A.M.; Davison, G.W.; Murphy, M.H.; Nadeem, N.; Trinick, T.; Duly, E.; Novials, A.; McEneny, J. Effect of alpha-lipoic acid and exercise training on cardiovascular disease risk in obesity with impaired glucose tolerance. *Lipids Health Dis.* **2011**, *10*, 217. [CrossRef] [PubMed]
113. Packer, L.; Kraemer, K.; Rimbach, G. Molecular aspects of lipoic acid in the prevention of diabetes complications. *Nutrition* **2001**, *17*, 888–895. [CrossRef]
114. Brufani, M. Acido α-lipoico farmaco o integratore. Una panoramica sulla farmacocinetica, le formulazioni disponibili e le evidenze cliniche nelle complicanze del diabete. *Prog. Nutr.* **2014**, *16*, 62–74.
115. Ou, P.; Tritschler, H.J.; Wolff, S.P. Thioctic (lipoic) acid: A therapeutic metal-chelating antioxidant? *Biochem. Pharmacol.* **1995**, *50*, 123–126. [CrossRef]
116. Ziegler, D.; Ametov, A.; Barinov, A.; Dyck, J.P.; Gurieva, I.; Low, P.A.; Munzel, U.; Yakhno, N.; Raz, I.; Novosadova, M.; et al. Oral treatment with alpha-lipoic acid improves symptomatic diabetic polyneuropathy: The Sydney 2 trial. *Diabetes Care* **2006**, *29*, 2365–2370. [CrossRef] [PubMed]
117. Castro, M.C.; Villagarcía, H.G.; Massa, M.L.; Francini, F. Alpha-lipoic acid and its protective role in fructose induced endocrine-metabolic disturbances. *Food Funct.* **2019**, *10*, 16–25. [CrossRef]
118. Singh, U.; Jialal, I. Alpha-lipoic acid supplementation and diabetes. *Nutr. Rev.* **2008**, *66*, 646–657. [CrossRef] [PubMed]
119. Keith, D.J.; Butler, J.A.; Bemer, B.; Dixon, B.; Johnson, S.; Garrard, M.; Sudakin, D.L.; Christensen, J.M.; Pereira, C.; Hagen, T.M. Age and gender dependent bioavailability of R- and R,S-alpha-lipoic acid: A Pilot study. *Pharmacol. Res.* **2012**, *66*, 199–206. [CrossRef] [PubMed]
120. Solmonson, A.; De Berardinis, R.J. Lipoic acid metabolism and mitochondrial redox regulation. *J. Biol. Chem.* **2018**, *293*, 7522–7530. [CrossRef] [PubMed]
121. Ziegler, D. Thioctic acid for patients with symptomatic diabetic polyneuropathy: A critical review. *Treat. Endocrinol.* **2004**, *3*, 173–189. [CrossRef]
122. Henriksen, E.J. Exercise training and the antioxidant alpha-lipoic acid in the treatment of insulin resistance and type 2 diabetes. *Free Radic. Biol. Med.* **2006**, *40*, 3–12. [CrossRef]
123. Ciftci, H.; Bakal, U. The effect of lipoic acid on macro and trace metal levels in living tissues exposed to oxidative stress. *Anti Cancer Agents Med. Chem.* **2009**, *9*, 560–568. [CrossRef]
124. Akiba, S.; Matsugo, S.; Packer, L.; Konishi, T. Assay of protein-bound lipoic acid in tissues by a new enzymatic method. *Anal. Biochem.* **1998**, *258*, 299–304. [CrossRef] [PubMed]
125. Golbidi, S.; Badran, M.; Laher, I. Diabetes and alpha lipoic Acid. *Front. Pharmacol.* **2011**, *2*, 69. [CrossRef]
126. Smith, A.R.; Shenvi, S.V.; Widlansky, M.; Suh, J.H.; Hagen, T.M. Lipoic acid as a potential therapy for chronic diseases associated with oxidative stress. *Curr. Med. Chem.* **2004**, *11*, 1135–1146. [CrossRef]
127. Han, D.; Sen, C.K.; Roy, S.; Kobayashi, M.S.; Tritschler, H.J.; Packer, L. Protection against glutamate-induced cytotoxicity in C6 glial cells by thiol antioxidants. *Am. J. Physiol. Integr. Comp. Physiol.* **1997**, *273*, 1771–1778. [CrossRef]
128. Wray, D.W.; Nishiyama, S.K.; Harris, R.A.; Zhao, J.; McDaniel, J.; Fjeldstad, A.S.; Witman, M.A.; Ives, S.J.; Barrett-O'Keefe, Z.; Richardson, R.S. Acute reversal of endothelial dysfunction in the elderly after antioxidant consumption. *Hypertension* **2012**, *59*, 818–824. [CrossRef] [PubMed]
129. Priyadarsini, K. The Chemistry of Curcumin: From Extraction to Therapeutic Agent. *Molecules* **2014**, *19*, 20091–20112. [CrossRef] [PubMed]
130. Nelson, K.M.; Dahlin, J.L.; Bisson, J.; Graham, J.; Pauli, G.F.; Walters, M.A. The Essential Medicinal Chemistry of Curcumin: Miniperspective. *J. Med. Chem.* **2017**, *60*, 1620–1637. [CrossRef]
131. Prasad, S.; Gupta, S.C.; Tyagi, A.K.; Aggarwal, B.B. Curcumin, a component of golden spice: From bedside to bench and back. *Biotechnol. Adv.* **2014**, *32*, 1053–1064. [CrossRef]
132. Jin, T.; Song, Z.; Weng, J.; Fantus, I.G. Curcumin and other dietary polyphenols: Potential mechanisms of metabolic actions and therapy for diabetes and obesity. *Am. J. Physiol. Endocrinol. Metab.* **2018**, *314*, E201–E205. [CrossRef]
133. Mohammadi, A.; Sahebkar, M.; Iranshahi, M.; Amini, M.; Khojasteh, R.; Ghayour-Mobarhan, M.; Ferns, G.A. Effects of supplementation with curcuminoids on dyslipidemia in obese patients: A randomized crossover trial. *Phytother. Res. PTR* **2013**, *27*, 374–379. [CrossRef] [PubMed]
134. Ganjali, S.; Sahebkar, A. Investigation of the effects of curcumin on serum cytokines in obese individuals: A randomized controlled trial. *Sci. World J.* **2014**, *2014*, 6. [CrossRef]
135. Kim, C.Y.; Le, T.; Chen, C.; Cheng, J.X.; Kim, K.H. Curcumin inhibits adipocyte differentiation through modulation of mitotic clonal expansion. *J. Nutr. Biochem.* **2011**, *22*, 910–920. [CrossRef]
136. Ahn, J.; Lee, H.; Kim, S.; Ha, T. Curcumin induced suppression of adipogenic differentiation is accompanied by activation of Wnt/beta-catenin signaling. *Am. J. Physiol. Cell Physiol.* **2010**, *298*, C1510–C1516. [CrossRef]
137. Woo, H.M.; Kang, J.H.; Kawada, T.; Yoo, H.; Sung, M.K.; Yu, R. Active spice-derived components can inhibit inflammatory responses of adipose tissue in obesity by suppressing inflammatory actions of macrophages and release of monocyte chemoattractant protein-1 from adipocytes. *Life Sci.* **2007**, *80*, 926–931. [CrossRef] [PubMed]
138. Asai, A.; Miyazawa, T. Dietary curcuminoids prevent high-fat diet-induced lipid accumulation in rat liver and epididymal adipose tissue. *J. Nutr.* **2001**, *131*, 2932–2935. [CrossRef] [PubMed]

139. Wang, S.; Moustaid, M.N.; Chen, L.; Mo, H.; Shastri, A.; Su, R.; Bapat, P.; Kwun, I.; Shen, C.L. Novel insights of dietary polyphenols and obesity. *J. Nutr. Biochem.* **2014**, *25*, 1–18. [CrossRef]
140. Tian, L.; Song, Z.; Shao, W.; Du, W.W.; Zhao, L.R.; Zeng, K.; Yang, B.B.; Jin, T. Curcumin represses mouse 3T3-L1 cell adipogenic differentiation via inhibiting miR-17-5p and stimulating the Wnt signalling pathway effector Tcf7l2. *Cell Death Dis.* **2017**, *8*, e2559. [CrossRef]
141. Wang, S.; Wang, X.; Ye, Z.; Xu, C.; Zhang, M.; Ruan, B.; Wei, M.; Jiang, Y.; Zhang, Y.; Wang, L.; et al. Curcumin promotes browning of white adipose tissue in a norepinephrine-dependent way. *Biochem. Biophys. Res. Commun.* **2015**, *466*, 247–253. [CrossRef]
142. Stevens, J.F.; Maier, C.S. The chemistry of gut microbial metabolism of polyphenols. *Phytochem. Rev.* **2016**, *15*, 425–444. [CrossRef]
143. Weisberg, S.P.; Leibel, P.R.; Tortoriello, D.V. Dietary curcumin significantly improves obesity-associated inflammation and diabetes in mouse models of diabesity. *Endocrinology* **2008**, *149*, 3549–3558. [CrossRef] [PubMed]
144. Shao, W.; Yu, Z.; Chiang, Y.; Yang, Y.; Chai, T.; Foltz, W.; Lu, H.; Fantus, I.G.; Jin, T. Curcumin prevents high fat diet induced insulin resistance and obesity via attenuating lipogenesis in liver and inflammatory pathway in adipocytes. *PLoS ONE* **2012**, *7*, 28784. [CrossRef]
145. Liddle, M.; Hull, C.; Liu, C.; Powell, D. Contact urticaria from curcumin. *Dermatitis* **2006**, *17*, 196–197. [CrossRef] [PubMed]
146. Chand, S.; Hair, C.; Beswick, L. A rare case of turmeric-induced hepatotoxicity. *Intern. Med. J.* **2020**, *50*, 258–259. [CrossRef]
147. Bastard, J.P.; Maachi, M.; Lagathu, C.; Kim, M.J.; Caron, M.; Vidal, H.; Capeau, J.; Feve, B. Recent advances in the relationship between obesity, inflammation, and insulin resistance. *Eur. Cytokine Netw.* **2006**, *17*, 4–12. [PubMed]
148. Boden, M.J.; Brandon, A.E.; Tid-Ang, J.D.; Preston, E.; Wilks, D.; Stuart, E.; Cleasby, M.E.; Turner, N.; Cooney, G.J.; Kraegen, E.W. Overexpression of manganese superoxide dismutase ameliorates high-fat diet-induced insulin resistance in rat skeletal muscle. *Am. J. Physiol. Endocrinol. Metab.* **2012**, *303*, E798–E805. [CrossRef]
149. Fu, S.; Watkins, S.M.; Hotamisligil, G.S. The role of endoplasmic reticulum in hepatic lipid homeostasis and stress signaling. *Cell Metab.* **2012**, *15*, 623–634. [CrossRef] [PubMed]
150. Glass, C.K.; Olefsky, J.M. Inflammation and lipid signaling in the etiology of insulin resistance. *Cell Metab.* **2012**, *15*, 635–645. [CrossRef]
151. Arruda, A.P.; Pers, B.M.; Parlakgül, G.; Güney, E.; Inouye, K.; Hotamisligil, G.S. Chronic enrichment of hepatic endoplasmic reticulum-mitochondria contact leads to mitochondrial dysfunction in obesity. *Nat. Med.* **2014**, *20*, 1427–1435. [CrossRef]
152. Montgomery, M.K.; Turner, N. Mitochondrial dysfunction and insulin resistance: An update. *Endocr. Connect.* **2015**, *4*, R1–R15. [CrossRef]
153. Haber, C.A.; Lam, T.K.; Yu, Z.; Gupta, N.; Goh, T.; Bogdanovic, E.; Giacca, A.; Fantus, I.G. N-acetylcysteine and taurine prevent hyperglycemia-induced insulin resistance in vivo: Possible role of oxidative stress. *Am. J. Physiol. Endocrinol. Metab.* **2003**, *285*, E744–E753. [CrossRef] [PubMed]
154. Hotamisligil, G.S. Inflammation, metaflammation and immunometabolic disorders. *Nature* **2017**, *542*, 177–185. [CrossRef]
155. Dinh, T.C.; Thi, P.T.N.; Minh, L.B.; Minh, T.V.T.; Bac, N.D.; Van, T.N.; Pham, V.H.; Show, P.L.; Tao, Y.; Nhu, N.V.T. The effects of green tea on lipid metabolism and its potential applications for obesity and related metabolic disorders—An existing update. *Diabetes Metab. Syndr.* **2019**, *13*, 1667–1673. [CrossRef] [PubMed]
156. Grosso, G.; Godos, J.; Galvano, F.; Giovannucci, E.L. Coffee, Caffeine, and Health Outcomes: An Umbrella Review. *Annu. Rev. Nutr.* **2017**, *37*, 131–156. [CrossRef] [PubMed]
157. Noschang, C.G.; Krolow, R.; Pettenuzzo, L.F.; Avila, M.C.; Fachin, A.; Arcego, D.; von Pozzer Toigo, E.P.; Crema, L.M.; Diehl, L.A.; Vendite, D.; et al. Interactions between chronic stress and chronic consumption of caffeine on the enzymatic antioxidant system. *Neurochem. Res.* **2009**, *34*, 1568–1574. [CrossRef] [PubMed]
158. Abreu, R.V.; Silva-Oliveira, E.M.; Moraes, M.F.D.; Pereira, G.S.; Moraes-Santos, T. Chronic coffee and caffeine ingestion effects on the cognitive function and antioxidant system of rat brains. *Pharmacol. Biochem. Behav.* **2011**, *99*, 659–664. [CrossRef] [PubMed]
159. Amer, M.G.; Mazen, N.F.; Mohamed, A.M. Caffeine intake decreases oxidative stress and inflammatory biomarkers in experimental liver diseases induced by thioacetamide: Biochemical and histological study. *Int. J. Immunopathol. Pharmacol.* **2017**, *30*, 13–24. [CrossRef] [PubMed]
160. Santos, R.M.M.; Lima, D.R.A. Coffee consumption, obesity and type 2 diabetes: A mini review. *Eur. J. Nutr.* **2016**, *55*, 1345–1358. [CrossRef]
161. Farias-Pereira, R.; Park, C.S.; Park, Y. Mechanisms of action of coffee bioactive components on lipid metabolism. *Food Sci. Biotechnol.* **2019**, *2*, 1287–1296. [CrossRef]
162. Reto, M.; Figueira, M.E.; Filipe, H.M.; Almeida, C.M. Chemical composition of green tea (*Camellia sinensis*) infusions commercialized in Portugal. *Plant. Foods Hum. Nutr.* **2007**, *62*, 139–144. [CrossRef]
163. Graham, H.N. Green tea composition, consumption, and polyphenol chemistry. *Prev. Med.* **1992**, *21*, 334–350. [CrossRef]
164. Alschuler, L. Grean Tea: Healing tonic. *Am. J. Nat. Med.* **1998**, *5*, 28–31.
165. Suzuki, T.; Miyoshi, N.; Hayakawa, S.; Imai, S.; Isemura, M.; Nakamura, Y. Health benefits of tea consumption. In *Beverage Impacts on Health and Nutrition*; Willson, T., Temple, N.J., Eds.; Springer: Cham, Switzerland, 2016.
166. Serafini, M.; Ghiselli, A.; Ferro, L.A. In vivo antioxidant effect of green and black tea in man. *Eur. J. Clin. Nutr.* **1996**, *50*, 28–32. [PubMed]
167. Erba, D.; Riso, P.; Colombo, A.; Testolin, G. Supplementation of Jurkat T cells with green tea extract decreases oxidative damage due to iron treatment. *J. Nutr.* **1999**, *129*, 2130–2134. [CrossRef] [PubMed]

168. Jigisha, A.; Nishant, R.; Navin, K. Green tea: A magical herb with miraculous outcomes. *Int. Res. J. Pharm.* **2012**, *3*, 139–148.
169. Chatterjee, P.; Chandra, S.; Dey, P.; Bhattacharya, S. Evaluation of anti-inflammatory effects of green tea and black tea: A comparative in vitro study. *J. Adv. Pharm. Technol. Res.* **2012**, *3*, 136–138.
170. Tsai, C.; Hsu, Y.; Ting, H.; Huang, C.; Yen, C. The in vivo antioxidant and antifbrotic properties of green tea (*Camellia sinensis*, *Teaceae*). *Food Chem.* **2013**, *136*, 1337–1344. [CrossRef]
171. Newsome, B.J.; Petriello, M.C.; Han, S.G. Green tea diet decreases PCB 126-induced oxidative stress in mice by up-regulating antioxidant enzymes. *J. Nutr. Biochem.* **2014**, *25*, 126–135. [CrossRef]
172. Gupta, D.A.; Bhaskar, D.J.; Gupta, R.K. Green tea: A review on its natural anti-oxidant therapy and cariostatic benefits. *Biol. Sci. Pharm. Res.* **2014**, *2*, 8–12.
173. Ashihara, H.; Deng, W.W.; Mullen, W.; Crozier, A. Distribution and biosynthesis of flavan-3-ols in *Camellia sinensis* seedlings and expression of genes encoding biosynthetic enzymes. *Phytochemistry* **2010**, *71*, 559–566. [CrossRef] [PubMed]
174. Atomssa, T.; Cholap, A.V. Characterization and determination of catechins in green tea leaves using UV-visible spectrometer. *J. Eng. Technol. Res.* **2015**, *7*, 22–31.
175. Katiyar, S.K.; Matsui, M.S.; Elmets, C.A.; Mukhtar, H. Polyphenolic antioxidant (-)-epigallocatechin-3-gallate from green tea reduces UVB-induced inflammatory responses and infiltration of leukocytes in human skin. *Photochem. Photobiol.* **1999**, *69*, 148–153. [CrossRef]
176. Hofbauer, R.; Frass, M.; Gmeiner, B.; Handler, S.; Speiser, W.; Kapiotis, S. The green tea extract epigallocatechin gallate is able to reduce neutrophil transmigration through monolayers of endothelial cells. *Wien. Klin. Wochenschr.* **1999**, *111*, 278–282. [PubMed]
177. Nomura, S.; Ichinose, T.; Jinde, M.; Kawashima, Y.; Tachiyashiki, K.; Imaizumi, K. Tea catechins enhance the mRNA expression of uncoupling protein 1 in rat brown adipose tissue. *J. Nutr. Biochem.* **2008**, *19*, 840–847. [CrossRef]
178. Stohs, S.J.; Badmaev, V. A review of natural stimulant and non-stimulant thermogenic Agents. *Phytother. Res.* **2016**, *30*, 732–740. [CrossRef]
179. Türközü, D.; Tek, N.A. A minireview of effects of green tea on energy expenditure. *Crit. Rev. Food Sci. Nutr.* **2017**, *57*, 254–258. [CrossRef] [PubMed]
180. Skrzydlewska, E.; Ostrowska, J.; Stankiewicz, A.; Farbiszewski, R. Green tea as a potent antioxidant in alcohol intoxication. *Addict. Biol.* **2002**, *7*, 307–314. [CrossRef]
181. Yokozawa, T.; Nakagawa, T.; Kitani, K. Antioxidative activity of green tea polyphenol in cholesterol-fed rats. *J. Agric. Food Chem.* **2002**, *50*, 3549–3552. [CrossRef]
182. Tadayon, M.; Movahedi, S.; Abedi, P.; Syahpoosh, A. Impact of green tea extract on serum lipid of postmenopausal women: A randomized controlled trial. *J. Tradit. Complement. Med.* **2017**, *8*, 391–395. [CrossRef] [PubMed]
183. Sabu, M.; Smitha, K.; Kuttan, R. Anti-diabetic activity of green tea polyphenols and their role in reducing oxidative stress in experimental diabetes. *J. Ethnopharmacol.* **2002**, *83*, 109–116.
184. Li, Y.; Zhao, S.; Zhang, W.; Zhao, P.; He, B.; Wu, N.; Han, P. Epigallocatechin-3-O-gallate (EGCG) attenuates FFAs-induced peripheral insulin resistance through AMPK pathway and insulin signaling pathway in vivo. *Diabetes Res. Clin. Pract.* **2011**, *93*, 205–214. [CrossRef]
185. Wang, L.; Zeng, B.; Liu, Z.; Liao, Z.; Zhong, Q.; Gu, L.; Wei, H.; Fang, X. Green tea polyphenols modulate colonic microbiota diversity and lipid metabolism in high-fat diet treated HFA mice. *J. Food Sci.* **2018**, *83*, 864–873. [CrossRef]
186. Raederstorff, D.G.; Schlachter, M.F.; Elste, V.; Weber, P. Effect of EGCG on lipid absorption and plasma lipid levels in rats. *J. Nutr. Biochem.* **2003**, *14*, 326–332. [CrossRef]
187. Crespy, V.; Williamson, G. A review of the health effects of green tea catechins in in vivo animal models. *J. Nutr.* **2004**, *134*, 3431S–3440S. [CrossRef]
188. Wolfram, S.; Raederstorff, D.; Wang, Y.; Teixeira, S.R.; Elste, V.; Weber, P. Teavigotm (epigallocatechin gallate) supplementation prevents obesity in rodents by reducing adipose tissue mass. *Ann. Nutr. Metabol.* **2005**, *49*, 54–63. [CrossRef] [PubMed]
189. Wolfram, S.; Wang, Y.; Thielecke, F. Anti-obesity effects of green tea: From bedside to bench. *Mol. Nutr. Food Res.* **2006**, *50*, 176–187. [CrossRef]
190. Garrow, J.S. Magic bullets now uncontrolled. *BMJ* **2010**, *340*, c1351. [CrossRef]
191. Cefalu, W.T.; Ye, J.; Zuberi, A.; Ribnicky, D.M.; Raskin, I.; Liu, Z.; Wang, Z.Q.; Brantley, P.J.; Howard, L.; Lefevre, M. Botanicals and the metabolic syndrome. *Am. J. Clin. Nutr.* **2008**, *87*, S481–S487. [CrossRef]
192. Czemplik, M.; Kulma, A.; Wang, Y.F.; Szopa, J. Therapeutic strategies of plant-derived compounds for diabetes via regulation of monocyte chemoattractant protein-1. *Curr. Med. Chem.* **2017**, *24*, 1453–1468. [CrossRef] [PubMed]
193. Barzilay, J.I.; Abraham, L.; Heckbert, S.R.; Cushman, M.; Kuller, L.H.; Resnick, H.E.; Tracy, R.P. The relation of markers of inflammation to the development of glucose disorders in the elderly: The cardiovascular health study. *Diabetes* **2001**, *50*, 2384–2389. [CrossRef] [PubMed]
194. Seymour, E.M.; Lewis, S.K.; Urcuyo, L.D.E.; Tanone, I.I.; Kirakosyan, A.; Kaufman, P.B.; Bolling, S.F. Regular tart cherry intake alters abdominal adiposity, adipose gene transcription, and inflammation in obesity-prone rats fed a high fat diet. *J. Med. Food* **2009**, *12*, 935–942. [CrossRef]
195. Prior, R.L.; Wilkes, S.; Rogers, T.; Khanal, R.C.; Wu, X.; Hager, T.J.; Hager, A.; Howard, L. Dietary black raspberry anthocyanins do not alter development of obesity in mice fed an obesogenic high-fat diet. *J. Agric. Food Chem.* **2010**, *58*, 3977–3983. [CrossRef] [PubMed]

196. DeFuria, J.; Bennett, G.; Strissel, K.J.; Perfield, J.W.; Milbury, P.E.; Greenberg, A.S.; Obin, M.S. Dietary blueberry attenuates whole-body insulin resistance in high fat-fed mice by reducing adipocyte death and its inflammatory sequelae. *J. Nutr.* **2009**, *139*, 1510–1516. [CrossRef] [PubMed]
197. Ju, J.H.; Yoon, H.S.; Park, H.J.; Kim, M.Y.; Shin, H.K.; Park, K.Y.; Yang, J.O.; Sohn, M.S.; Do, M.S. Anti-obesity and antioxidative effects of purple sweet potato extract in 3t3-l1 adipocytes in vitro. *J. Med. Food* **2011**, *14*, 1097–1106. [CrossRef]
198. Zhao, Y.; Chen, B.; Shen, J.; Wan, L.; Zhu, Y.; Yi, T.; Xiao, Z. The beneficial effects of quercetin, curcumin, and resveratrol in obesity. *Oxid. Med. Cell Longev.* **2017**, *2017*, 1459497. [CrossRef]
199. Lasa, A.; Churruca, I.; Eseberri, I.; Andrés-Lacueva, C.; Portillo, M.P. Delipidating effect of resveratrol metabolites in 3T3-L1 adipocytes. *Mol. Nutr. Food Res.* **2012**, *56*, 1559–1568. [CrossRef] [PubMed]
200. Linus, P. Institute at Oregon State University, Micronutrient Information Center. Available online: http://lpi.oregonstate.edu/infocenter/contentnuts.html/ (accessed on 1 February 2021).
201. Kim, J.; Jang, D.S.; Kim, H.; Kim, J.S. Anti-lipase and lipolytic activities of ursolic acid isolated from the roots of Actinidia arguta. *Arch. Pharm. Res.* **2009**, *32*, 983–987. [CrossRef] [PubMed]
202. Liu, Y.; Tian, W.; Ma, X.; Ding, W. Evaluation of inhibition of fatty acid synthase by ursolic acid: Positive cooperation mechanism. *Biochem. Biophys. Res. Commun.* **2010**, *392*, 386–390. [CrossRef]
203. Jia, Y.; Bhuiyan, M.J.; Jun, H.J.; Lee, J.H.; Hoang, M.H.; Lee, H.J.; Kim, N.; Lee, D.; Hwang, K.Y.; Hwang, B.Y. Ursolic acid is a PPAR-alpha agonist that regulates hepatic lipid metabolism. *Bioorg. Med. Chem.* **2011**, *21*, 5876–5880. [CrossRef]
204. Kunkel, S.D.; Suneja, M.; Ebert, S.M.; Bongers, K.S.; Fox, D.K.; Malmberg, S.E.; Alipour, F.; Shields, R.K.; Adams, C.M. mRNA expression signatures of human skeletal muscle atrophy identify a natural compound that increases muscle mass. *Cell Metab.* **2011**, *13*, 627–638. [CrossRef]
205. Niki, E. Role of vitamin E as a lipid-soluble peroxyl radical scavenger: In vitro and in vivo evidence. *Free Radic. Biol. Med.* **2014**, *66*, 3–12. [CrossRef]
206. Li, Y.; Schellhorn, H.E. New developments and novel therapeutic perspectives for vitamin C. *J. Nutr.* **2007**, *137*, 2171–2184. [CrossRef]
207. Harding, A.H.; Wareham, N.J.; Bingham, S.A.; Khaw, K.; Luben, R.; Welch, A.; Forouhi, N.G. Plasma vitamin C level, fruit and vegetable consumption, and the risk of new-onset type 2 diabetes mellitus: The European prospective investigation of cancer—Norfolk prospective study. *Arch. Intern. Med.* **2008**, *168*, 1493–1499. [CrossRef] [PubMed]
208. Rotilio, G.; Calabrese, L.; Bossa, F.; Barra, D.; Agro, A.F.; Mondovi, B. Properties of the apoprotein and role of copper and zinc in protein conformation and enzyme activity of bovine superoxide dismutase. *Biochemistry* **1972**, *11*, 2182–2187. [CrossRef] [PubMed]
209. Gunasekara, P.; Hettiarachchi, M.; Liyanage, C.; Lekamwasam, S. Effects of zinc and multimineral vitamin supplementation on glycemic and lipid control in adult diabetes. *Diabetes Metab. Syndr. Obes.* **2011**, *4*, 53–60.
210. Lands, B. Historical perspectives on the impact of n-3 and n-6 nutrients on health. *Prog. Lipid Res.* **2014**, *55*, 17–29. [CrossRef]
211. Simopoulos, A.P. The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. *Exp. Biol. Med.* **2008**, *233*, 674–688. [CrossRef]
212. Jenkins, D.J. Fish oil and Omega-3 fatty acids: Fact sheet. *Can. Med. Assoc. J.* **2008**, *178*, 150. [CrossRef]
213. Brenna, J.T.; Salem, N.; Sinclair, A.J.; Cunnane, S.C. Alpha- Linolenic acid supplementation and conversion to n-3 long-chain polyunsaturated fatty acids in humans. *Prostaglandins Leukot. Essent. Fatty Acids* **2009**, *80*, 85–91. [CrossRef] [PubMed]
214. Munro, I.A.; Garg, M.L. Dietary supplementation with long chain omega-3 polyunsaturated fatty acids and weight loss in obese adults. *Obes. Res. Clin. Pract.* **2013**, *7*, 173–181. [CrossRef] [PubMed]
215. Huang, C.W.; Chien, Y.S.; Chen, Y.J.; Ajuwon, K.M.; Mersmann, H.M.; Ding, S.T. Role of n-3 Polyunsaturated fatty acids in ameliorating the obesity—-Induced metabolic syndrome in animal models and humans. *Int. J. Mol. Sci.* **2016**, *17*, 1689. [CrossRef] [PubMed]
216. Crane, F.L. Biochemical functions of coenzyme Q10. *J. Am. Coll. Nutr.* **2001**, *20*, 591–598. [CrossRef] [PubMed]
217. Sohet, F.M.; Delzenne, N.M. Is there a place for coenzyme Q in the management of metabolic disorders associated with obesity? *Nutr. Rev.* **2012**, *70*, 631–641. [CrossRef] [PubMed]

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Antioxidant Properties of Alpha-Lipoic (Thioctic) Acid Treatment on Renal and Heart Parenchyma in a Rat Model of Hypertension

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Abstract: Renal and cardiac impairments are frequent events in the presence of hypertension. Organ damage is mainly linked to oxidative stress due to high blood pressure and may be reduced by antioxidant supplementation. Alpha-lipoic acid (ALA) is one of most effective antioxidants. It is widely used as a nutritional supplement in a racemic mixture (+/−), even though the (+)-enantiomer is biologically active. This study was designed to investigate the effect of treatment with (+/−)-ALA and its enantiomers on renal and heart parenchyma in spontaneously hypertensive rats (SHR), using immunochemical and immunohistochemical techniques. The results confirmed that the oxidative mechanisms of organ alterations, due to hypertension, and characterized by glomerular and tubular lesions, left ventricular hypertrophy, and fibrosis but not by apoptosis were accompanied by proteins' and nucleic acids' oxidation. We found greater effectiveness of (+)-ALA compared to (+/−)-ALA in reducing oxidative stress, cardiac and renal damages in SHR. To conclude, these data propose (+)-ALA as one of the more appropriate antioxidant molecules to prevent renal and cardiac alterations associated with hypertension.

Keywords: alpha-lipoic acid; hypertension; kidney; heart; parenchyma

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1. Introduction

Hypertension is a multifactorial disease characterized by elevated blood pressure. It is a risk factor for renal and cardiovascular diseases [1,2]. Both the kidney and the heart are target organs in hypertension, and their structure and function become gradually impaired with longstanding hypertension. Predictably, as a consequence of hypertension, the concomitant presence of renal and heart failure in the same patient was reported [3]. Indeed, cardiorenal interactions occur through several pathways in both directions [4].

Multiple mechanisms are implicated in the determination of hypertension-induced kidney damage. These involve different cell types and anatomical structures in the kidney, including the glomeruli, tubulointerstitium, immune cells, and vasculature. Arterial hypertension is correlated with a progressive intimal thickening of muscular arteries and arterioles of the renal parenchyma, due to collagen deposition and spreading of elastic fibers and myofibroblasts [5]. Other histopathological alterations are interstitial fibrosis, associated with tubular atrophy, and arteriosclerosis of the afferent arterioles [1,5].

Concerning the mechanisms, the hypertrophy of the cardiac muscle is one of the most important maladaptive responses to augmented workload. It is associated with histological and ultrastructural modifications and alterations in the level of different enzymes [6]. In

addition to cardiomyocyte hypertrophy, myocardial microcirculation rarefaction, interstitial fibrosis, and myocyte apoptosis are reported [7].

Increasing relevance is attributed to the role of oxidative stress for what concerns the mechanisms of hypertension and cardiovascular and renal damage because it promotes endothelial dysfunction, vascular remodeling, and inflammation, leading to vascular damage [8,9]. Excess bioavailability of reactive oxygen species (ROS) often accompanied hypertension-induced structural abnormalities on mitochondria of the cardiomyocyte and renal parenchymal cells [10]. In experimental and human hypertension, an increase in the production of superoxide anions and hydrogen peroxide, a decrease in nitric oxide (NO) synthesis, and a reduction in endogenous antioxidant bioavailability have been observed [11,12]. Other biomarkers of oxidative stress in human hypertension are isoprostanes and malondialdehyde (MDA) [13].

The most effective treatment in the management of hypertension seems to be the administration of anti-hypertensive drugs with antioxidant properties [14]. Even if the data of clinical trials are discordant, the supplementation of natural antioxidants could also be a promising therapeutic tool for hypertension [15,16]. Experimental evidence suggests that early antioxidant treatment may induce beneficial effects on hypertension and cardiovascular and renal damage [17,18]. However, antioxidants are less effective in the advanced stage of hypertension, whereas the renin–angiotensin system (RAS) may still play a central pathogenetic role, even at later stages, as confirmed by the positive action of its blockade [19].

Previously, we demonstrated that the antioxidant alpha-lipoic acid (ALA) treatment reduced oxidative stress and prevented adhesion molecule expression in the cardiac and renal vascular endothelium of spontaneously hypertensive rats (SHR) [20]. The SHR is a reliable model of a spontaneously developing pressure load similar to human essential hypertension; thus, it is usually recognized that the SHR represents an analog of essential hypertension in humans [21,22]. Severe hypertension arises in 100% of SHR with a systolic blood pressure of 180 mmHg or higher [23].

In SHR, we already assessed the neuroprotective activity of ALA in central nervous system lesions consequent to peripheral nerve injury [24]. Recently, data from the spinal cord could explain the protection of ALA on the brain [25]. Finally, the use of ALA for therapeutic purposes was reviewed by [26].

Therefore, the current study aims to investigate the alterations in renal and heart parenchyma of SHR and the potential benefits of ALA on it, using immunochemical and immunohistochemical techniques.

2. Materials and Methods

2.1. Animals and Tissue Treatment

SHR has a genetic predisposition to develop arterial hypertension, allowing it to study causes, mechanisms, pathology, and behavioral consequences of the disease [27]. Twenty-week-old male SHR (n = 30) and age-matched normotensive Wistar–Kyoto rats (WKY) rats (n = 6) were used and treated, as previously described [20]. Animal manipulation was carried out according to the National and European Community Guidelines for Animal Care (DL 116/92, of application of the European Communities Council Directive, 86/609/EEC) and also following the ethical guidelines of the University of Florence. The guidelines deal with the treatment of animals under lab testing. These guidelines are consistent with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication No. 85-23, revised 1996; University of Florence assurance number: A5278-01). Experiments involving animals have been reported according to ARRIVE guidelines. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Concerning the treatment, alpha-lipoic acid (1,2-dithiolane-3-pentanoic acid or thioctic acid), exists in two enantiomers, namely (+)- and (–)-alpha-lipoic acid. It is due to the

presence of an asymmetric carbon in the C3 position. The former enantiomer represents the active form of the compound [20].

Lipoic acid, as (+/−)-compound, lysine salt (+)-enantiomer and (−)-enantiomer, was provided from Sintactica (Milan, Italy). Compounds were solubilized in NaOH-supplemented physiologic solution and buffered to 7.4 pH by adding HCl. Different racemic and lipoic acid salt compounds were solubilized in saline [25] and SHR rats were treated once a day for 30 days with an i.p. injection of 250 µmol/kg/day of (+/–)-ALA (n = 6); 125 µmol/kg/day of (+/–)-ALA (n = 6); 125 µmol/kg/day of (+)-ALA (n = 6) and 125 µmol/kg/day of (–)-ALA (n = 6). WKY (n = 6) and SHR (n = 6) rats were also treated with the same amounts of the vehicle as control. Food intake and body weight were monitored daily, while measurements of systolic blood pressure were performed once a week, in conscious rats, by tail-cuff methods using electronic sphygmomanometer, specific for small animals (Model: GIMA Italy, B3Plus). Animals were anesthetized and then perfused [20]. After, the kidney and heart were dissected out, weighed, fixed for 72 h in 4% paraformaldehyde, dehydrated with alcohol and embedded in paraffin wax. Longitudinal consecutive renal and heart sections were cut using a rotary microtome and processed for morphological analysis and immunohistochemistry (IHC), as detailed previously [20].

2.2. Histological Analysis, Immunohistochemistry and Immunofluorescence

Paraffin blocks were cut into 6–8 µm sections for Masson staining and immunohistochemistry. Kidney tissues were also stained with periodic acid-Schiff (PAS) to highlight basement membranes of glomerular capillary loops and tubular epithelium, and semiquantitative analysis of the glomerular intensity of PAS staining was performed. Moreover, glomerular and tubular injury scores were examined in Masson's stained sections as follows. For the glomerular injury score (GIS), approximately 60 random glomeruli from each specimen were examined and each glomerulus was graded from 1 to 4 [28]. Then, each score was calculated according to the formula GIS = [(1 × number of grade 2 glomeruli) + (2 × number of grade 3 glomeruli) + (3 × number of grade 4 glomeruli)] × 100/(number of glomeruli observed) [29,30]. The tubular injury was defined using the scoring system from 0 to 4 [31]. Then, the tubular injury score (TIS) was calculated according to the formula TIS = [(1 × number of grade 1 dark tubules profiles area) + (2 × number of grade 2 dark tubules profiles area) + (3 × number of grade 3 dark tubules profiles area) + (4 × number of grade 4 dark tubules profiles area)]/(surface area examined) [30]. Cardiac fibrosis analysis was performed by measuring the presence of accumulation of connective tissue in the parenchyma of the left ventricle and in the adventitia of the coronary arteries in Masson trichrome-stained sections from each heart [32].

Immunohistochemistry anti-8-hydroxy-2′-deoxyguanosine (anti-8-oxo-dG) was performed both in the kidney and heart using a mouse monoclonal antibody (Trevigen, Gaithersburg, MD, USA, Cat. No. 4354-MC-050) diluted 1:250. This mouse monoclonal antibody specifically binds to 8-oxo-dG within DNA, as detailed [25]. The product of the immune reaction was then revealed by exposing slides with anti-mouse biotinylated polyclonal antibody (Merck-Millipore, Burlington, MA, USA, Cat. No. AP124B) (diluted 1:200). The colored reaction product was developed with 3′,3′-diaminobenzidine tetrahydrochloride (DAB) solution (Vector Laboratories, Inc., Burlingame, CA, USA). The sections were observed, and images were captured with the microscope (Leica) and evaluated using an IAS 2000 (Delta Sistemi, Rome, Italy). The number of 8-oxo-dG positive cells was counted per high-power field (HPF) images (40× magnification).

Finally, the Membstain Apoptosis kit Direct (MBL International Corporation, Woburn, MA, USA, Cat. No. 8445) based on in situ labeling of nuclear DNA fragmentation (Tolt-mediated dUTP nick-end labeling, TUNEL staining) was used following the company instruction. The sections were analyzed under a microscope a fluorescence (Olympus Italia, Segrate, Italy), using a 465 nm excitation filter. The number of TUNEL positive cells was counted per HPF images (20× and 40× magnification for heart and kidney, respectively).

2.3. Protein Extraction and Western Blot Analysis

Protein lysate was obtained homogenizing the tissue using lysis buffer as previously detailed [32]. For 8% and 12% SDS-PAGE, 40 µg total protein sample was deposited in each well. The separated protein was transferred to a nitrocellulose membrane, which was blocked with bovine serum albumin (BSA) in PBS 0.1% Tween-20 for 1 h at room temperature and then incubated with anti-caspase-3 (Cell Signaling Technology, Danvers, MA, USA, 1:1000) and a GAPDH antibody as an internal reference (Cell Signaling Technology, Danvers, MA, USA, 1:1000) at 4 °C overnight. Then, the membranes were transferred to room temperature and incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (Bethyl Laboratories, Inc., Montgomery, TX, USA, dilution 1:5000).

Moreover, the protein oxidation status was investigated using the OxyBlot Protein Oxidation Detection kit (Merck-Millipore, Burlington, MA, USA, Cat. No. S7150). The detection was performed using the LiteAblot PLUS kit (EuroClone, Milan, Italy). Band intensities were measured by densitometry with IAS 2000 image analyzer (Biosystem, Rome, Italy). Blots are representative of one of three separate experiments.

2.4. Statistical Analysis

All data were expressed as mean ± S.E.M. The statistical comparisons were determined using Student's t-tests for WKY and SHR control (C) and one-way ANOVA for SHR C and groups of SHR with ALA, followed by the Newman–Keuls post hoc tests for multi-groups, with p-value < 0.05 being considered statistically significant.

3. Results

No significant differences were observed between WKY and SHR or SHR treated with ALA in body weight. Neither hypertension nor pharmacologic treatments modified kidney weight values. The ratio of heart weight to body weight was increased in control SHR rats (3.37 ± 0.12) compared to control WKY rats (3.05 ± 0.06). The antioxidant treatment did not change this phenomenon. At sacrifice, the systolic blood pressure values were higher in control SHR rats (215 ± 3 mmHg) compared to the normotensive WKY rats (148 ± 9 mmHg). In SHR rats, 30 days of treatment with any stereoisomer of ALA did not significantly affect systolic blood pressure levels [20].

3.1. Kidney

To assess the overall level of oxidative modification of proteins in the kidney of rats, we carried out Western blot analyses using the OxyBlot Protein Oxidation Detection kit. A consistent increase in protein oxidation was detected in the renal cortex of SHR versus normotensive WKY rats (Figure 1a,b), suggesting that protein oxidation is related to hypertensive status. The administrations of (+/−)-ALAs and of (−)-ALA 125 µmol/kg/day did not lead to a reduction in oxidation levels of renal proteins compared to hypertensive control (Figure 1a,b). On the contrary, a significant decrease was found in SHR treated with (+)-ALA 125 µmol/kg/day (Figure 1a,b). To determine if there was an increase in oxidative DNA damage, IHC anti-8-oxo-dG was performed in the sections of the kidney (Figure 1c). Figure 1c showed representative images of 8-oxo-dG staining more cells of proximal and distal convoluted tubules than nuclei of the glomerular mesangium. The SHR rats showed an increase of 8-oxo-dG positive nuclei, and intensity of immunoreaction, due to oxidative stress, which also involves nucleic acids (Figure 1c,d). Only the treatment with antioxidant (+)-ALA 125 µmol/kg/day decreased the number of positive cells and the intensity of immunoreaction compared to SHR control rats (Figure 1c,d). Indeed, the administration of (+/−)-ALA 250, (+/−)-ALA 125 µmol/kg/day and (−)-ALA 125 µmol/kg/day did not induce a reduction in the number of 8-oxo-dG positive cells (Figure 1c,d).

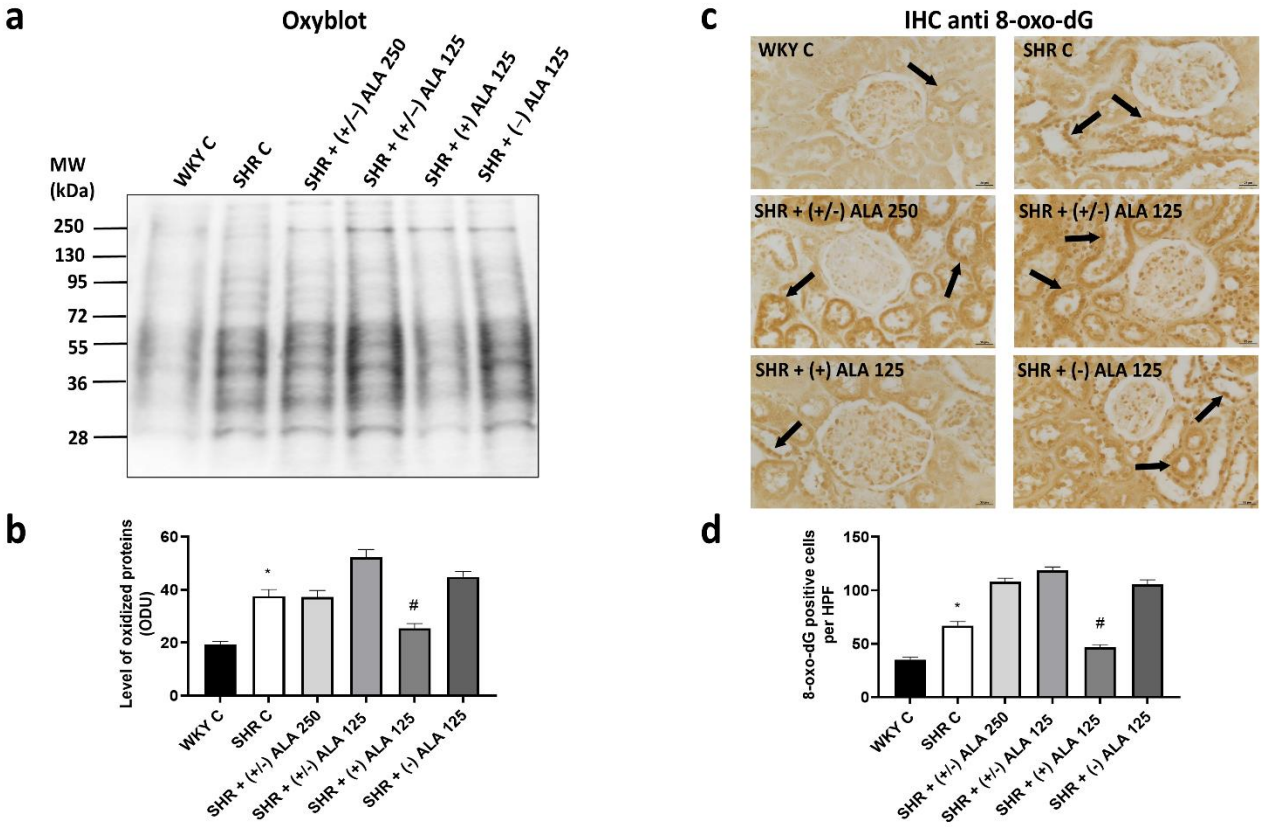

Figure 1. Oxidative stress in the kidney. (**a**) Lysates of kidney from WKY C, SHR C, SHR (+/−)-alpha-lipoic acid (ALA) 250 µmol/kg/day, SHR + (+/−) ALA 125 µmol/kg/day, SHR + (+) ALA 125 µmol/kg/day, SHR + (−) ALA 125 µmol/kg/day were immunoblotted using the Oxyblot kit; (**b**) The bar graph reports the level of oxidized proteins measured in optical density unit (ODU); (**c**) Sections of kidney processed for the 8-oxo-dG immunohistochemistry (IHC). Arrow: positive nucleus. Calibration bar 25 µm; (**d**) The bar graph shows the quantification of the number of positive cells per high-power field (HPF) (40×). Data are mean ± S.E.M. * $p < 0.05$ vs. WKY C; # $p < 0.05$ vs. SHR C.

The presence of oxidative damage in the kidney induced by hypertension did not imply an increase in apoptotic processes. As shown in Figure 2a,b, the expression of caspase-3 (molecular weight 34 kDa) in SHR rats did not change compared to WKY control rats, as well as in the treated groups. Even if an increasing tendency of caspase-3 in animals treated with (+/−)-ALAs and (−)-ALA 125 µmol/kg/day was found compared to the SHR control group, the differences were not statistically significant. Moreover, there was no increase in TUNEL positive cells per field in SHR compared to normotensive WKY rats (Figure 2c,d).

In hypertensive rats, PAS staining of the kidney showed glomerular structural abnormalities, and several tubules exhibited PAS droplets (Figure 3). As showed by quantification of its staining intensity (Figure 3b), the administrations of (+/−)-ALA 125 µmol/kg/day and (−)-ALA 125 µmol/kg/day in SHR rats did not influence the particularly heavy PAS labeling found in SHR control animals. On the contrary, the compounds (+/−)-ALA 250 µmol/kg/day and (+)-ALA 125 µmol/kg/day were demonstrated to be the most effective. These data were in the direction of the semiquantitative analysis on Masson's trichrome staining.

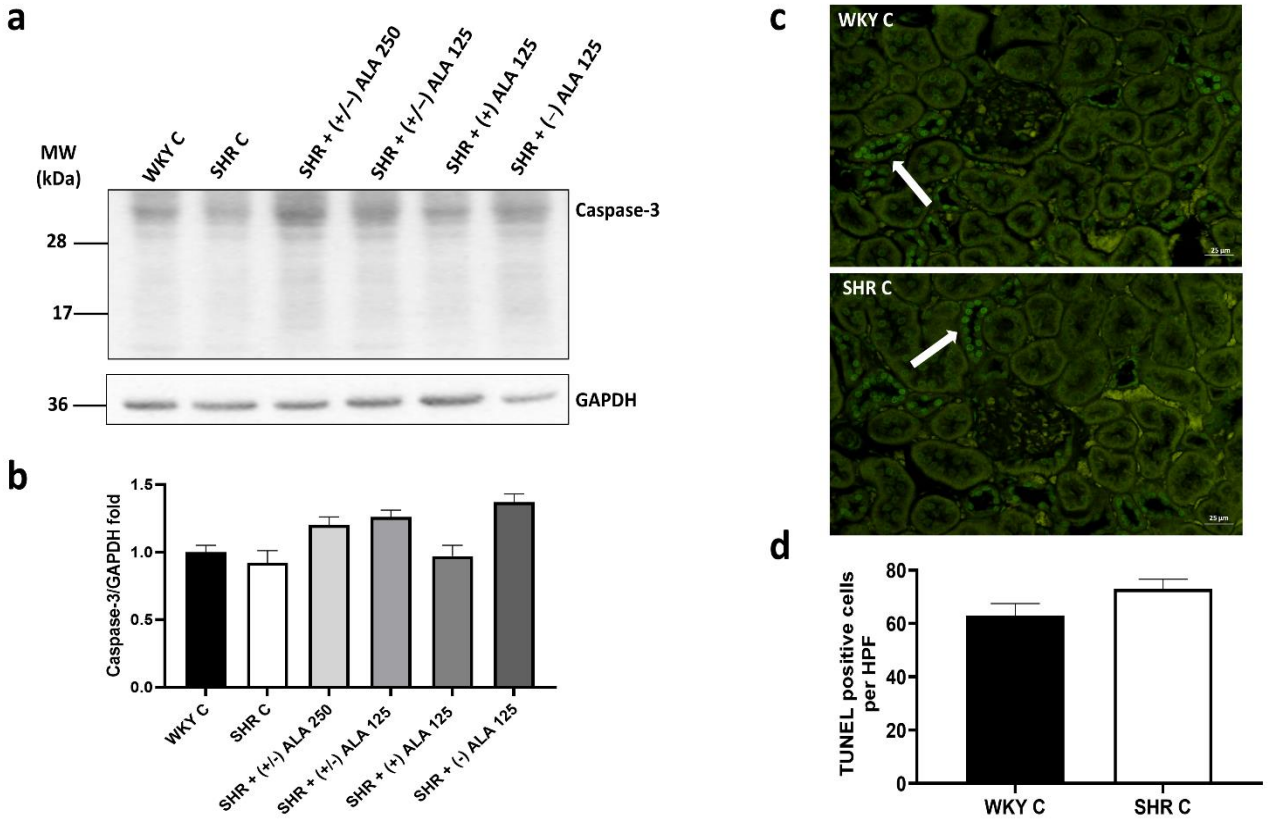

Figure 2. Apoptosis in the kidney. (**a**) Lysates of kidney from WKY C, SHR C, SHR + (+/−) alpha-lipoic acid (ALA) 250 µmol/kg/day, SHR + (+/−) ALA 125 µmol/kg/day, SHR + (+) ALA 125 µmol/kg/day, SHR + (−) ALA 125 µmol/kg/day were immunoblotted using specific anti caspase-3; (**b**) The bar graph indicates the ratio of densitometric analysis of bands and GAPDH levels, used as loading control, considering the WKY C group as reference. Blots are representative of one of three separate experiments; (**c**) Sections of kidney from WKY C and SHR C processed for TUNEL staining. Arrow: apoptotic nucleus. Calibration bar 25 µm; (**d**) Bar graph shows the quantification of the number of positive cells per high-power field (HPF) (40×). Data are mean ± S.E.M.

In SHR, the renal morphological and morphometric data showed parenchymal changes consisting of glomerular lesions, characterized by basal wall thickening and atrophy (Figure 4a,b). Besides, tubular alterations were observed both in proximal and distal tubules, characterized by degenerative tubular epithelium, which was thinner and necrotic (Figure 5a). Quantitative analyses of glomerular and tubular damages showed an increase in both GIS and TIS values (Figures 4b and 5b, respectively) in hypertensive rats compared to normotensive ones. The treatments with higher dose of racemic ALA prevented partially the GIS (Figure 4b). Indeed, the most active compound was (+)-ALA 125 µmol/kg/day both at the glomerular and tubular level (Figures 4b and 5b), while (−)-ALA 125 µmol/kg/day was not effective in preventing the damage induced by hypertension (Figures 4b and 5b).

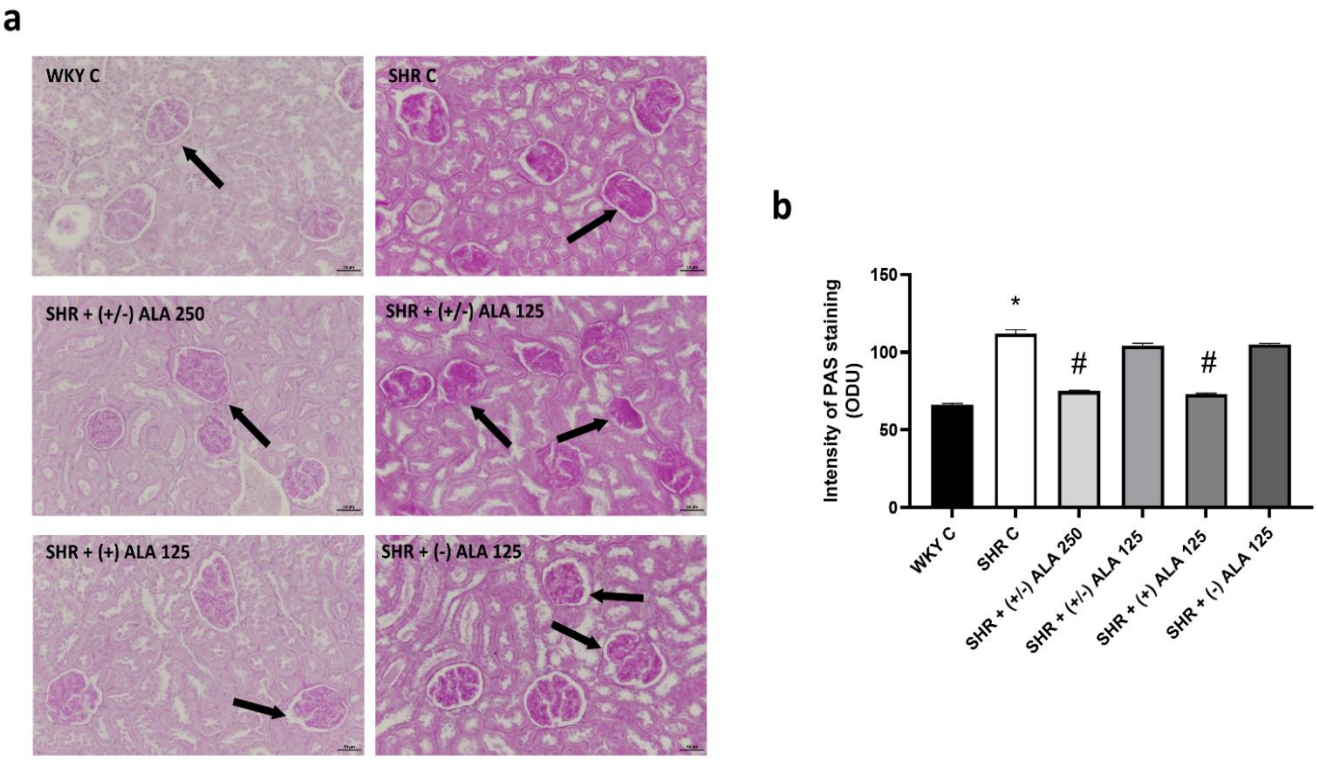

Figure 3. Renal glomerular morphology. (**a**) Renal tissue of WKY C, SHR C, SHR + (+/−) alpha lipoic acid (ALA) 250 µmol/kg/day, SHR + (+/−) ALA 125 µmol/kg/day, SHR + (+) ALA 125 µmol/kg/day, SHR + (−) ALA 125 µmol/kg/day were stained using periodic acid-Schiff (PAS) technique. Arrow: damaged glomerulus. Calibration bar: 50 µm; (**b**) The bar graph shows the quantification of the intensity of PAS staining measured in optical density unit (ODU). Data are mean ± S.E.M. * $p < 0.05$ vs. WKY C; # $p < 0.05$ vs. SHR C.

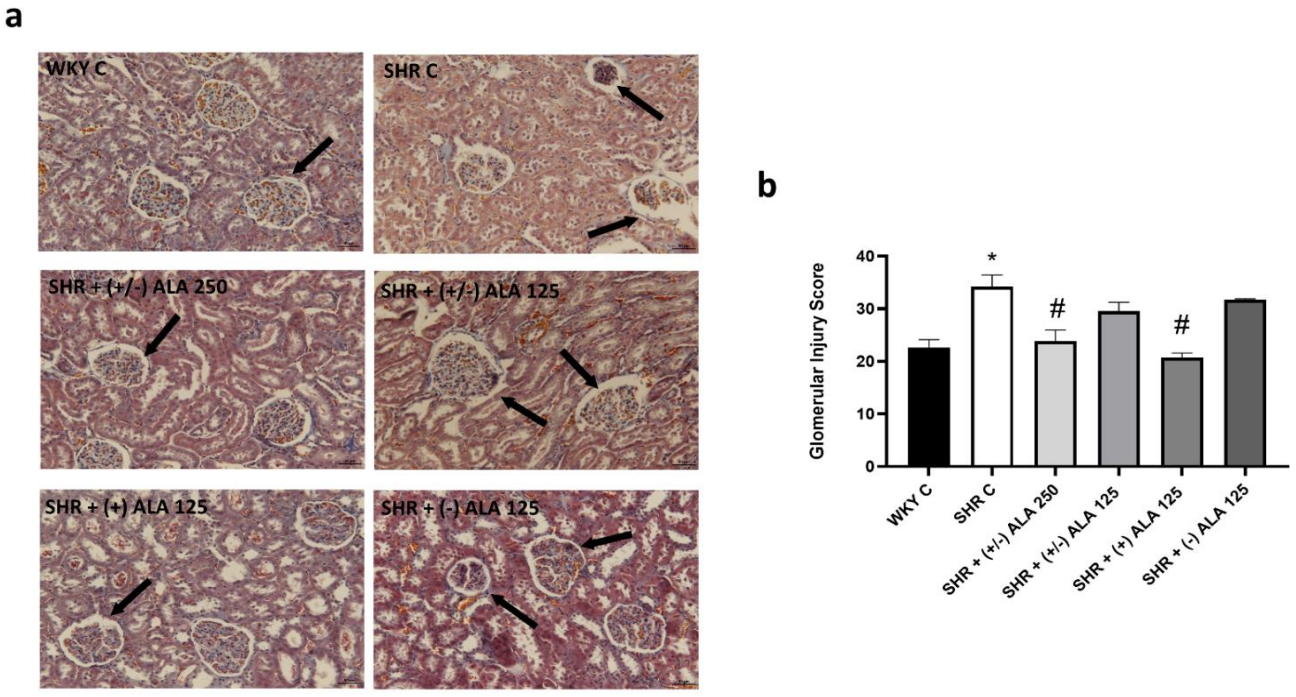

Figure 4. Renal glomerular morphology. (**a**) Renal tissue of WKY C, SHR C, SHR + (+/−) alpha lipoic acid (ALA) 250 µmol/kg/day, SHR + (+/−) ALA 125 µmol/kg/day, SHR + (+) ALA 125 µmol/kg/day, SHR + (−) ALA 125 µmol/kg/day were stained using Masson's trichrome technique. Arrow: damaged glomerulus. Calibration bar: 50 µm; (**b**) Morphometric analysis to assess the glomerular injury score. Data are mean ± S.E.M. * $p < 0.05$ vs. WKY C; # $p < 0.05$ vs. SHR C.

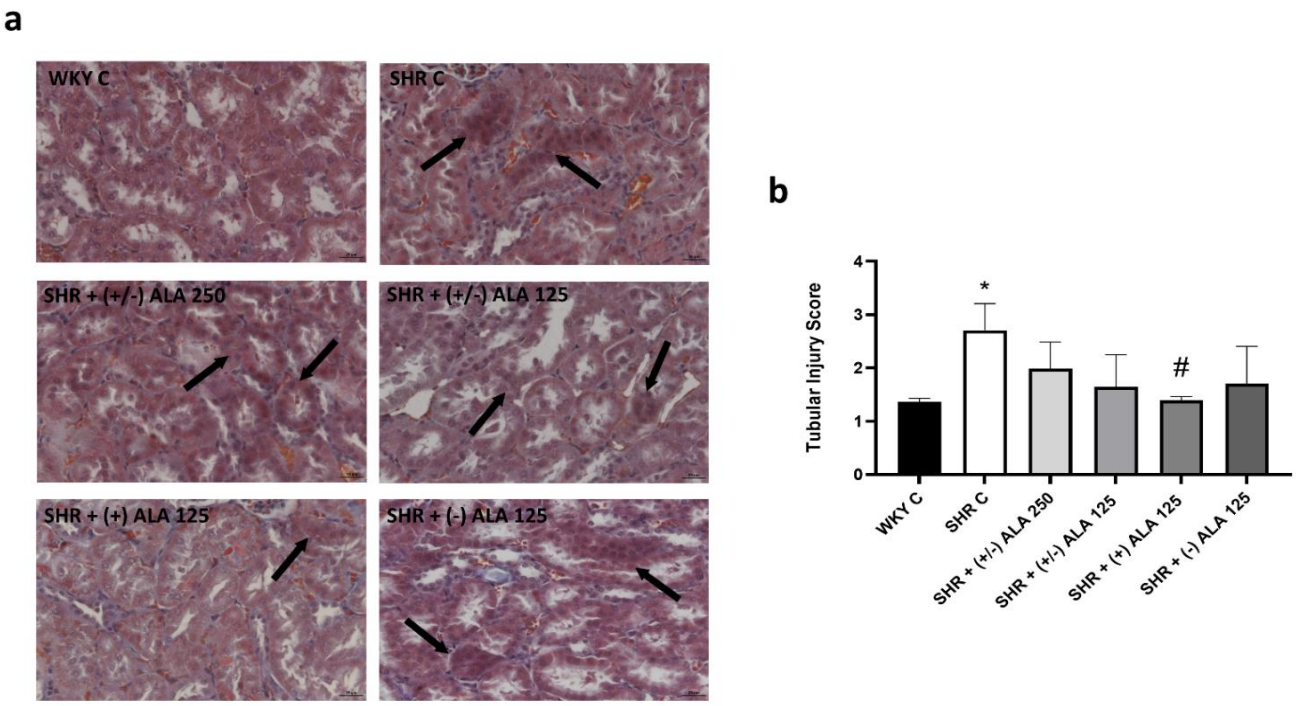

Figure 5. Renal tubules morphology. (**a**) Renal tissue of WKY C, SHR C, SHR + (+/−) alpha lipoic acid (ALA) 250 µmol/kg/day, SHR + (+/−) ALA 125 µmol/kg/day, SHR + (+) ALA 125 µmol/kg/day, SHR + (−) ALA 125 µmol/kg/day were stained using Masson's trichrome technique. Arrow: damaged tubules. Calibration bar: 25 µm; (**b**) Morphometric analysis to assess the tubular injury score. Data are mean ± S.E.M. * $p < 0.05$ vs. WKY C; # $p < 0.05$ vs. SHR C.

3.2. Heart

The results of Oxyblot kit showed an increase of oxidized proteins in the ventricles of the heart of SHR versus normotensive WKY rats (Figure 6a,b), as well as in kidneys, suggesting that protein oxidation was also related to hypertensive status. Treatment with antioxidants partially decreased the level of oxidized proteins, as shown in rats injected with (+/−)-ALA 250 µmol/kg/day and (+)-ALA 125 µmol/kg/day (Figure 6a,b). In line with the Western blot analysis was the IHC for 8-oxo-dG staining the nuclei of cardiomyocytes (Figure 6c). SHR rats showed an increase both in the number of positive nuclei, and intensity of immunoreaction, due to oxidative stress, which also involved nucleic acids (Figure 6c,d). These nuclei were larger in hypertensive rats than in normotensive ones. The treatment with the antioxidants (+/−)-ALA 250 µmol/kg/day and (+)-ALA 125 µmol/kg/day decreased the number of positive cells and the intensity of the immunoreaction compared to hypertensive control animals, while (+/−)-ALA 125 µmol/kg/day and the (−)-ALA did not induce changes in the number of 8-oxo-dG positive cells per field (Figure 6c,d).

As well as in the kidney, the apoptosis pathway was not initiated in the heart. As shown in Figure 7a,b the expression of caspase-3 (molecular weight 34 kDa) in SHR rats did not change compared to WKY control rats, as well as in the treated groups. Moreover, there was no increase in TUNEL positive cells in SHR compared to normotensive WKY rats (Figure 7c,d).

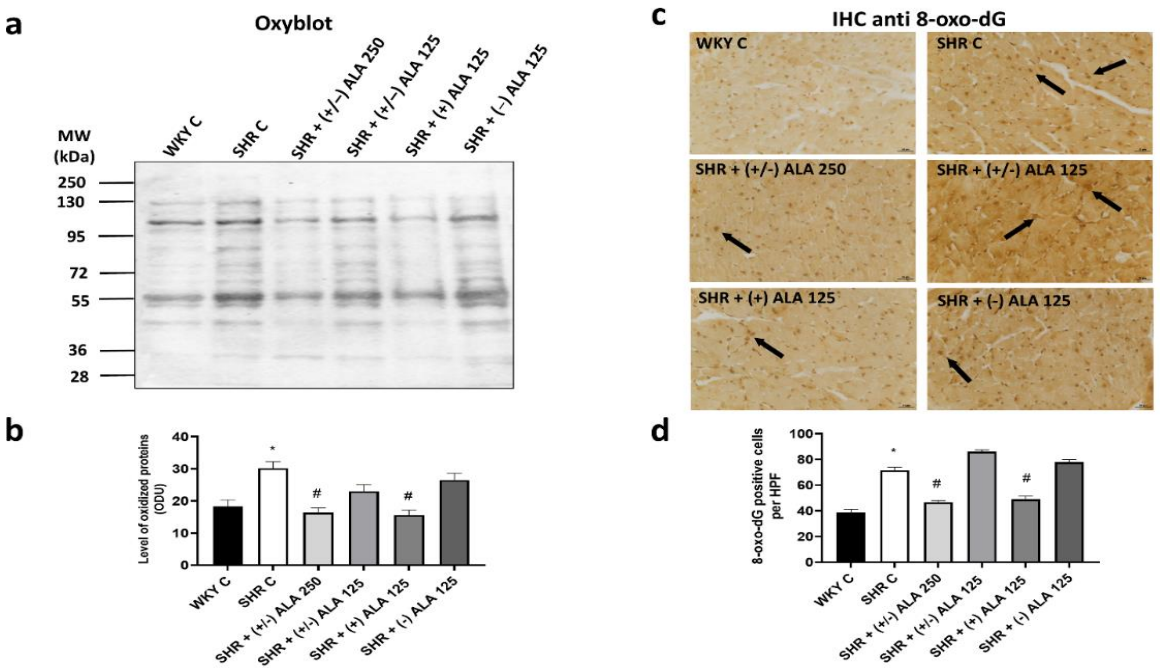

Figure 6. Oxidative stress in heart. (**a**) Lysates of heart from WKY C, SHR C, SHR + (+/−) alpha lipoic acid (ALA) 250 μmol/kg/day, SHR + (+/−) ALA 125 μmol/kg/day, SHR + (+) ALA 125 μmol/kg/day, SHR + (−) ALA 125 μmol/kg/day were immunoblotted using the Oxyblot kit; (**b**) The bar graph reports the level of oxidized proteins measured in optical density unit (ODU); (**c**) Sections of heart processed for the 8-oxo-dG immunohistochemistry (IHC). Arrow: positive nucleus. Calibration bar 25 μm; (**d**) The bar graph shows the quantification of the number of positive cells per high-power field (HPF) (40×). Data are mean ± S.E.M. * $p < 0.05$ vs. WKY C; # $p < 0.05$ vs. SHR C.

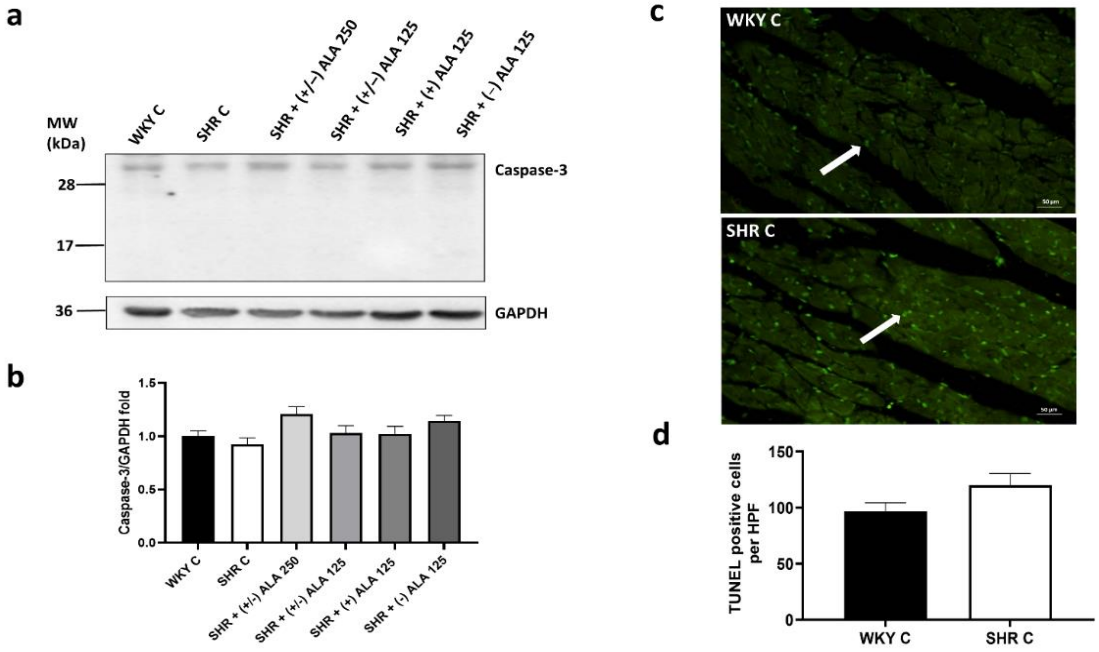

Figure 7. Apoptosis in heart. (**a**) Lysates of heart from WKY C, SHR C, SHR + (+/−) alpha lipoic acid (ALA) 250 μmol/kg/day, SHR + (+/−) ALA 125 μmol/kg/day, SHR + (+) ALA 125 μmol/kg/day, SHR + (−) ALA 125 μmol/kg/day were immunoblotted using specific anti caspase-3; (**b**) The bar graph indicates the ratio of densitometric analysis of bands and GAPDH levels used as loading control, considering the WKY C group as reference. Blots are representative of one of three separate experiments; (**c**) Sections of heart from WKY C and SHR C processed for TUNEL staining. Arrow: apoptotic nucleus. Calibration bar 50 μm; (**d**) The bar graph shows the quantification of the number of positive cells per high-power field (HPF) (20×). Data are mean ± S.E.M.

Morphometric analyses of the myocardium, focused at the subendocardial level, showed a clear connective tissue accumulated between the cardiomyocytes in SHR rats (Figure 8a). Moreover, an increase of cardiomyocyte areas was reported in SHR rats compared to WKY rats (Figure 8b), as well as hypertrophy of the left ventricular wall, but not the right one. This phenomenon was reduced only by treatment of (+)-ALA 125 µmol/kg/day (Figure 8b), which significantly decreased left ventricular fibrosis (Figure 8c) while the (−)-ALA 125 µmol/kg/day did not have any positive effect either on cardiac hypertrophy nor fibrosis (Figure 8b,c).

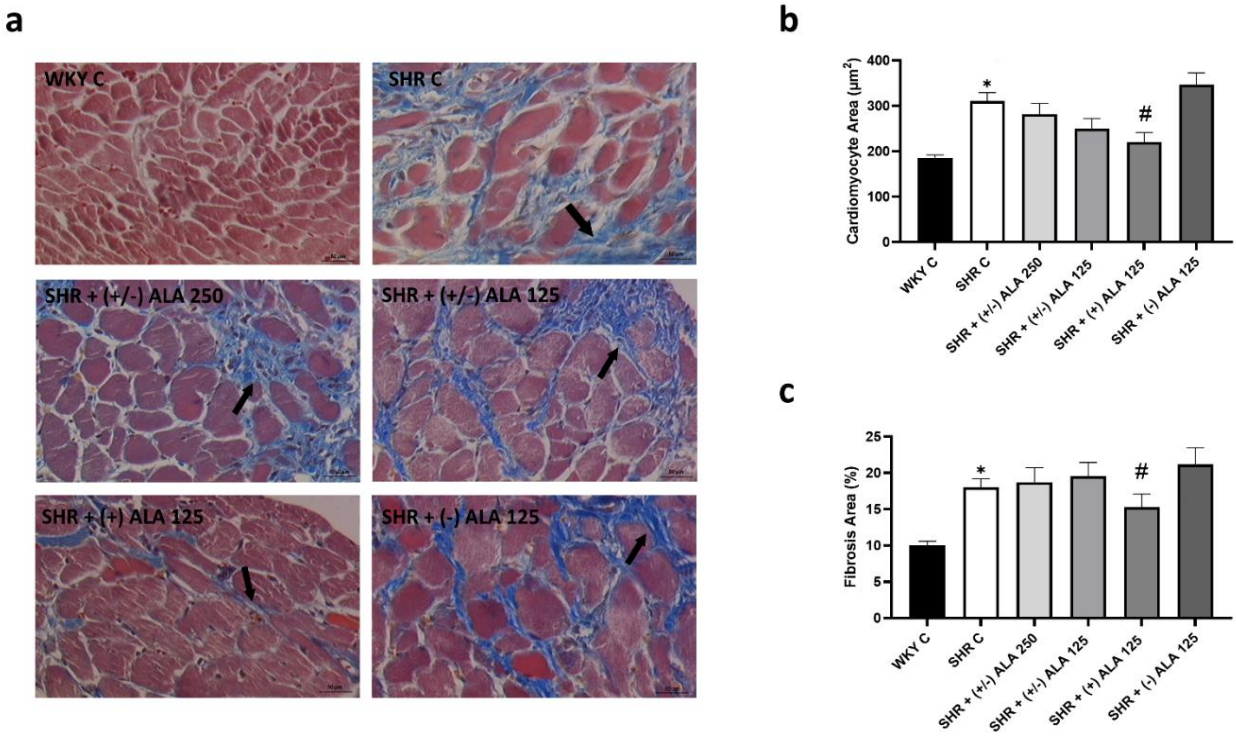

Figure 8. Heart morphology. (**a**) Cardiac tissue of WKY C, SHR C, SHR + (+/−) alpha lipoic acid (ALA) 250 µmol/kg/day, SHR + (+/−) ALA 125 µmol/kg/day, SHR + (+) ALA 125 µmol/kg/day, SHR + (−) ALA 125 µmol/kg/day were stained using Masson's trichrome technique. Arrow: damaged cardiomyocytes. Calibration bar: 50 µm; (**b**,**c**) Morphometric analysis to evaluate the areas of cardiomyocytes and cardiac fibrosis, respectively. Data are mean ± S.E.M. * $p < 0.05$ vs. WKY C; # $p < 0.05$ vs. SHR C.

4. Discussion

Arterial hypertension can be generated by numerous factors, such as lifestyle, physiological and genetic causes [33]. High blood pressure induced mechanical damage along with the vascular system, heart, and kidneys, which are the main organs negatively impaired [33,34]. Indeed, heart failure, cardiac hypertrophy and renal dysfunction are some features of hypertensive end-organ damage developing in SHR rats [34,35].

Our data supported the coexistence of cardiac and renal damage in SHR [20,23,30,36–38]. Histologically, we confirmed glomerular and tubular lesions in hypertensive conditions [1,5,23]. Basal wall thickening and atrophy were found at the glomerular level, while the degenerative tubular epithelium was thinner and necrotic. The attempts of reparative mechanisms lead to end-organ damage, principally due to fibrosis [33]. Concerning this, excessive matrix deposition, remodeling, and cardiac hypertrophy become maladaptive responses to prolonged and abnormal hemodynamic stress, due to hypertension [6,7,39]. In accordance, we showed left ventricular hypertrophy and increased fibrosis (histologic hallmarks of cardiac failure) in SHR rats.

Even if caspase-mediated myocardial apoptosis was reported in SHR [40], here we did not have any differences, probably related to duration or severity of hypertension [41].

Moreover, the inflammatory pathway and oxidative stress can be triggered by hypertension. Despite the evidence regarding oxidative stress involvement in hypertension, the mechanisms involved are not well defined [42]. Hypertension is related to the overproduction of ROS, together with decreased NO bioavailability and reduced antioxidant capacity in the brain, vasculature, and kidney [11,12,42,43]. The ROS generated in cardiovascular cells induce pathological vascular injury in blood vessels associated with the accumulation of extracellular matrix protein, endothelial dysfunction, and inflammation, typical features of vascular phenotype in hypertension [8,9,44]. Many experimental models have reported the relationship between oxidative stress and hypertension [45–47]. As reviewed by [48] in SHR models, ROS such as superoxide and hydrogen peroxide (H_2O_2), as well as oxidation markers such as tyrosine nitration of proteins, production, and excretion of 8-isoprostaglandin F2α (8-iso), MDA, and thiobarbituric acid reactive substances, are increased in vessels, heart, and kidneys. Moreover, renal blood flow and glomerular filtration are reduced in SHR, similarly in obesity-prone and Dahl salt-sensitive rats [48]. Interestingly, hypertensive males have been reported to have greater levels of oxidative stress compared with females [49]. In addition, SHRs exhibit sex differences in blood pressure, with males having higher blood pressure than females [50]; for this reason, we chose males. Previously, an increase of lipid peroxidation and nucleic acid oxidation in plasma, kidney, and heart of SHR rats has been demonstrated [20,24]. Not only the presence of oxidative stress but also elevated endothelial adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) expression were found in the heart and the renal endothelium of hypertensive rats [20].

These data highlighted the importance of antioxidant compounds as a support to conventional anti-hypertensive treatments. Clinical randomized studies demonstrated the potential anti-hypertensive effect of antioxidant molecules in the diet both in hypertensive and normotensive subjects [15]. Moreover, prenatal use of natural antioxidants may reverse programming progressions and avoid hypertension of developmental origin [51]. Initially, similar dietary approaches showed a reduction in cardiovascular morbidity and mortality in hypertensive subjects, showing benefits [52,53]; however, later, large clinical trials, including the SU.VI.MAX study showed no improvement in blood pressure with antioxidant therapy [54].

Generally used antioxidants include Vitamins A, C, and E, L-arginine, flavonoids, and mitochondria-targeted agents (Coenzyme Q10, acetyl-L-carnitine, and ALA) [15]. ALA is a dithiol compound produced from octanoic acid in mitochondria. The in vitro and in vivo properties of ALA have been widely revised [26,55–57], in particular its antioxidant potential, acting as metal chelators, free radical scavengers, repairer of oxidized injury and regenerator of antioxidants defense, such as glutathione (GSH) and catalase activity, vitamins C and E [26,58,59]. The contribution in metabolic pathways related to mitochondria, in cell signaling that may improve coupling of endothelial nitric oxide synthase (eNOS), and in anti-inflammatory actions are other relevant benefits found with ALA supplementation [55,60]. For such reasons, this compound has received great consideration as an antioxidant in the management of diabetic problems such as neuropathy, retinopathy, and other vascular diseases [61]. ALA was also effective in preventing acute kidney injury [62], glomerular injury caused by diabetes mellitus [63], and the toxic effects of acetaminophen [64], cisplatin [65], and methotrexate [66] on the kidney in rats. In addition, in unilateral ureteral obstruction mice treated with ALA, only moderate, histologically identifiable renal injury and minimal fibrosis were reported [67]. At the molecular level, the expression of extracellular matrix (ECM) proteins, fibrogenic and inflammatory factors (transforming growth factor-β1 and monocyte chemoattractant protein-1), and epithelial-to-mesenchymal transition markers (E-cadherin and alpha-smooth muscle actin) was much lower in ALA-treated than in non-treated mice [67]. Regarding results in SHR rats, renal histopathological examination showed that ALA supplementation ameliorated glomerular damage and prevented vascular damage [68]. Moreover, ALA-treated animals showed a significant reduction in urine protein levels, N-acetyl-β-(D)-glucosaminidase activity,

and significant increase of creatinine clearance, particularly in SHR at 28 weeks [68]. In patients with autosomal dominant polycystic kidney disease, ALA was suggested as an anti-inflammatory and antioxidant nutraceutical compound with few side effects to reduce the related cardiovascular risk factors [69]. Moreover, the long-term intermittent treatment with ALA prevented body weight gain and reduced metabolic and cardiac alterations (i.e., atherosclerosis), corroborating its protective properties on the cardiovascular system [70,71]. Besides, studies in diabetic rats and other different hypertensive animal models revealed the potential for ALA supplementation to reduce blood pressure [72–75] and to improve baroreflex sensitivity in rats with renovascular hypertension [76]. In line with our previous published data [20], Vasdev et al. [72] showed no effect of ALA (at 500 mg/kg feed for nine weeks) on body weight during supplementation in SHR rats. Contrary to our results, the ALA supplementation did attenuate hypertension measured by tail-cuff methodology [72], as was similarly reported by Midaoui and Champlain [77] in rats fed with the glucose solution and ALA (500 mg/kg feed). Concerning the mechanisms, they speculated that ALA increased the free sulfhydryl groups of calcium channels, inducing a decrease in cytosolic free calcium, vascular tone, and hypertension [72]. In addition, the authors postulated that the antihypertensive effects of ALA are associated with an attenuation of oxidative stress in the aortic vessel and the preservation of glutathione peroxidase activity in the plasma of glucose-treated rats [77].

Moreover, ALA was recognized to have antiviral effects by reducing not only oxidative stress but also the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) activation [78]. Besides, ALA increased the human host defense activating ATP-dependent K+ channels, and thus the increased K+ in the cell raised the intracellular pH. As a consequence, the entry of the virus into the cell decreased. For these reasons, ALA can increase human host defense against SARS-CoV-2 by increasing intracellular pH and reducing oxidative stress [78].

Even if this compound demonstrated a powerful antioxidant activity in vitro, a short half-life and a modest oral bioavailability were found in vivo [79]. Indeed, therapeutic efficacy is relatively low due to its pharmacokinetic limitation: hepatic degradation, reduced solubility, and gastric instability. However, ALA's liquid preparations and new amphiphilic matrices' formulations have significantly enhanced ALA bioavailability and, consequently, therapeutic efficacy [26]. Better pharmacokinetic parameters were found in the R-enantiomer of ALA compared to S-ALA [80]. According to other studies [25,81,82], we found greater effectiveness of (+)-ALA compared to (+/−)-ALA in reducing oxidative stress, demonstrated by the reduction of oxidized proteins levels, and by 8-oxo-dG expression, as well as cardiac and renal damages induced by hypertension. However, the limitation of our study is that we did not assess the effects of various forms of ALA on SHR renal and cardiac functions yet. Thus, further studies are necessary. The advantage in using (+)-ALA, as compared to the racemic form, may be linked to an amplified bioavailability and biological activity of this enantiomer that enhanced the antioxidant activity in SHR animals. The supplementation of lipoic acid as natural antioxidant has already been demonstrated to be safe in clinical trials [83], and to have multiple beneficial effects [57]. In particular, (+)-ALA showed the most pronounced activity, with extremely few acute and subchronic toxicities, compared to all the forms of ALA [84].

5. Conclusions

Gathering up data highlights the greater effectiveness of (+)-ALA compared to racemic form in reducing oxidative stress, cardiac and renal damages in SHR. To conclude, these findings propose (+)-ALA as one of the more appropriate antioxidant molecules for slowing down organ alterations associated with hypertension.

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References

1. Sievers, L.K.; Eckardt, K.U. Molecular Mechanisms of Kidney Injury and Repair in Arterial Hypertension. *Int. J. Mol. Sci.* **2019**, *20*, 2138. [CrossRef]
2. Sliwa, K.; Stewart, S.; Gersh, B.J. Hypertension: A global perspective. *Circulation* **2011**, *123*, 2892–2896. [CrossRef]
3. Messerli, F.H.; Rimoldi, S.F.; Bangalore, S. The Transition from Hypertension to Heart Failure: Contemporary Update. *JACC Heart Fail.* **2017**, *5*, 543–551. [CrossRef]
4. Bock, J.S.; Gottlieb, S.S. Cardiorenal syndrome: New perspectives. *Circulation* **2010**, *121*, 2592–2600. [CrossRef] [PubMed]
5. Hill, G.S. Hypertensive nephrosclerosis. *Curr. Opin. Nephrol. Hypertens.* **2008**, *17*, 266–270. [CrossRef] [PubMed]
6. Iliev, A.; Kotov, G.; Dimitrova, I.N.; Landzhov, B. Hypertension-induced changes in the rat myocardium during the development of cardiac hypertrophy—A comparison between the left and the right ventricle. *Acta Histochem.* **2019**, *121*, 16–28. [CrossRef] [PubMed]
7. Santos, M.; Shah, A.M. Alterations in cardiac structure and function in hypertension. *Curr. Hypertens. Rep.* **2014**, *16*, 428. [CrossRef]
8. Mennuni, S.; Rubattu, S.; Pierelli, G.; Tocci, G.; Fofi, C.; Volpe, M. Hypertension and kidneys: Unraveling complex molecular mechanisms underlying hypertensive renal damage. *J. Hum. Hypertens.* **2014**, *28*, 74–79. [CrossRef] [PubMed]
9. Eirin, A.; Lerman, A.; Lerman, L.O. Mitochondrial injury and dysfunction in hypertension-induced cardiac damage. *Eur. Heart J.* **2014**, *35*, 3258–3266. [CrossRef]
10. Rubattu, S.; Pagliaro, B.; Pierelli, G.; Santolamazza, C.; Di Castro, S.; Mennuni, S.; Volpe, M. Pathogenesis of target organ damage in hypertension: Role of mitochondrial oxidative stress. *Int. J. Mol. Sci.* **2014**, *16*, 823–839. [CrossRef] [PubMed]
11. González, J.; Valls, N.; Brito, R.; Rodrigo, R. Essential hypertension and oxidative stress: New insights. *World J. Cardiol.* **2014**, *6*, 353–366. [CrossRef]
12. Brito, R.; Castillo, G.; González, J.; Valls, N.; Rodrigo, R. Oxidative stress in hypertension: Mechanisms and therapeutic opportunities. *Exp. Clin. Endocrinol. Diabetes* **2015**, *123*, 325–335. [CrossRef]
13. Montezano, A.C.; Dulak-Lis, M.; Tsiropoulou, S.; Harvey, A.; Briones, A.M.; Touyz, R.M. Oxidative stress and human hypertension: Vascular mechanisms, biomarkers, and novel therapies. *Can. J. Cardiol.* **2015**, *31*, 631–641. [CrossRef]
14. Sorriento, D.; De Luca, N.; Trimarco, B.; Iaccarino, G. The Antioxidant Therapy: New Insights in the Treatment of Hypertension. *Front. Physiol.* **2018**, *9*, 258. [CrossRef]
15. Kizhakekuttu, T.J.; Widlansky, M.E. Natural antioxidants and hypertension: Promise and challenges. *Cardiovasc. Ther.* **2010**, *28*, e20–e32. [CrossRef] [PubMed]
16. Baradaran, A.; Nasri, H.; Rafieian-Kopaei, M. Oxidative stress and hypertension: Possibility of hypertension therapy with antioxidants. *J. Res. Med. Sci.* **2014**, *19*, 358–367. [PubMed]
17. Manning, R.D., Jr.; Tian, N.; Meng, S. Oxidative stress and antioxidant treatment in hypertension and the associated renal damage. *Am. J. Nephrol.* **2005**, *25*, 311–317. [CrossRef] [PubMed]
18. Rafiq, K.; Nishiyama, A. Is antioxidant therapy effective for advanced hypertension and renal injury? *J. Hypertens.* **2014**, *32*, 475–476. [CrossRef]
19. Zanchetti, A. Challenges in hypertension: Prevalence, definition, mechanisms and management. *J. Hypertens.* **2014**, *32*, 451–453. [CrossRef] [PubMed]
20. Tayebati, S.K.; Tomassoni, D.; Di Cesare Mannelli, L.; Amenta, F. Effect of treatment with the antioxidant alpha-lipoic (thioctic) acid on heart and kidney microvasculature in spontaneously hypertensive rats. *Clin. Exp. Hypertens.* **2016**, *38*, 30–38. [CrossRef] [PubMed]
21. Okamoto, K.; Tabei, R.; Yamori, Y.; Ooshima, A. Spontaneously hypertensive rat as a useful model for hypertension research. *Jikken Dobutsu.* **1973**, *22*, 289–298.
22. Taddei, S.; Virdis, A.; Ghiadoni, L.; Salvetti, G.; Bernini, G.; Magagna, A.; Salvetti, A. Age-related reduction of NO availability and oxidative stress in humans. *Hypertension* **2001**, *38*, 274–279. [CrossRef] [PubMed]

23. Zhang, J.; Fallahzadeh, M.K.; McCullough, P.A. Aging Male Spontaneously Hypertensive Rat as an Animal Model for the Evaluation of the Interplay between Contrast-Induced Acute Kidney Injury and Cardiorenal Syndrome in Humans. *Cardiorenal. Med.* **2016**, *7*, 1–10. [CrossRef]
24. Tomassoni, D.; Amenta, F.; Di Cesare Mannelli, L.; Ghelardini, C.; Nwankwo, I.E.; Pacini, A.; Tayebati, S.K. Neuroprotective activity of thioctic acid in central nervous system lesions consequent to peripheral nerve injury. *Biomed. Res. Int.* **2013**, *2013*, 985093. [CrossRef] [PubMed]
25. Pacini, A.; Tomassoni, D.; Trallori, E.; Micheli, L.; Amenta, F.; Ghelardini, C.; Di Cesare Mannelli, L.; Traini, E. Comparative Assessment of the Activity of Racemic and Dextrorotatory Forms of Thioctic (Alpha-Lipoic) Acid in Low Back Pain: Preclinical Results and Clinical Evidences from an Open Randomized Trial. *Front. Pharmacol.* **2021**, *12*, 607572. [CrossRef]
26. Salehi, B.; Yılmaz, Y.B.; Antika, G.; Tumer, T.B.; Mahomoodally, M.F.; Lobine, D.; Akram, M.; Riaz, M.; Capanoglu, E.; Sharopov, F.; et al. Insights on the Use of α-Lipoic Acid for Therapeutic Purposes. *Biomolecules* **2019**, *9*, 356. [CrossRef]
27. Tayebati, S.K.; Tomassoni, D.; Amenta, F. Spontaneously hypertensive rat as a model of vascular brain disorder: Microanatomy, neurochemistry and behavior. *J. Neurol. Sci.* **2012**, *322*, 241–249. [CrossRef] [PubMed]
28. Raij, L.; Azar, S.; Keane, W. Mesangial immune injury, hypertension, and progressive glomerular damage in Dahl rats. *Kidney Int.* **1984**, *26*, 137–143. [CrossRef]
29. Komatsu, K.; Frohlich, E.D.; Ono, H.; Ono, Y.; Numabe, A.; Willis, G.W. Glomerular dynamics and morphology of aged spontaneously hypertensive rats. Effect of angiotesin-converting enzyme inhibition. *Hypertension* **1995**, *25*, 207–213. [CrossRef] [PubMed]
30. Sabbatini, M.; Leonardi, A.; Testa, R.; Vitaioli, L.; Amenta, F. Effect of calcium antagonists on glomerular arterioles in spontaneously hypertensive rats. *Hypertension* **2000**, *35*, 775–779. [CrossRef]
31. Chen, J.; Chen, J.K.; Conway, E.M.; Harris, R.C. Survivin mediates renal proximal tubule recovery from AKI. *J. Am. Soc. Nephrol.* **2013**, *24*, 2023–2033. [CrossRef]
32. Martinelli, I.; Tomassoni, D.; Moruzzi, M.; Roy, P.; Cifani, C.; Amenta, F.; Tayebati, S.K. Cardiovascular Changes Related to Metabolic Syndrome: Evidence in Obese Zucker Rats. *Int. J. Mol. Sci.* **2020**, *21*, 2035. [CrossRef]
33. Muñoz-Durango, N.; Fuentes, C.A.; Castillo, A.E.; González-Gómez, L.M.; Vecchiola, A.; Fardella, C.E.; Kalergis, A.M. Role of the Renin-Angiotensin-Aldosterone System beyond Blood Pressure Regulation: Molecular and Cellular Mechanisms Involved in End-Organ Damage during Arterial Hypertension. *Int. J. Mol. Sci.* **2016**, *17*, 797. [CrossRef] [PubMed]
34. Pinto, Y.M.; Paul, M.; Ganten, D. Lessons from rat models of hypertension: From Goldblatt to genetic engineering. *Cardiovasc. Res.* **1998**, *39*, 77–88. [CrossRef]
35. Lerman, L.O.; Kurtz, T.W.; Touyz, R.M.; Ellison, D.H.; Chade, A.R.; Crowley, S.D.; Mattson, D.L.; Mullins, J.J.; Osborn, J.; Eirin, A.; et al. Animal Models of Hypertension: A Scientific Statement from the American Heart Association. *Hypertension* **2019**, *73*, e87–e120. [CrossRef] [PubMed]
36. Sabbatini, M.; Vitaioli, L.; Baldoni, E.; Amenta, F. Nephroprotective effect of treatment with calcium channel blockers in spontaneously hypertensive rats. *J. Pharmacol. Exp. Ther.* **2000**, *294*, 948–954. [PubMed]
37. Sabbatini, M.; Leonardi, A.; Testa, R.; Tomassoni, D.; Vitaioli, L.; Amenta, F. Effects of dihydropyridine-type Ca2+ antagonists on the renal arterial tree in spontaneously hypertensive rats. *J. Cardiovasc. Pharmacol.* **2002**, *39*, 39–48. [CrossRef] [PubMed]
38. Amenta, F.; Peleg, E.; Tomassoni, D.; Sabbatini, M.; Rosenthal, T. Effect of treatment with lercanidipine on heart of Cohen-Rosenthal diabetic hypertensive rats. *Hypertension* **2003**, *41*, 330–335. [CrossRef]
39. Shimizu, I.; Minamino, T. Physiological and pathological cardiac hypertrophy. *J. Mol. Cell. Cardiol.* **2016**, *97*, 245–262. [CrossRef]
40. Deng, X.U.; Xia, K.E.; Chen, P.O.; Sheikh, M.S.A.; Yang, D.F.; Li, S.M.; Yang, T.L. Reversion of left ventricle remodeling in spontaneously hypertensive rats by valsartan is associated with the inhibition of caspase-3, -8 and -9 activities. *Biomed. Rep.* **2015**, *3*, 533–536. [CrossRef]
41. Wang, Q.; Cui, Y.; Lin, N.; Pang, S. Correlation of cardiomyocyte apoptosis with duration of hypertension, severity of hypertension and caspase-3 expression in hypertensive rats. *Exp. Ther. Med.* **2019**, *17*, 2741–2745. [CrossRef]
42. Loperena, R.; Harrison, D.G. Oxidative Stress and Hypertensive Diseases. *Med. Clin. N. Am.* **2017**, *101*, 169–193. [CrossRef] [PubMed]
43. Paravicini, T.M.; Touyz, R.M. NADPH oxidases, reactive oxygen species, and hypertension: Clinical implications and therapeutic possibilities. *Diabetes Care* **2008**, *31*, S170–S180. [CrossRef] [PubMed]
44. Sprague, A.H.; Khalil, R.A. Inflammatory cytokines in vascular dysfunction and vascular disease. *Biochem. Pharmacol.* **2009**, *78*, 539–552. [CrossRef]
45. Touyz, R.M. Reactive oxygen species, vascular oxidative stress, and redox signaling in hypertension: What is the clinical significance? *Hypertension* **2004**, *44*, 248–252. [CrossRef] [PubMed]
46. Dinh, Q.N.; Drummond, G.R.; Sobey, C.G.; Chrissobolis, S. Roles of inflammation, oxidative stress, and vascular dysfunction in hypertension. *Biomed. Res. Int.* **2014**, *2014*, 406960. [CrossRef] [PubMed]
47. Govender, M.M.; Nadar, A. A subpressor dose of angiotensin II elevates blood pressure in a normotensive rat model by oxidative stress. *Physiol. Res.* **2015**, *64*, 153–159. [CrossRef]
48. Lassègue, B.; Griendling, K.K. Reactive oxygen species in hypertension; An update. *Am. J. Hypertens.* **2004**, *17*, 852–860. [CrossRef]
49. Lopez-Ruiz, A.; Sartori-Valinotti, J.; Yanes, L.L.; Iliescu, R.; Reckelhoff, J.F. Sex differences in control of blood pressure: Role of oxidative stress in hypertension in females. *Am. J. Physiol. Heart Circ. Physiol.* **2008**, *295*, H466–H474. [CrossRef]

50. Reckelhoff, J.F. Gender differences in the regulation of blood pressure. *Hypertension* **2001**, *37*, 1199–1208. [CrossRef]
51. Hsu, C.N.; Tain, Y.L. Early Origins of Hypertension: Should Prevention Start Before Birth Using Natural Antioxidants? *Antioxidants* **2020**, *9*, 1034. [CrossRef] [PubMed]
52. Parikh, A.; Lipsitz, S.R.; Natarajan, S. Association between a DASH-like diet and mortality in adults with hypertension: Findings from a population-based follow-up study. *Am. J. Hypertens.* **2009**, *22*, 9–16. [CrossRef]
53. Mullan, B.A.; Young, I.S.; Fee, H.; McCance, D.R. Ascorbic acid reduces blood pressure and arterial stiffness in type 2 diabetes. *Hypertension* **2002**, *40*, 804–809. [CrossRef] [PubMed]
54. Czernichow, S.; Bertrais, S.; Blacher, J.; Galan, P.; Briançon, S.; Favier, A.; Safar, M.; Hercberg, S. Effect of supplementation with antioxidants upon long-term risk of hypertension in the SU.VI.MAX study: Association with plasma antioxidant levels. *J. Hypertens.* **2005**, *23*, 2013–2018. [CrossRef] [PubMed]
55. Smith, A.R.; Shenvi, S.V.; Widlansky, M.; Suh, J.H.; Hagen, T.M. Lipoic acid as a potential therapy for chronic diseases associated with oxidative stress. *Curr. Med. Chem.* **2004**, *11*, 1135–1146. [CrossRef]
56. Shay, K.P.; Moreau, R.F.; Smith, E.J.; Smith, A.R.; Hagen, T.M. Alpha-lipoic acid as a dietary supplement: Molecular mechanisms and therapeutic potential. *Biochim. Biophys. Acta* **2009**, *1790*, 1149–1160. [CrossRef]
57. Tibullo, D.; Li Volti, G.; Giallongo, C.; Grasso, S.; Tomassoni, D.; Anfuso, C.D.; Lupo, G.; Amenta, F.; Avola, R.; Bramanti, V. Biochemical and clinical relevance of alpha lipoic acid: Antioxidant and anti-inflammatory activity, molecular pathways and therapeutic potential. *Inflamm. Res.* **2017**, *66*, 947–959. [CrossRef]
58. Biewenga, G.P.; Haenen, G.R.; Bast, A. The pharmacology of the antioxidant lipoic acid. *Gen. Pharmacol.* **1997**, *29*, 315–331. [CrossRef]
59. Camiolo, G.; Tibullo, D.; Giallongo, C.; Romano, A.; Parrinello, N.L.; Musumeci, G.; Di Rosa, M.; Vicario, N.; Brundo, M.V.; Amenta, F.; et al. α-Lipoic Acid Reduces Iron-induced Toxicity and Oxidative Stress in a Model of Iron Overload. *Int. J. Mol. Sci.* **2019**, *20*, 609. [CrossRef]
60. Zhang, W.J.; Frei, B. Alpha-lipoic acid inhibits TNF-alpha-induced NF-kappaB activation and adhesion molecule expression in human aortic endothelial cells. *FASEB J.* **2001**, *15*, 2423–2432. [CrossRef]
61. Rochette, L.; Ghibu, S.; Muresan, A.; Vergely, C. Alpha-lipoic acid: Molecular mechanisms and therapeutic potential in diabetes. *Can J. Physiol. Pharmacol.* **2015**, *93*, 1021–1027. [CrossRef]
62. Zhang, J.; McCullough, P.A. Lipoic Acid in the Prevention of Acute Kidney Injury. *Nephron* **2016**, *134*, 133–140. [CrossRef]
63. Takaoka, M.; Ohkita, M.; Kobayashi, Y.; Yuba, M.; Matsumura, Y. Protective effect of alpha-lipoic acid against ischaemic acute renal failure in rats. *Clin. Exp. Pharmacol. Physiol.* **2002**, *29*, 189–194. [CrossRef] [PubMed]
64. Abdel-Zaher, A.O.; Abdel-Hady, R.H.; Mahmoud, M.M.; Farrag, M.M.Y. The potential protective role of alpha-lipoic acid against acetaminophen-induced hepatic and renal damage. *Toxicology* **2008**, *243*, 261–270. [CrossRef] [PubMed]
65. Lee, Y.M.; Bae, S.Y.; Won, N.H.; Pyo, H.J.; Kwon, Y.J. Alpha-lipoic acid attenuates cisplatin-induced tubulointerstitial injuries through inhibition of mitochondrial bax translocation in rats. *Nephron. Exp. Nephrol.* **2009**, *113*, e104–e112. [CrossRef]
66. Armagan, I.; Bayram, D.; Candan, I.A.; Yigit, A.; Celik, E.; Armagan, H.H.; Uğuz, A.C. Effects of pentoxifylline and alpha lipoic acid on methotrexate-induced damage in liver and kidney of rats. *Environ. Toxicol. Pharmacol.* **2015**, *39*, 1122–1131. [CrossRef] [PubMed]
67. Cho, H.S.; Kim, J.H.; Jang, H.N.; Lee, T.W.; Jung, M.H.; Kim, T.H.; Chang, S.H.; Park, D.J. Alpha-lipoic acid ameliorates the epithelial mesenchymal transition induced by unilateral ureteral obstruction in mice. *Sci. Rep.* **2017**, *7*, 46065. [CrossRef] [PubMed]
68. Govindasamy, C.; Sirajudeen, K.N.S. Effect of alpha-lipoic acid supplementation on blood pressure, renal oxidant-antioxidant status and renal damage in spontaneously hypertensive rats. *Asian Pac. J. Trop. Biomed.* **2019**, *9*, 415–423. [CrossRef]
69. Lai, S.; Petramala, L.; Muscaritoli, M.; Cianci, R.; Mazzaferro, S.; Mitterhofer, A.P.; Pasquali, M.; D'Ambrosio, V.; Carta, M.; Ansuini, M.; et al. α-lipoic acid in patients with autosomal dominant polycystic kidney disease. *Nutrition* **2020**, *71*, 110594. [CrossRef]
70. Cheserek, M.J.; Wu, G.; Li, L.; Li, L.; Karangwa, E.; Shi, Y.; Le, G. Cardioprotective effects of lipoic acid, quercetin and resveratrol on oxidative stress related to thyroid hormone alterations in long-term obesity. *J. Nutr. Biochem.* **2016**, *33*, 36–44. [CrossRef]
71. Pop, C.; Ștefan, M.G.; Muntean, D.M.; Stoicescu, L.; Gal, A.F.; Kiss, B.; Morgovan, C.; Loghin, F.; Rochette, L.; Lauzier, B.; et al. Protective Effects of a Discontinuous Treatment with Alpha-Lipoic Acid in Obesity-Related Heart Failure with Preserved Ejection Fraction, in Rats. *Antioxidants* **2020**, *9*, 1073. [CrossRef]
72. Vasdev, S.; Ford, C.A.; Parai, S.; Longerich, L.; Gadag, V. Dietary alpha-lipoic acid supplementation lowers blood pressure in spontaneously hypertensive rats. *J. Hypertens.* **2000**, *18*, 567–573. [CrossRef]
73. Koçak, G.; Aktan, F.; Canbolat, O.; Ozoğul, C.; Elbeğ, S.; Yildizoglu-Ari, N.; Karasu, C.; ADIC Study Group—Antioxidants in Diabetes-Induced Complications. Alpha-lipoic acid treatment ameliorates metabolic parameters, blood pressure, vascular reactivity and morphology of vessels already damaged by streptozotocin-diabetes. *Diabetes Nutr. Metab.* **2000**, *13*, 308–318.
74. Takaoka, M.; Kobayashi, Y.; Yuba, M.; Ohkita, M.; Matsumura, Y. Effects of alpha-lipoic acid on deoxycorticosterone acetate-salt-induced hypertension in rats. *Eur. J. Pharmacol.* **2001**, *424*, 121–129. [CrossRef]
75. Dudek, M.; Razny, K.; Bilska-Wilkosz, A.; Iciek, M.; Sapa, J.; Wlodek, L.; Filipek, B. Hypotensive effect of alpha-lipoic acid after a single administration in rats. *Anatol. J. Cardiol.* **2016**, *16*, 306–309. [CrossRef]

76. Queiroz, T.M.; Guimarães, D.D.; Mendes-Junior, L.G.; Braga, V.A. α-lipoic acid reduces hypertension and increases baroreflex sensitivity in renovascular hypertensive rats. *Molecules* **2012**, *17*, 13357–13367. [CrossRef]
77. Midaoui, A.E.; de Champlain, J. Prevention of hypertension, insulin resistance, and oxidative stress by alpha-lipoic acid. *Hypertension* **2002**, *39*, 303–307. [CrossRef]
78. Cure, E.; Cure, M.C. Alpha-lipoic acid may protect patients with diabetes against COVID-19 infection. *Med. Hypotheses.* **2020**, *143*, 110185. [CrossRef]
79. Teichert, J.; Kern, J.; Tritschler, H.J.; Ulrich, H.; Preiss, R. Investigations on the pharmacokinetics of alpha-lipoic acid in healthy volunteers. *Int. J. Clin. Pharmacol. Ther.* **1998**, *36*, 625–628.
80. Brufani, M.; Figliola, R. (R)-α-lipoic acid oral liquid formulation: Pharmacokinetic parameters and therapeutic efficacy. *Acta Biomed.* **2014**, *85*, 108–115. [PubMed]
81. Maglione, E.; Marrese, C.; Migliaro, E.; Marcuccio, F.; Panico, C.; Salvati, C.; Citro, G.; Quercio, M.; Roncagliolo, F.; Torello, C.; et al. Increasing bioavailability of (R)-alpha-lipoic acid to boost antioxidant activity in the treatment of neuropathic pain. *Acta Biomed.* **2015**, *86*, 226–233.
82. Mrakic-Sposta, S.; Vezzoli, A.; Maderna, L.; Gregorini, F.; Montorsi, M.; Moretti, S.; Greco, F.; Cova, E.; Gussoni, M. R(+)-Thioctic Acid Effects on Oxidative Stress and Peripheral Neuropathy in Type II Diabetic Patients: Preliminary Results by Electron Paramagnetic Resonance and Electroneurography. *Oxid. Med. Cell. Longev.* **2018**, *2018*, 1767265. [CrossRef] [PubMed]
83. Fogacci, F.; Rizzo, M.; Krogager, C.; Kennedy, C.; Georges, C.M.G.; Knežević, T.; Liberopoulos, E.; Vallée, A.; Pérez-Martínez, P.; Wenstedt, E.F.E.; et al. Safety Evaluation of α-Lipoic Acid Supplementation: A Systematic Review and Meta-Analysis of Randomized Placebo-Controlled Clinical Studies. *Antioxidants* **2020**, *9*, 1011. [CrossRef]
84. Lucarini, E.; Trallori, E.; Tomassoni, D.; Amenta, F.; Ghelardini, C.; Pacini, A.; Di Cesare Mannelli, L. Toxicological Profile of the Pain-Relieving Antioxidant Compound Thioctic Acid in Its Racemic and Enantiomeric Forms. *Antioxidants* **2020**, *9*, 749. [CrossRef]

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Anti-Inflammatory and Antioxidant Properties of Tart Cherry Consumption in the Heart of Obese Rats

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Simple Summary: Obesity is a well-known condition responsible for being a risk factor for cardiovascular disease progression. The intake of bioactive phytochemicals, contained in red fruits, is attracting great attention since their benefits have been attributed mostly to their possible antioxidant properties. We aimed to assess the potential effects from the daily supplementation of tart cherries, both seeds and juice, in obese animals. Our results showed that tart cherries reduced oxidative stress and mitigated the inflammation in the hearts of obese rats. Indeed, we propose this fruit in the prevention of cardiovascular diseases related to obesity.

Abstract: Obesity is a risk factor for cardiovascular diseases, frequently related to oxidative stress and inflammation. Dietary antioxidant compounds improve heart health. Here, we estimate the oxidative grade and inflammation in the heart of dietary-induced obese (DIO) rats after exposure to a high-fat diet compared to a standard diet. The effects of tart cherry seed powder and seed powder plus tart cherries juice were explored. Morphological analysis and protein expressions were performed in the heart. The oxidative status was assessed by the measurement of protein oxidation and 4-hydroxynonenal in samples. Immunochemical and Western blot assays were performed to elucidate the involved inflammatory markers as proinflammatory cytokines and cellular adhesion molecules. In the obese rats, cardiomyocyte hypertrophy was accompanied by an increase in oxidative state proteins and lipid peroxidation. However, the intake of tart cherries significantly changed these parameters. An anti-inflammatory effect was raised from tart cherry consumption, as shown by the downregulation of analyzed endothelial cell adhesion molecules and cytokines compared to controls. Tart cherry intake should be recommended as a dietary supplement to prevent or counteract heart injury in obese conditions.

Keywords: obesity; heart; tart cherries; inflammation; oxidative stress; cardiovascular diseases

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1. Introduction

Most evidence supports a connection between obesity and cardiovascular diseases (CVD), including coronary heart disease, heart failure, hypertension, stroke, atrial fibrillation, and sudden cardiac death [1–3]. The cardiovascular system is structurally and functionally modified to accommodate excess body weight. Consequently, the increase in dysregulated adipokine secretion enhances inflammation and perturbates vascular homeostasis. Insulin resistance, hyperglycemia, hypertension, and dyslipidemia are recognized

as concomitant risk factors in the association between obesity and CVD, and the consequences are often attributed to pro-inflammatory and pro-thrombotic conditions as well as endothelial dysfunction and platelet activation [2–5].

It has been found that feeding a diet rich in fat and carbohydrates leads to significant oxidative stress and inflammation in obese subjects [6]. Concerning the mechanisms in obese persons, the adipose tissue secretion of adipokines and cytokines or chemokines is dysregulated. These bioactive molecules participate in the regulation of appetite and energy homeostasis, lipid metabolism (tumor necrosis factor-alpha, TNF-α), insulin sensitivity (TNF-α, adiponectin, resistin, visfatin), immunity (TNF-α, monocyte chemoattractant protein-1, MCP-1 and interleukin-6, IL-6), angiogenesis, blood pressure, and hemostasis (plasminogen activator inhibitor, PAI-1) [4,7,8]. Additionally, in obesity, chronic low-grade of inflammation is a central source of oxidative stress. Mitochondrial dysfunction leads to the alteration of free radical production and fatty acid oxidation; both have been implicated in the pathogenesis of obesity and its associated risk factors [9,10]. Therefore, a diet rich in antioxidants protects the cell from free radical injury by counteracting and scavenging them [11]. For instance, cherries contain different polyphenolic compounds that have a beneficial impact on human health [12].

Considerable interest has been shown in diets enriched with natural bioactive substances and their capacity for preserving or improving cardiovascular health [13,14]. High consumption of vegetables and fruits has been directly connected with a reduced incidence of CVD [15], mostly due to the abundance and variability of bioactive composites within. Among them, anthocyanins (members of the flavonoid group) have emerged as beneficial in animal and human studies [16,17]. As reviewed by Mazza [18], many studies have revealed that anthocyanins show an extensive variety of biological actions, such as antioxidant [19,20], anti-inflammatory [21,22], and anti-carcinogenic activities [23]; induction of apoptosis [24]; and neuroprotective effects [25,26]. Moreover, anthocyanins show a diversity of properties on blood vessels [27,28] and platelets [29] that may diminish the incidence of coronary heart disease [30]. Interestingly, anthocyanins may decrease the cardiovascular risk associated with endothelial dysfunction and inflammatory responses to a typical high-fat "Western" meal [31].

Because obesity is characterized by both a chronic state of oxidative stress and low-grade inflammation, here we explored the cardiac potential alterations in a diet-induced obesity (DIO) animal model, in which rats were fed a high-fat diet (HFD) and then the influence of tart cherry seed and juice intake were detected, assessing oxidative stress and inflammatory markers.

2. Materials and Methods

2.1. Animal and Blood Parameters

The cardiac samples were collected from the same male Wistar rats (n = 44; 225–250 g) described in the paper by Micioni Di Bonaventura et al. [26]. Institutional Guidelines, conformed with the Italian Ministry of Health (protocol number 1610/2013) and associated guidelines from the European Communities Council Directive were followed. Animals were divided into: CHOW rats (n = 8, standard diet, 7% fat) and DIO rats (n = 36, HFD, 45% fat) [26]. In the DIO group, 6 rats were excluded because they were resistant [26]. The effects of *Prunus cerasus* L. supplementation were assessed in DIO animals, and the concentration of anthocyanins tested, as well as the preparation of seed powder and juice from tart cherries, were previously detailed [26,32]. The composition of both juice and seeds has been already cited elsewhere [32]. In the paper by Cocci et al. [32], the fatty acid composition of seeds was assessed. After 17 weeks of HFD, animals were sacrificed, and heart weights were recorded. Blood parameters were reported previously as well as systolic blood pressure [26,32–36]. DS and DJS groups presented a decrease in systolic blood pressure in comparison with DIO rats. The consumption of tart cherry counteracted only the hyperglycemia but not the hyperinsulinemia. Moreover, the *Prunus cerasus* L. diminished the triglyceride levels compared to the DIO control rats [26,32–34].

2.2. Morphological Aspects

After tissues were excised, hearts were fixed in 4% paraformaldehyde; after they were dehydrated by graded alcohols and embedded in paraffin. These samples were cut using the microtome to prepare longitudinal tissue sections 8 µm-thick. Sections were deparaffinized immersing in xylene, and rehydrated through graded alcohols, followed by staining of hematoxylin and eosin (Diapath S.p.A., Martinengo, BG, Italy, Ref. 010263), silver impregnation (Diapath S.p.A., Martinengo, BG, Italy, Ref. 010211), and Masson's trichrome (Diapath S.p.A., Martinengo, BG, Italy, Ref. 010210). Cardiomyocyte cross-sectional area and fibrosis were measured, as described previously [37,38].

2.3. Western Blot and Quantification

For Western blots (WB), cardiac samples were lysed in lysis buffer, whose composition has been already detailed in [38]. After centrifugation, the supernatants were collected. Proteins were measured, separated by 8–12% SDS-PAGE, and transferred to a nitrocellulose membrane. Membranes were probed with the indicated antibodies, including anti-intracellular adhesion molecule-1 (ICAM-1), anti-vascular cell adhesion molecule-1 (VCAM-1), anti-platelet endothelial cell adhesion molecule-1 (PECAM-1), anti-endothelial-leukocyte adhesion molecule-1 (E-selectin), anti-nuclear factor kappa-light-chain-enhancer of activated B cells subunit p50 (NF-κB p50), anti-TNF-α, anti-interleukin-1β (IL-1β), anti-IL-6 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and anti-caspase-3 (9662, Cell Signaling Technology, Danvers, MA, USA) at 4 °C overnight. β-actin (A2228, Sigma-Aldrich Co., St. Louis, MO, USA) was used as loading control. Optimal antibodies concentration was previously established [26,38]. After incubation with horseradish-peroxidase (HRP)-conjugated secondary antibodies (Bethyl Laboratories, Inc., Montgomery, TX, USA), followed by enhanced chemiluminescence (ECL) method, protein signals were measured. The densitometric analysis of bands were performed [38]. Finally, anti 4-Hydroxynonenal antibody (4-HNE) (sc-130083, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was used to assess the lipid peroxidation in cardiac homogenates. Moreover, the protein carbonyl levels were analyzed using Oxyblot kit, as detailed [38]. For 4-HNE and oxyblot, the images were quantified by measuring the intensity of the whole protein lane.

2.4. Immunohistochemistry and Image Analysis

Cardiac sections were processed for immunohistochemistry (IHC) analysis, as previously described [38]. The primary antibodies used for WB, were also incubated for IHC in tissues sections overnight at 4 °C: anti-ICAM-1 (sc-8439), anti-VCAM-1 (sc-8304), anti-PECAM-1 (sc-1506), anti-E-selectin (sc-14011), anti-TNF-α (sc-52746), anti-IL-1β (sc-7884), and anti-IL-6 (sc-1265) (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Optimal antibodies concentration has already been established [38]. The sections were incubated in biotinylated secondary antibody (Bethyl Laboratories, Inc., Montgomery, TX, USA) and then with VECTASTAIN ABC HRP kit, according to the manufacturer's protocol. 3,3′-diaminobenzidine tetrahydrochloride (DAB) substrate was then applied on the sections. Both these kits were purchased by Vector Laboratories (Burlingame, CA, USA). Finally, slides were counterstained with hematoxylin, and observed under a light microscope. Cardiac pictures were captured at 40×. The mean intensity of immunostaining was recorded as previously described [26].

2.5. Immunofluorescence and Quantification

For confocal microscopy, the sections were incubated with antibody specific for NF-κB (p50) (sc-114, Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The secondary antibody was conjugated with Alexa Fluor 594 (red) and sections were counterstained with DAPI. Slides were observed with confocal microscope (Nikon, Corporation, Tokyo, Japan). Pictures were captured and the percentage of NF-κB (p50) positive area was measured with Nikon NIS Element software.

2.6. Data Analysis

The statistical significance of the differences was performed with GraphPad Prism program (version 8.0) by analysis of variance (ANOVA) followed by Tukey's post hoc test. Data represented as mean ± standard error of mean (SEM). Statistical significance was set at $p < 0.05$.

3. Results

3.1. Weight and Heart Morphology

Tart cherry seeds and juice consumption did not alter body weight or feeding performance [26,33,34]. Heart weights were measured, and no remarkable differences were found among the different experimental groups (Figure 1A). Morphological measures of the myocardium performed on the sections stained with hematoxylin and eosin exhibited an increase in the ventricular cardiomyocyte area of DIO animals compared to CHOW rats (Figure 1B,C). The consumption of tart cherries restored the area of cardiomyocytes (Figure 1B,C).

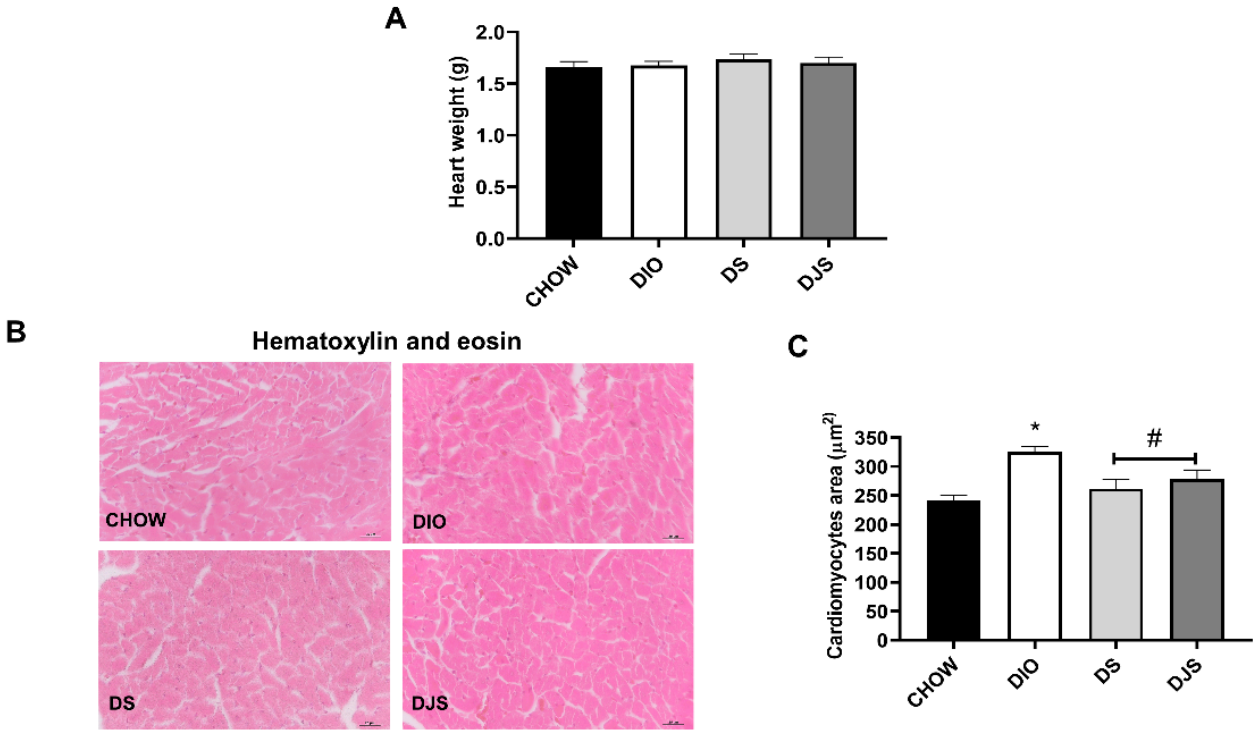

Figure 1. Heart weight and assessment of cardiomyocyte hypertrophy. (**A**) Whole heart weight; (**B**,**C**) Representative pictures of hematoxylin and eosin staining and quantification of cardiomyocyte cross-sectional area CHOW rats ($n = 8$), fed with standard diet; DIO rats ($n = 9$), fed with high-fat diet; DS ($n = 12$), DIO rats supplemented with tart cherry seeds; DJS ($n = 9$), DS rats supplemented with tart cherry juice. Magnification 40×. Scale bar 25 μm. Data are mean ± SEM. * $p < 0.05$ vs. CHOW rats; # $p < 0.05$ vs. DIO rats.

In cardiac sections of DIO rats, processed with the silver impregnation, a slight increase in the reticulin fibers was evident (Figure 2A). The quantification of the silver-stained area, expressed as a percentage, demonstrated no differences among the groups (Figure 2B). Collagen and extracellular matrix were not increased at the level of subendocardial area of the obese rats as showed in Masson's trichrome stained sections (Figure 2C). The amounts of fibrosis were low in all the animal groups without differences between the groups (Figure 2D).

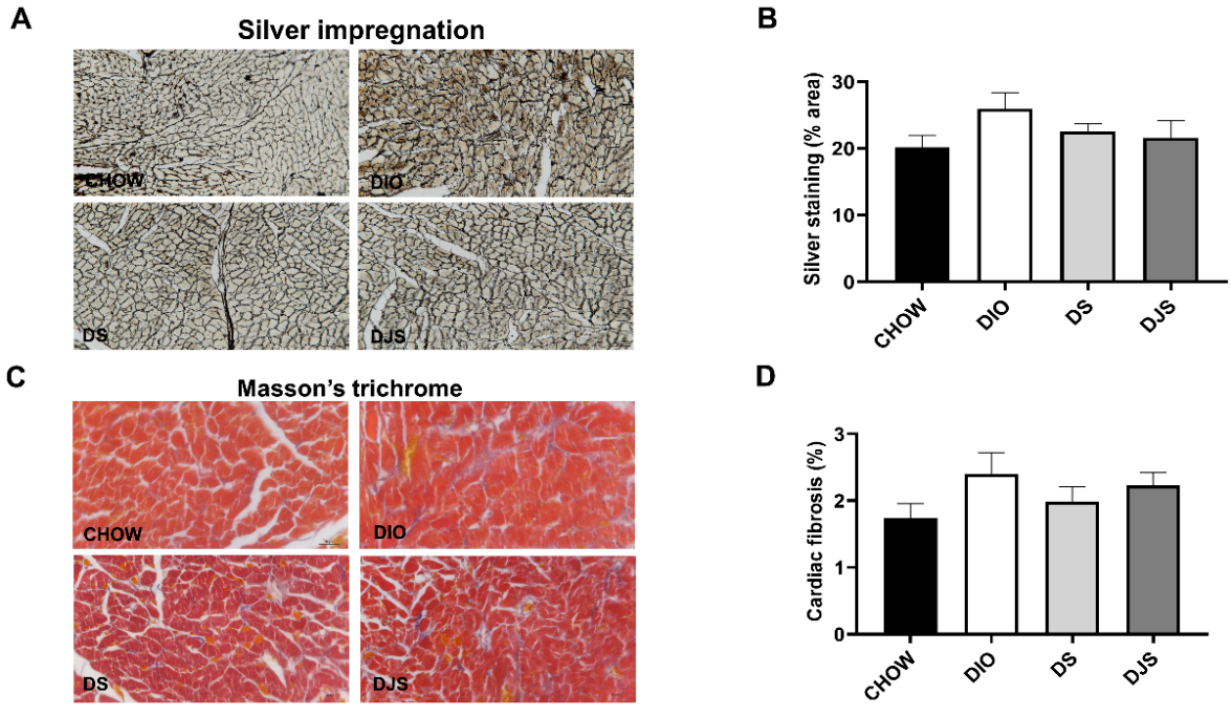

Figure 2. Assessment of cardiac fibrosis. (**A**,**B**) Representative pictures of silver impregnation stained cardiac sections and quantification of silver staining area. Magnification 20×. Scale bar 50 µm; (**C**,**D**) Representative pictures of Masson's trichrome staining and quantification of fibrosis. Magnification 40×. Scale bar 25 µm. CHOW rats (n = 8), fed with standard diet; DIO rats (n = 9), fed with high-fat diet; DS (n = 12), DIO rats supplemented with tart cherry seeds; DJS (n = 9), DS rats supplemented with tart cherry juice. Data are mean ± SEM.

3.2. Oxidative Stress and Apoptosis

Previously, a decrease in oxidative stress was reported with the intake of tart cherries in the serum and liver of obese rats [33]. In accordance, the quantification of oxyblot assay and the WB analysis for 4-HNE in heart homogenates demonstrated an increase in oxidation state of proteins and lipid peroxidation, respectively, in DIO samples compared to control rats (Figure 3A,B). Furthermore, the tart cherries were able to inhibit both these conditions (Figure 3A,B). In addition, we investigated the potential modulation of apoptosis induced by diet and tart cherry supplementation. The data showed no difference in the full-length caspase-3 (35 kDa) levels, without cleaved caspase-3 (17 kDa) expression in all the samples (Figure 3C). In Supplementary Figure S1, we showed the full-length WB gels (Figure S1A–C).

3.3. Inflammation

As the NF-κB is a transcription factor responsible for triggering the immune response, and the most prevalent activated form of NF-κB is a heterodimer, consisting of a p50 or p52, its expression was investigated. Moreover, ICAM-1, VCAM-1, PECAM-1, E-selectin, TNF-α, IL-1β, and IL-6 levels were studied since NF-κB activation increases the expression of the adhesion molecules as well as the production of pro-inflammatory cytokines [39]. As shown in WB (Figure 4A) and immunofluorescence quantification (Figure 4B), a substantial upregulation of this transcription factor was reported in DIO rats, and those levels were remarkably lowered, both in DS and in DJS rats. We showed the full-length WB of NF-κB in Figure S1D.

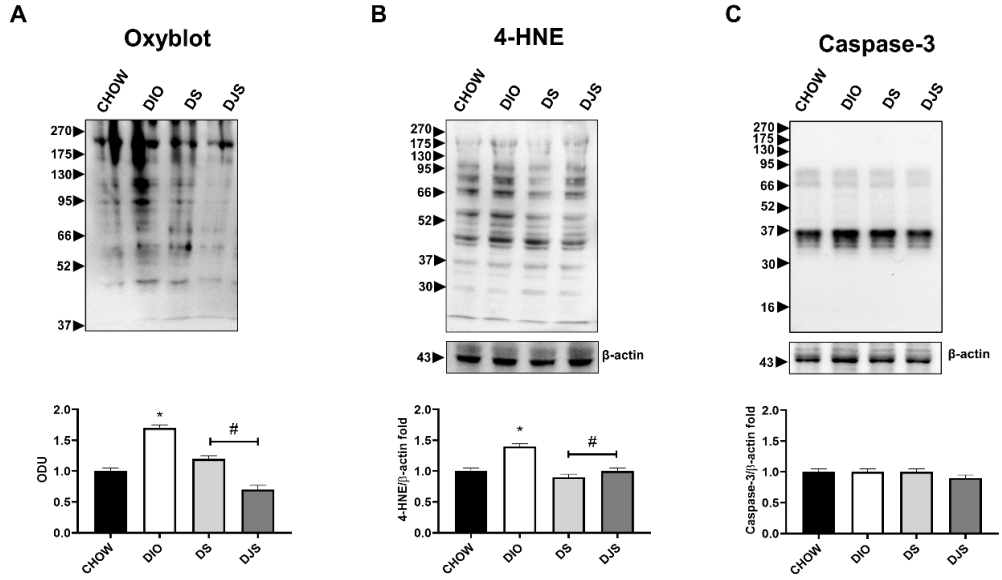

Figure 3. Oxidative stress and apoptosis. (**A**) Oxyblot in cardiac samples and the graph shows the measurements of optical density expressed as arbitrary optical density unit (ODU). Cardiac lysates were immunoblotted with anti-4-Hydroxynonenal (4-HNE) (**B**) and anti-Caspase-3 (**C**). Graphs show the densitometric ratios of bands and β-actin expression, used to normalize the data. CHOW rats (n = 8), reference group fed with standard diet; DIO rats (n = 9), fed with high-fat diet; DS (n = 12), DIO rats supplemented with tart cherry seeds; DJS (n = 9), DS rats supplemented with tart cherry juice. Data are mean ± SEM. * $p < 0.05$ vs. CHOW rats; # $p < 0.05$ vs. DIO rats.

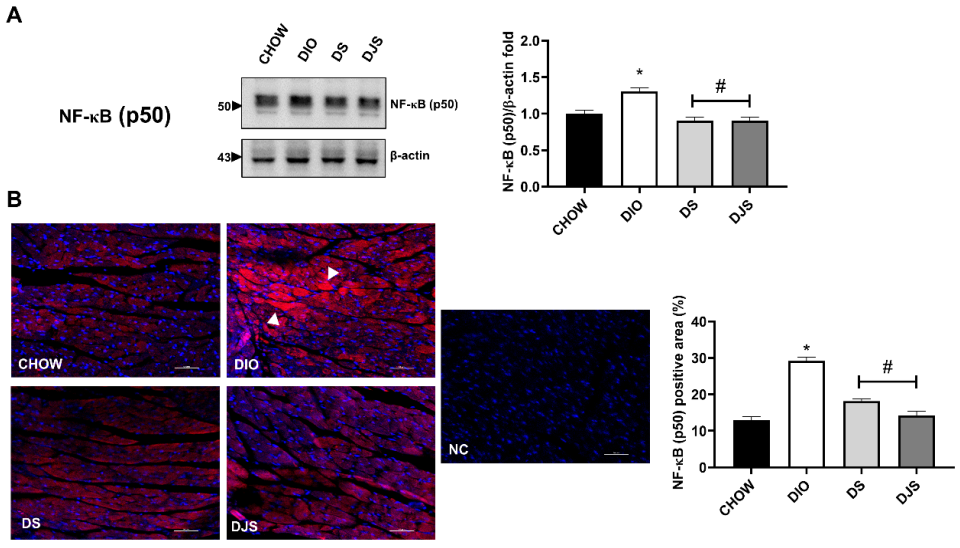

Figure 4. Measurement of nuclear factor kappa-light-chain-enhancer of activated B cells subunit p50 (NF-κB p50). (**A**) Cardiac lysates from rats were immunoblotted using specific anti NF-κB (p50). Graph shows the ratio of densitometric analysis of bands and β-actin expression used to normalize the data, taking CHOW rats as a reference group; (**B**) Confocal image of representative immunofluorescent staining for NF-κB (p50) in the heart and quantification expressed as percentage (%) of NF-κB (p50) positive area. Magnification 10× zoom 3. Scale bar 10 μm. NC, Negative control. Arrowheads indicate the more immunoreactive cardiomyocytes. CHOW rats (n = 8), fed with standard diet; DIO rats (n = 9), fed with high-fat diet; DS (n = 12), DIO rats supplemented with tart cherry seeds; DJS (n = 9), DS rats supplemented with tart cherry juice. Data are mean ± SEM. * $p < 0.05$ vs. CHOW rats; # $p < 0.05$ vs. DIO rats.

3.3.1. Adhesion Molecules

WB, performed on heart tissue lysates, showed relevant changes in ICAM-1 and PECAM-1 expression at 90 and 130 kDa, respectively (Figure 5A,C). No difference was evident for VCAM-1 and E-selectin expressions in cardiac homogenates (Figure 5B,D). In supplementary Figure S2, we showed the full-length WB gels of these adhesion molecules (Figure S2A–D).

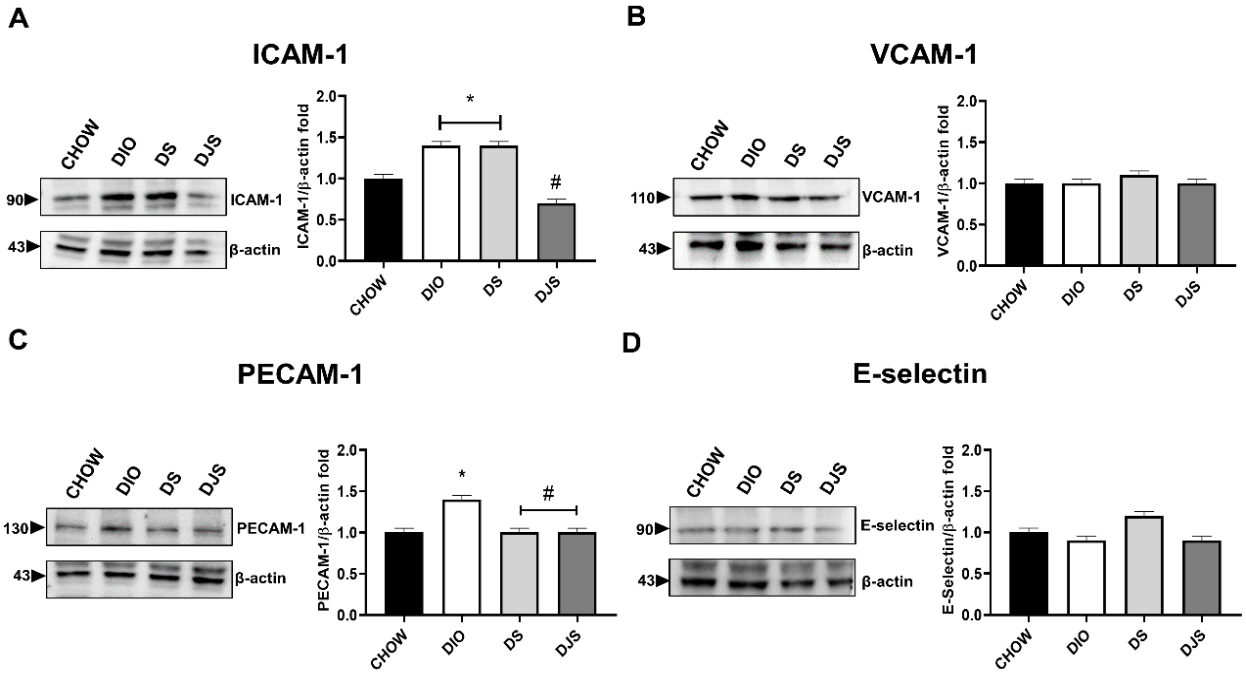

Figure 5. Western blot analysis of inflammatory adhesion molecules. Cardiac lysates were immunoblotted using antibodies against intracellular adhesion molecule-1 (ICAM-1) (**A**); vascular cell adhesion molecule-1 (VCAM-1) (**B**); platelet endothelial cell adhesion molecule-1 (PECAM-1) (**C**); E-selectin (**D**). Graphs show the densitometric ratios of bands and β-actin expression used to normalize the data. CHOW rats (n = 8), reference group fed with standard diet; DIO rats (n = 9), fed with high-fat diet; DS (n = 12), DIO rats supplemented with tart cherry seeds; DJS (n = 9), DS rats supplemented with tart cherry juice. Data are mean ± SEM. * $p < 0.05$ vs. CHOW rats; # $p < 0.05$ vs. DIO rats.

In the IHC, the adhesion molecules analyzed were found expressed in the blood vessels. As shown by representative pictures of immunohistochemical staining for VCAM-1 (Figure 6A) and PECAM-1 (Figure 6B), no significant difference occurred between lean and obese animals also supplemented with tart cherry seeds or juice. It was demonstrated that VCAM-1 was present also in the cardiomyocytes (Figure 6A), as reported elsewhere [38,40].

3.3.2. Cytokines

TNF-α, IL-1β, and IL-6 are the most well-recognized intermediaries of the early inflammatory reply. Inflammation consisting of enhanced cytokines levels was found in the DIO in comparison with CHOW rats. In HFD fed rats, the expressions of TNF-α (26 kDa) and IL-6 (21 kDa) were downregulated both in DS and DJS groups (Figure 7A,C). Finally, IL-1β was reduced significantly by only the association of seeds and juice (Figure 7B). In supplementary Figure S3, we showed the full-length WB gels of these cytokines (Figure S3A–C).

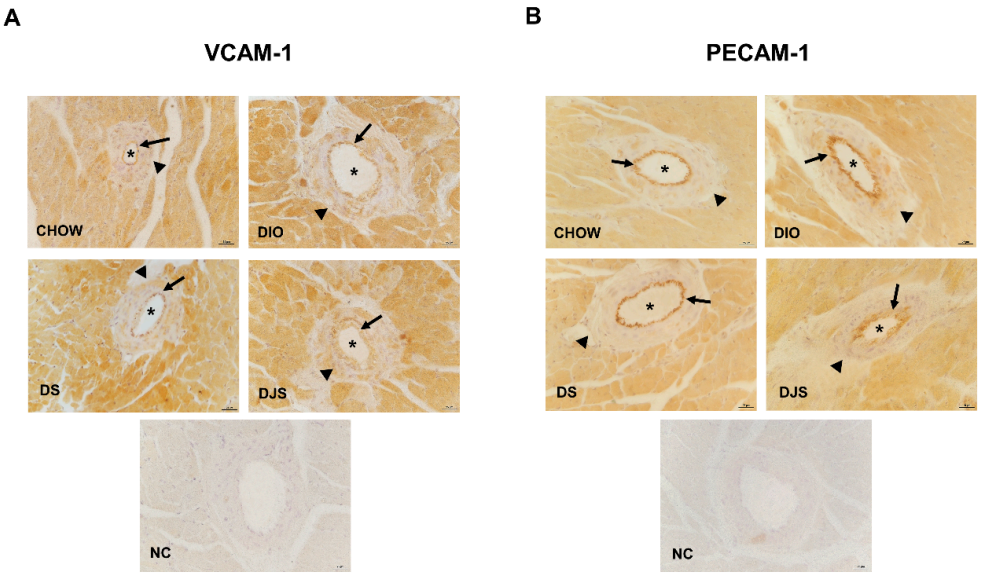

Figure 6. Immunohistochemical analysis of inflammatory adhesion molecules. Representative pictures of heart sections processed for the immunohistochemistry of vascular cell adhesion molecule-1 (VCAM-1) (**A**) and platelet endothelial cell adhesion molecule-1 (PECAM-1) (**B**). The immunoreaction is located in the endothelium (arrows) while the tunica media (arrowheads) is negative. The lumen of vessels is marked with an asterisk (*). Magnification 40×. Scale bar 25 µm. NC, Negative control. CHOW rats (n = 8), fed with standard diet; DIO rats (n = 9), fed with high-fat diet; DS (n = 12), DIO rats supplemented with tart cherry seeds; DJS (n = 9), DS rats supplemented with tart cherry juice.

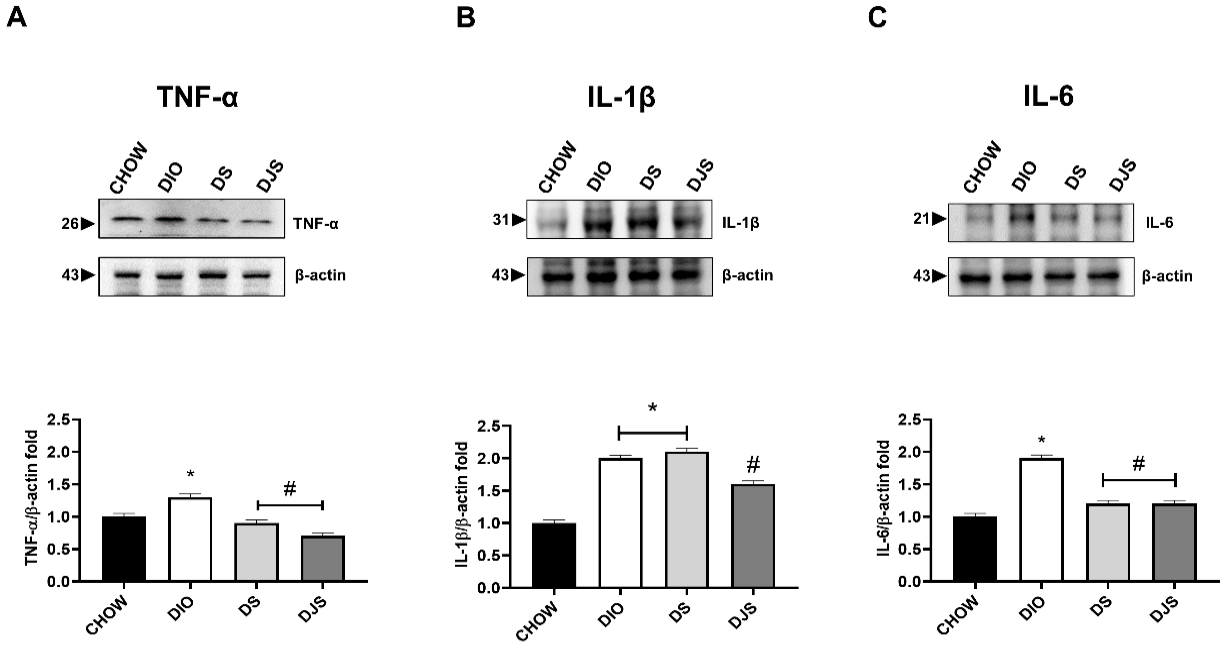

Figure 7. Western blot analysis of inflammatory cytokines. Cardiac lysates were immunoblotted using antibodies against tumor necrosis factor-α (TNF-α) (**A**), anti-interleukin-1β (IL-1β) (**B**), and interleukin-6 (IL-6) (**C**). Graphs show the densitometric ratios of bands and β-actin expression used to normalize the data. CHOW rats (n = 8), reference group fed with standard diet; DIO rats (n = 9), fed with high-fat diet; DS (n = 12), DIO rats supplemented with tart cherry seeds; DJS (n = 9), DS rats supplemented with tart cherry juice. Data are mean ± SEM. * $p < 0.05$ vs. CHOW rats; # $p < 0.05$ vs. DIO rats.

The protein quantification in WB was according to the immunohistochemistry measurements expressed as mean immunoreaction intensities of the mentioned cytokines (Figure 8A,C,E). In particular, the immunoreaction for TNF-α (Figure 8B), IL-1β (Figure 8D), and IL-6 (Figure 8F) was well defined in the damaged cardiomyocytes of DIO rats. Representative pictures showed a clear reduction in TNF-α and IL-6 with seeds as well as with seeds plus juice supplementation compared to the obese DIO rats (Figure 8B,F).

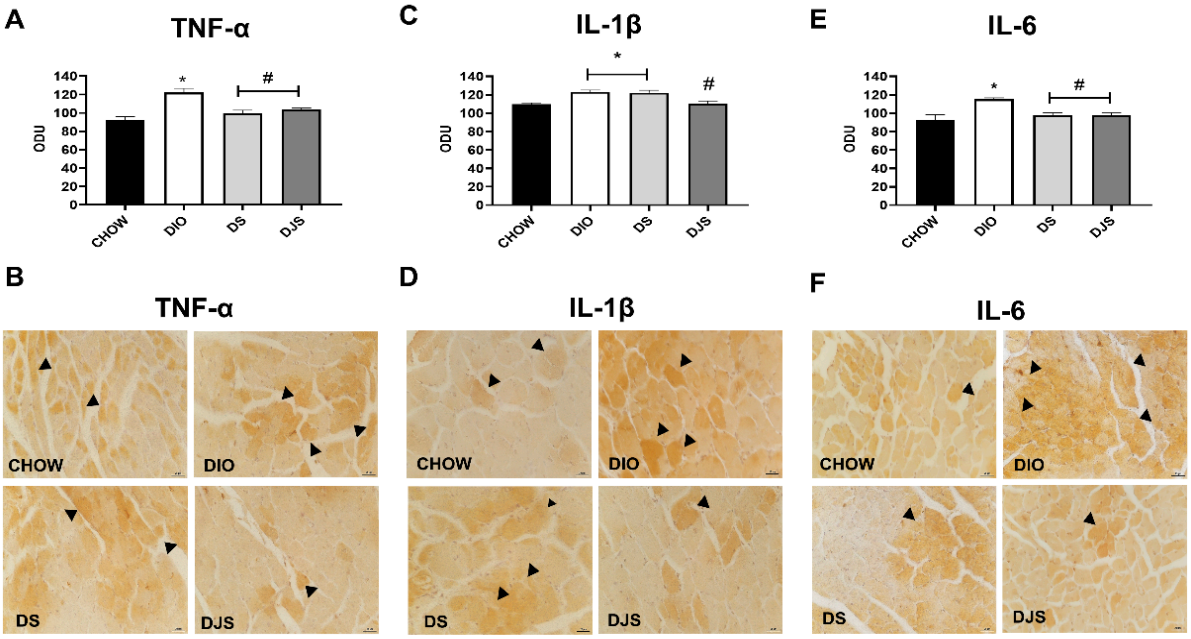

Figure 8. Immunohistochemical analysis of inflammatory cytokines. Graphs indicate the mean intensities of area immunoreaction of tumor necrosis factor-α (TNF-α) (**A**), anti-interleukin-1β (IL-1β) (**C**), and interleukin-6 (IL-6) (**E**) measured in the arbitrary optical density unit (ODU). Data are mean ± SEM. * $p < 0.05$ vs. CHOW rats; # $p < 0.05$ vs. DIO rats. Immunohistochemical representative pictures of heart sections processed for TNF-α (**B**), IL-1β (**D**), and IL-6 (**F**). The immunoreactive cardiomyocytes are indicated with arrowheads. Magnification 40×. Scale bar 25 µm. CHOW rats ($n = 8$), fed with standard diet; DIO rats ($n = 9$), fed with high-fat diet; DS ($n = 12$), DIO rats supplemented with tart cherry seeds; DJS ($n = 9$), DS rats supplemented with tart cherry juice.

4. Discussion

Obesity-related health complications are increasing, and the consumption of HFD is considered a major cause of these complications. Clarifying mechanisms involved in obesity-related cardiac impairments requires suitable animal models. Rodents as genetic models of obesity, such as the Zucker obese rat, the ob/ob rat, and the spontaneously hypertensive obese rat, have been studied for cardiovascular research [38,41,42]. In the present study, rats with HFD developed an obese phenotype, impaired blood parameters, and pressure, accompanied by cardiovascular alterations. The cardiovascular abnormalities presented in other diet-induced obese models were hypertension, tachycardia, reduced cardiac contractility, increased end-diastolic pressure left ventricular hypertrophy, and increased collagen deposition [43]. The progress of cardiac fibrosis has been found in many obese animal models, and it is often accompanied by diastolic dysfunction. The severity of cardiac fibrosis varies, depending on the age, species, and strain of the animals, the underlying mechanism of obesity, and the presence of concomitant pathophysiological conditions (e.g., metabolic dysfunction and hypertension) [44]. Our results on histological sections of cardiac tissue showed cardiomyocyte hypertrophy but not fibrosis in the myocardium of DIO rats. Accordingly, HFD may even be less effective in the induction of cardiac fibrotic alterations [44]. In male C57/BL6J mice, Calligaris et al. reported that feeding with an HFD for 16 months was required to develop substantial cardiac fibrosis

and hypertrophy [45]. In addition, 20-week-old obese Zucker diabetic fatty/spontaneously hypertensive heart failure F1 hybrid (ZSF1) male rats showed important alterations in major systemic biomarkers of cardiovascular function without histopathological modifications in the heart [46].

Oxidative stress also causes myocardial tissue damage and inflammation, contributing to heart failure progression [47]. The signaling, mediated by the activation of inflammatory markers or NF-κB and other transcription factors as central regulators of inflammation, is the key issue to understanding oxidative stress responses in obesity [9]. Previous works have suggested an enhanced NF-κB activation in obese individuals and experimental animals fed with HFD [9,48,49]. The reply of endothelial cells to NF-κB activation and inflammation consists in the induction of adhesion molecules, promoting binding and transmigration of leukocytes, while instantaneously improving their thrombogenic potential [50]. Lee et al. [51] reported that cardiac TNF-α protein level and serum IL-6 were remarkably augmented in db/db mice and were linked with endothelial dysfunction in the coronary microvasculature. In another study, coronary endothelial dysfunction resulted from elevated plasma concentration and the expression of TNF-α and its receptor (TNFR1) in coronary arterioles of db/db mice [52]. Moreover, TNF-α gene knock-out or treatment with a TNF-α neutralizing antibody improved endothelial function of coronary arterioles in diabetic mice [52]. In our study, obesity-induced damage to the heart was associated with NF-κB activation and increased in ICAM-1, PECAM-1, TNF-α, IL-1β, and IL-6 expression levels. Collectively, our current findings in the heart support the previous study in obese Zucker rats [38] and further suggest the interplay among inflammation and oxidative stress in the obese myocardium. As summarized by Adrielle Lima Vieira et al. [53], reports indicate high levels of circulating cell adhesion molecules in obesity, especially in the presence of visceral adipose tissue accumulation [54–56]. Even if other studies described an intensification of VCAM-1, ICAM-1, and E-selectin in obese condition [55–57], and specifically in the aorta of obese Zucker rats at 15 weeks of age [58], our data displayed no differences in E-selectin and VCAM-1 in DIO compared to CHOW rats. Perhaps in obesity, the modulation of the vascular adhesion proteins expression may happen in the peripheral arteries rather than the coronary arteries [38]. Studies proposed a link between the levels of these molecules and the anthropometric markers of obesity, but they are still controversial [53]. Finally, the HFD-fed rats did not show the induction of caspase-3 activation either in the liver [33] or in the heart: this indicates that the hepatocytes and cardiomyocytes alterations were not related with cell death and/or fibrosis [33,39].

Tart cherry supplementation reduced the systolic blood pressure, glycemic values, oxidative stress, and inflammation, confirming its positive effects on the risk factors related to obesity and metabolic syndrome [59–63]. For instance, we have already reported that in rats fed with seeds and juice, a remarkable decrease in systolic blood pressure in obese rats was found [26,33,34]. This effect has been attributed to anthocyanins, which exhibit several biological effects, including vasodilatory capacity. In fact, the nitric oxide (NO) pathway could be responsible for the relaxation response of coronary arteries to red fruit extracts [64], and anthocyanins condensed tannin-containing fractions showed more vasodilation property than other polyphenols [17]. In hypertensive rodents supplemented with blueberry-enriched anthocyanins, a reduction in blood pressure was assessed. The anthocyanins exhibited NO-dependent vasodilation via endothelium triggered by acetylcholine. Moreover, endothelium-dependent relaxation is another vasodilator outcome derived from anthocyanins [64,65]. In clinical trials, the circulating phenolic compounds of sour cherries were able to counteract the hypertension [66]. In addition, the anti-hypertensive capacity of sour cherry could be attributed to its anti-inflammatory and antioxidant capacities [67]. Interestingly, there are two key components in the tart cherry seeds: the oleic and linoleic acids, which can protect the endothelium [68]. These fatty acids could elucidate the anti-inflammatory properties also observed in the cerebral areas of DIO rats [26]. In obese and diabetic conditions, a diet rich in oleic acid can induce the positive effect reversing the negative consequences of inflammatory cytokines [69]. Oleic acid has been proposed

to protect against cardiovascular insulin resistance, improving endothelial injury in response to proinflammatory activation, and reducing apoptosis in vascular smooth muscle cells. All these properties may help to maintain plaque stability and to ameliorate the atherosclerotic process [70]. Furthermore, it has been reported that the intake of sour cherry seed kernel extract improved postischemic recovery of cardiac function during reperfusion. Moreover, other potential action mechanisms of proanthocyanidin, trans-resveratrol, and flavonoid components of the extract could be responsible for the cardioprotection in ischemic-reperfused myocardium [71]. Finally, preclinical evidence showed benefits in dietary supplementation of the cited antioxidant compounds on fat accumulation [11].

Here, we demonstrated a clear reduction in protein carbonyl levels and 4-HNE in rodents HFD supplemented with tart cherries, compared to DIO rats. In particular, the upregulation in 4-HNE because of oxidative stress was detected in numerous cardiac pathologies (e.g., diabetic cardiomyopathy). 4-HNE damages the myocardium, interfering with mitochondria and making adducts [72]. Previously, we reported not only a decrease in oxidative stress both in the serum and in the liver [33], but also in inflammation both in the brain [26] and in the adipose tissue [34] of HFD fed rats supplemented with tart cherries. The same animal model was also explored by Seymour and co-workers [62], who reported that the intake of tart cherries reduces retroperitoneal IL-6 and TNF-α mRNA expression, NF-κB activity, and plasma IL-6 and TNF-α concentrations. These results were consistent with our data, demonstrating that certain inflammatory biomarkers were remarkably decreased in cardiac samples of obese rats following tart cherry seed and juice consumption.

Since diverse study designs have been carried out, in human or animal models, and at many dosages of tart cherry, the results are quite variable. Beneficial effects of tart cherry supplementations were found in female rats, protecting early age-related bone loss and increasing bone mineralization [73]; therefore, it would be interesting to extend the study to female rats. Aside from our current results in male rats, there is wide evidence that demonstrates that tart cherry ingestion provides anti-inflammatory and anti-oxidative activities both in vivo and in vitro [63,67,74–79].

5. Conclusions

Our findings support the dual antioxidant and anti-inflammatory actions of tart cherries, which may represent an interesting therapeutic strategy to provide a dietary supplement for people either at high risk or with established obesity-related cardiovascular disease.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/biology11050646/s1. Figure S1: Full-length WB images for Figures 3 and 4; Figure S2: Full-length WB images for Figure 5; Figure S3: Full-length WB images for Figure 7.

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References

1. Ortega, F.B.; Lavie, C.J.; Blair, S.N. Obesity and Cardiovascular Disease. *Circ. Res.* **2016**, *118*, 1752–1770. [CrossRef] [PubMed]
2. Koliaki, C.; Liatis, S.; Kokkinos, A. Obesity and cardiovascular disease: Revisiting an old relationship. *Metabolism* **2019**, *92*, 98–107. [CrossRef] [PubMed]
3. Battineni, G.; Sagaro, G.G.; Chintalapudi, N.; Amenta, F.; Tomassoni, D.; Tayebati, S.K. Impact of Obesity-Induced Inflammation on Cardiovascular Diseases (CVD). *Int. J. Mol. Sci.* **2021**, *22*, 4798. [CrossRef] [PubMed]
4. Odrowaz-Sypniewska, G. Markers of pro-inflammatory and pro-thrombotic state in the diagnosis of metabolic syndrome. *Adv. Med. Sci.* **2007**, *52*, 246–250.
5. Santilli, F.; Guagnano, M.T.; Vazzana, N.; La Barba, S.; Davi, G. Oxidative stress drivers and modulators in obesity and cardiovascular disease: From biomarkers to therapeutic approach. *Curr. Med. Chem.* **2015**, *22*, 582–595. [CrossRef]
6. Patel, C.; Ghanim, H.; Ravishankar, S.; Sia, C.L.; Viswanathan, P.; Mohanty, P.; Dandona, P. Prolonged reactive oxygen species generation and nuclear factor-kappaB activation after a high-fat, high-carbohydrate meal in the obese. *J. Clin. Endocrinol. Metab.* **2007**, *92*, 4476–4479. [CrossRef]
7. Makki, K.; Froguel, P.; Wolowczuk, I. Adipose tissue in obesity-related inflammation and insulin resistance: Cells, cytokines, and chemokines. *ISRN Inflamm.* **2013**, *2013*, 139239. [CrossRef]
8. Jung, U.J.; Choi, M.S. Obesity and its metabolic complications: The role of adipokines and the relationship between obesity, inflammation, insulin resistance, dyslipidemia and nonalcoholic fatty liver disease. *Int. J. Mol. Sci.* **2014**, *15*, 6184–6223. [CrossRef]
9. Bondia-Pons, I.; Ryan, L.; Martinez, J.A. Oxidative stress and inflammation interactions in human obesity. *J. Physiol. Biochem.* **2012**, *68*, 701–711. [CrossRef]
10. Di Domenico, M.; Pinto, F.; Quagliuolo, L.; Contaldo, M.; Settembre, G.; Romano, A.; Coppola, M.; Ferati, K.; Bexheti-Ferati, A.; Sciarra, A.; et al. The Role of Oxidative Stress and Hormones in Controlling Obesity. *Front. Endocrinol. (Lausanne)* **2019**, *10*, 540. [CrossRef]
11. Roy, P.; Tomassoni, D.; Traini, E.; Martinelli, I.; Micioni Di Bonaventura, M.V.; Cifani, C.; Amenta, F.; Tayebati, S.K. Natural Antioxidant Application on Fat Accumulation: Preclinical Evidence. *Antioxidants* **2021**, *10*, 858. [CrossRef] [PubMed]
12. Kelley, D.S.; Adkins, Y.; Laugero, K.D. A Review of the Health Benefits of Cherries. *Nutrients* **2018**, *10*, 368. [CrossRef] [PubMed]
13. Wallace, T.C. Anthocyanins in cardiovascular disease. *Adv. Nutr.* **2011**, *2*, 1–7. [CrossRef] [PubMed]
14. Samtiya, M.; Aluko, R.E.; Dhewa, T.; Moreno-Rojas, J.M. Potential Health Benefits of Plant Food-Derived Bioactive Components: An Overview. *Foods* **2021**, *10*, 839. [CrossRef]
15. Nöthlings, U.; Schulze, M.B.; Weikert, C.; Boeing, H.; van der Schouw, Y.T.; Bamia, C.; Benetou, V.; Lagiou, P.; Krogh, V.; Beulens, J.W.; et al. Intake of vegetables, legumes, and fruit, and risk for all-cause, cardiovascular, and cancer mortality in a European diabetic population. *J. Nutr.* **2008**, *138*, 775–781. [CrossRef]
16. Mozos, I.; Flangea, C.; Vlad, D.C.; Gug, C.; Mozos, C.; Stoian, D.; Luca, C.T.; Horbańczuk, J.O.; Horbańczuk, O.K.; Atanasov, A.G. Effects of Anthocyanins on Vascular Health. *BioMolecules* **2021**, *11*, 811. [CrossRef]
17. Reis, J.F.; Monteiro, V.V.; de Souza Gomes, R.; do Carmo, M.M.; da Costa, G.V.; Ribera, P.C.; Monteiro, M.C. Action mechanism and cardiovascular effect of anthocyanins: A systematic review of animal and human studies. *J. Transl. Med.* **2016**, *14*, 315. [CrossRef]
18. Mazza, G.J. Anthocyanins and heart health. *Ann. Ist. Super. Sanita* **2007**, *43*, 369–374.
19. Tsuda, T.; Horio, F.; Osawa, T. The role of anthocyanins as an antioxidant under oxidative stress in rats. *Biofactors* **2000**, *13*, 133–139. [CrossRef]
20. Zheng, W.; Wang, S.Y. Oxygen radical absorbing capacity of phenolics in blueberries, cranberries, chokeberries, and lingonberries. *J. Agric. Food Chem.* **2003**, *51*, 502–509. [CrossRef]
21. Youdim, K.A.; McDonald, J.; Kalt, W.; Joseph, J.A. Potential role of dietary flavonoids in reducing microvascular endothelium vulnerability to oxidative and inflammatory insults. *J. Nutr. Biochem.* **2002**, *13*, 282–288. [CrossRef]
22. Wang, J.; Mazza, G. Inhibitory effects of anthocyanins and other phenolic compounds on nitric oxide production in LPS/IFN-activated RAW 264.7 macrophages. *J. Agric. Food Chem.* **2002**, *50*, 850–857. [CrossRef] [PubMed]
23. Lin, B.W.; Gong, C.C.; Song, H.F.; Cui, Y.Y. Effects of anthocyanins on the prevention and treatment of cancer. *Br. J. Pharmacol.* **2017**, *174*, 1226–1243. [CrossRef] [PubMed]
24. Katsube, N.; Iwashita, K.; Tsushida, T.; Yamaki, K.; Kobori, M. Induction of apoptosis in cancer cells by Bilberry (*Vaccinium myrtillus*) and the anthocyanins. *J. Agric. Food Chem.* **2003**, *51*, 68–75. [CrossRef]
25. Galli, R.L.; Shukitt-Hale, B.; Youdim, K.A.; Joseph, J.A. Fruit polyphenolics and brain aging: Nutritional interventions targeting age-related neuronal and behavioral deficits. *Ann. N. Y. Acad. Sci.* **2002**, *959*, 128–132. [CrossRef]
26. Micioni Di Bonaventura, M.V.; Martinelli, I.; Moruzzi, M.; Micioni Di Bonaventura, E.; Giusepponi, M.E.; Polidori, C.; Lupidi, G.; Tayebati, S.K.; Amenta, F.; Cifani, C.; et al. Brain alterations in high fat diet induced obesity: Effects of tart cherry seeds and juice. *Nutrients* **2020**, *12*, 623. [CrossRef]
27. Andriambeloson, E.; Magnier, C.; Haan-Archipoff, G.; Lobstein, A.; Anton, R.; Beretz, A.; Stoclet, J.C.; Andriantsitohaina, R. Natural dietary polyphenolic compounds cause endothelium-dependent vasorelaxation in rat thoracic aorta. *J. Nutr.* **1998**, *128*, 2324–2333. [CrossRef]
28. Martin, S.; Giannone, G.; Andriantsitohaina, R.; Martinez, M.C. Delphinidin, an active compound of red wine, inhibits endothelial cell apoptosis via nitric oxide pathway and regulation of calcium homeostasis. *Br. J. Pharmacol.* **2003**, *139*, 1095–1102. [CrossRef]

29. Demrow, H.S.; Slane, P.R.; Folts, J.D. Administration of wine and grape juice inhibits in vivo platelet activity and thrombosis in stenosed canine coronary arteries. *Circulation* **1995**, *91*, 1182–1188. [CrossRef]
30. Renaud, S.; de Lorgeril, M. Wine, alcohol, platelets, and the French paradox for coronary heart disease. *Lancet* **1992**, *339*, 1523–1526. [CrossRef]
31. do Rosario, V.A.; Chang, C.; Spencer, J.; Alahakone, T.; Roodenrys, S.; Francois, M.; Weston-Green, K.; Hölzel, N.; Nichols, D.S.; Kent, K.; et al. Anthocyanins attenuate vascular and inflammatory responses to a high fat high energy meal challenge in overweight older adults: A cross-over, randomized, double-blind clinical trial. *Clin. Nutr.* **2021**, *40*, 879–889. [CrossRef] [PubMed]
32. Cocci, P.; Moruzzi, M.; Martinelli, I.; Maggi, F.; Micioni Di Bonaventura, M.V.; Cifani, C.; Mosconi, G.; Tayebati, S.K.; Damiano, S.; Lupidi, G.; et al. Tart cherry (*Prunus cerasus* L.) dietary supplement modulates visceral adipose tissue CB1 mRNA levels along with other adipogenesis-related genes in rat models of diet-induced obesity. *Eur. J. Nutr.* **2021**, *60*, 2695–2707. [CrossRef] [PubMed]
33. Martinelli, I.; Micioni Di Bonaventura, M.V.; Moruzzi, M.; Amantini, C.; Maggi, F.; Gabrielli, M.G.; Fruganti, A.; Marchegiani, A.; Dini, F.; Marini, C.; et al. Effects of *Prunus cerasus* L. seeds and juice on liver steatosis in an animal model of diet-induced obesity. *Nutrients* **2020**, *12*, 1308. [CrossRef] [PubMed]
34. Moruzzi, M.; Klöting, N.; Blüher, M.; Martinelli, I.; Tayebati, S.K.; Gabrielli, M.G.; Roy, P.; Micioni Di Bonaventura, M.V.; Cifani, C.; Lupidi, G.; et al. Tart Cherry Juice and Seeds Affect Pro-Inflammatory Markers in Visceral Adipose Tissue of High-Fat Diet Obese Rats. *Molecules* **2021**, *26*, 1403. [CrossRef] [PubMed]
35. Roy, P.; Martinelli, I.; Moruzzi, M.; Maggi, F.; Amantini, C.; Di Bonaventura, M.V.M.; Cifani, C.; Amenta, F.; Tayebati, S.K.; Tomassoni, D. Ion channels alterations in the forebrain of high-fat diet fed rats. *Eur. J. Histochem.* **2021**, *65*, 3305. [CrossRef]
36. Martinelli, I.; Tayebati, S.K.; Roy, P.; Micioni Di Bonaventura, M.V.; Moruzzi, M.; Cifani, C.; Amenta, F.; Tomassoni, D. Obesity-Related Brain Cholinergic System Impairment in High-Fat-Diet-Fed Rats. *Nutrients* **2022**, *14*, 1243. [CrossRef]
37. Qin, F.; Siwik, D.A.; Pimentel, D.R.; Morgan, R.J.; Biolo, A.; Tu, V.H.; Kang, Y.J.; Cohen, R.A.; Colucci, W.S. Cytosolic H_2O_2 mediates hypertrophy, apoptosis, and decreased SERCA activity in mice with chronic hemodynamic overload. *Am. J. Physiol. Heart Circ. Physiol.* **2014**, *306*, H1453–H1463. [CrossRef]
38. Martinelli, I.; Tomassoni, D.; Moruzzi, M.; Roy, P.; Cifani, C.; Amenta, F.; Tayebati, S.K. Cardiovascular Changes Related to Metabolic Syndrome: Evidence in Obese Zucker Rats. *Int. J. Mol. Sci.* **2020**, *21*, 2035. [CrossRef]
39. Tak, P.P.; Firestein, G.S. NF-kappaB: A key role in inflammatory diseases. *J. Clin. Investig.* **2001**, *107*, 7–11. [CrossRef]
40. Uosaki, H.; Fukushima, H.; Takeuchi, A.; Matsuoka, S.; Nakatsuji, N.; Yamanaka, S.; Yamashita, J.K. Efficient and scalable purification of cardiomyocytes from human embryonic and induced pluripotent stem cells by VCAM1 surface expression. *PLoS ONE* **2011**, *6*, e23657. [CrossRef]
41. Mukaddam-Daher, S.; Menaouar, A.; El-Ayoubi, R.; Gutkowska, J.; Jankowski, M.; Velliquette, R.A.; Ernsberger, P. Cardiac effects of moxonidine in spontaneously hypertensive obese rats. *Ann. N Y Acad. Sci.* **2003**, *1009*, 244–250. [CrossRef] [PubMed]
42. Conti, M.; Renaud, I.M.; Poirier, B.; Michel, O.; Belair, M.F.; Mandet, C.; Bruneval, P.; Myara, I.; Chevalier, J. High levels of myocardial antioxidant defense in aging nondiabetic normotensive Zucker obese rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2004**, *286*, R793–R800. [CrossRef] [PubMed]
43. Carroll, J.F.; Zenebe, W.J.; Strange, T.B. Cardiovascular function in a rat model of diet-induced obesity. *Hypertension* **2006**, *48*, 65–72. [CrossRef] [PubMed]
44. Cavalera, M.; Wang, J.; Frangogiannis, N.G. Obesity, metabolic dysfunction, and cardiac fibrosis: Pathophysiological pathways, molecular mechanisms, and therapeutic opportunities. *Transl. Res.* **2014**, *164*, 323–335. [CrossRef]
45. Calligaris, S.D.; Lecanda, M.; Solis, F.; Ezquer, M.; Gutiérrez, J.; Brandan, E.; Leiva, A.; Sobrevia, L.; Conget, P. Mice long-term high-fat diet feeding recapitulates human cardiovascular alterations: An animal model to study the early phases of diabetic cardiomyopathy. *PLoS ONE* **2013**, *8*, e60931. [CrossRef] [PubMed]
46. Stolina, M.; Luo, X.; Dwyer, D.; Han, C.Y.; Chen, R.; Zhang, Y.; Xiong, Y.; Chen, Y.; Yin, J.; Shkumatov, A.; et al. The evolving systemic biomarker milieu in obese ZSF1 rat model of human cardiometabolic syndrome: Characterization of the model and cardioprotective effect of GDF15. *PLoS ONE* **2020**, *15*, e0231234. [CrossRef]
47. Aimo, A.; Castiglione, V.; Borrelli, C.; Saccaro, L.F.; Franzini, M.; Masi, S.; Emdin, M.; Giannoni, A. Oxidative stress and inflammation in the evolution of heart failure: From pathophysiology to therapeutic strategies. *Eur. J. Prev. Cardiol.* **2020**, *27*, 494–510. [CrossRef]
48. Jovanovic, A.; Sudar-Milovanovic, E.; Obradovic, M.; Pitt, S.J.; Stewart, A.J.; Zafirovic, S.; Stanimirovic, J.; Radak, D.; Isenovic, E.R. Influence of a High-Fat Diet on Cardiac iNOS in Female Rats. *Curr. Vasc. Pharmacol.* **2017**, *15*, 491–500. [CrossRef]
49. Carlsen, H.; Haugen, F.; Zadelaar, S.; Kleemann, R.; Kooistra, T.; Drevon, C.A.; Blomhoff, R. Diet-induced obesity increases NF-kappaB signaling in reporter mice. *Genes Nutr.* **2009**, *4*, 215–222. [CrossRef]
50. Mussbacher, M.; Salzmann, M.; Brostjan, C.; Hoesel, B.; Schoergenhofer, C.; Datler, H.; Hohensinner, P.; Basílio, J.; Petzelbauer, P.; Assinger, A.; et al. Cell Type-Specific Roles of NF-κB Linking Inflammation and Thrombosis. *Front. Immunol.* **2019**, *10*, 85. [CrossRef]
51. Lee, S.; Park, Y.; Zhang, C. Exercise Training Prevents Coronary Endothelial Dysfunction in Type 2 Diabetic Mice. *Am. J. Biomed. Sci.* **2011**, *3*, 241–252. [CrossRef] [PubMed]
52. Zhang, C.; Park, Y.; Picchi, A.; Potter, B.J. Maturation-induces endothelial dysfunction via vascular inflammation in diabetic mice. *Basic Res. Cardiol.* **2008**, *103*, 407–416. [CrossRef] [PubMed]

53. Vieira, R.A.L.; de Freitas, R.N.; Volp, A.C. Adhesion molecules and chemokines; relation to anthropometric, body composition, biochemical and dietary variables. *Nutr. Hosp.* **2014**, *30*, 223–236. [CrossRef]
54. Couillard, C.; Ruel, G.; Archer, W.R.; Pomerleau, S.; Bergeron, J.; Couture, P.; Lamarche, B.; Bergeron, N. Circulating levels of oxidative stress markers and endothelial adhesion molecules in men with abdominal obesity. *J. Clin. Endocrinol. Metab.* **2005**, *90*, 6454–6459. [CrossRef]
55. El-Baz, F.K.; Aly, H.F.; Abd-Alla, H.I. The ameliorating effect of carotenoid rich fraction extracted from *Dunaliella salina* microalga against inflammation-associated cardiac dysfunction in obese rats. *Toxicol. Rep.* **2019**, *7*, 118–124. [CrossRef]
56. Mulhem, A.; Moulla, Y.; Klöting, N.; Ebert, T.; Tönjes, A.; Fasshauer, M.; Dietrich, A.; Schön, M.R.; Stumvoll, M.; Richter, V.; et al. Circulating cell adhesion molecules in metabolically healthy obesity. *Int. J. Obes. (Lond)* **2021**, *45*, 331–336. [CrossRef]
57. Zanni, M.V.; Stanley, T.L.; Makimura, H.; Chen, C.Y.; Grinspoon, S.K. Effects of TNF-alpha antagonism on E-selectin in obese subjects with metabolic dysregulation. *Clin. Endocrinol. (Oxf)* **2010**, *73*, 48–54. [CrossRef]
58. Porres, J.M.; Constantino, J.; Kapravelou, G.; Lopez-Chaves, C.; Galisteo, M.; Aranda, P.; López-Jurado, M.; Martínez, R. The combined treatment with lentil protein hydrolysate and a mixed training protocol is an efficient lifestyle intervention to manage cardiovascular and renal alterations in obese Zucker rats. *Eur. J. Nutr.* **2020**, *59*, 3473–3490. [CrossRef]
59. Wang, H.T.; Liu, C.F.; Tsai, T.H.; Chen, Y.L.; Chang, H.W.; Tsai, C.Y.; Leu, S.; Zhen, Y.Y.; Chai, H.T.; Chung, S.Y.; et al. Effect of obesity reduction on preservation of heart function and attenuation of left ventricular remodeling, oxidative stress and inflammation in obese mice. *J. Transl. Med.* **2012**, *10*, 145. [CrossRef]
60. Piccolella, S.; Fiorentino, A.; Pacifico, S.; D'Abrosca, B.; Uzzo, P.; Monaco, P. Antioxidant properties of sour cherries (*Prunus cerasus* L.): Role of colorless phytochemicals from the methanolic extract of ripe fruits. *J. Agric. Food Chem.* **2008**, *56*, 1928–1935. [CrossRef]
61. Seymour, E.M.; Singer, A.A.; Kirakosyan, A.; Urcuyo-Llanes, D.E.; Kaufman, P.B.; Bolling, S.F. Altered hyperlipidemia, hepatic steatosis, and hepatic peroxisome proliferator-activated receptors in rats with intake of tart cherry. *J. Med. Food* **2008**, *11*, 252–259. [CrossRef] [PubMed]
62. Seymour, E.M.; Lewis, S.K.; Urcuyo-Llanes, D.E.; Tanone, I.I.; Kirakosyan, A.; Kaufman, P.B.; Bolling, S.F. Regular tart cherry intake alters abdominal adiposity, adipose gene transcription, and inflammation in obesity-prone rats fed a high fat diet. *J. Med. Food* **2009**, *12*, 935–942. [CrossRef] [PubMed]
63. Jayarathne, S.; Stull, A.J.; Miranda, A.; Scoggin, S.; Claycombe-Larson, K.; Kim, J.H.; Moustaid-Moussa, N. Tart Cherry Reduces Inflammation in Adipose Tissue of Zucker Fatty Rats and Cultured 3T3-L1 Adipocytes. *Nutrients* **2018**, *10*, 1576. [CrossRef] [PubMed]
64. Bell, D.R.; Gochenaur, K. Direct vasoactive and vasoprotective properties of anthocyanin-rich extracts. *J. Appl. Physiol.* **2006**, *100*, 1164–1170. [CrossRef] [PubMed]
65. Kalea, A.Z.; Clark, K.; Schuschke, D.A.; Klimis-Zacas, D.J. Vascular reactivity is affected by dietary consumption of wild blueberries in the Sprague-Dawley rat. *J. Med. Food* **2009**, *12*, 21–28. [CrossRef] [PubMed]
66. Keane, K.M.; George, T.W.; Constantinou, C.L.; Brown, M.A.; Clifford, T.; Howatson, G. Effects of Montmorency tart cherry (*Prunus cerasus* L.) consumption on vascular function in men with early hypertension. *Am. J. Clin. Nutr.* **2016**, *103*, 1531–1539. [CrossRef]
67. Chai, S.C.; Davis, K.; Zhang, Z.; Zha, L.; Kirschner, K.F. Effects of Tart Cherry Juice on Biomarkers of Inflammation and Oxidative Stress in Older Adults. *Nutrients* **2019**, *11*, 228. [CrossRef]
68. Carrillo, C.; Cavia Mdel, M.; Alonso-Torre, S. Role of oleic acid in immune system; mechanism of action; a review. *Nutr. Hosp.* **2012**, *27*, 978–990. [CrossRef]
69. Vassiliou, E.K.; Gonzalez, A.; Garcia, C.; Tadros, J.H.; Chakraborty, G.; Toney, J.H. Oleic acid and peanut oil high in oleic acid reverse the inhibitory effect of insulin production of the inflammatory cytokine TNF-alpha both in vitro and in vivo systems. *Lipids Health Dis.* **2009**, *8*, 25. [CrossRef]
70. Perdomo, L.; Beneit, N.; Otero, Y.F.; Escribano, Ó.; Díaz-Castroverde, S.; Gómez-Hernández, A.; Benito, M. Protective role of oleic acid against cardiovascular insulin resistance and in the early and late cellular atherosclerotic process. *Cardiovasc. Diabetol.* **2015**, *14*, 75. [CrossRef]
71. Bak, I.; Lekli, I.; Juhasz, B.; Nagy, N.; Varga, E.; Varadi, J.; Gesztelyi, R.; Szabo, G.; Szendrei, L.; Bacskay, I.; et al. Cardioprotective mechanisms of *Prunus cerasus* (sour cherry) seed extract against ischemia-reperfusion-induced damage in isolated rat hearts. *Am. J. Physiol. Heart Circ. Physiol.* **2006**, *291*, H1329–H1336. [CrossRef] [PubMed]
72. Deshpande, M.; Mali, V.R.; Pan, G.; Xu, J.; Yang, X.P.; Thandavarayan, R.A.; Palaniyandi, S.S. Increased 4-hydroxy-2-nonenal-induced proteasome dysfunction is correlated with cardiac damage in streptozotocin-injected rats with isoproterenol infusion. *Cell Biochem. Funct.* **2016**, *34*, 334–342. [CrossRef] [PubMed]
73. Smith, B.J.; Crockett, E.K.; Chongwatpol, P.; Graef, J.L.; Clarke, S.L.; Rendina-Ruedy, E.; Lucas, E.A. Montmorency tart cherry protects against age-related bone loss in female C57BL/6 mice and demonstrates some anabolic effects. *Eur. J. Nutr.* **2019**, *58*, 3035–3046. [CrossRef] [PubMed]
74. Traustadóttir, T.; Davies, S.S.; Stock, A.A.; Su, Y.; Heward, C.B.; Roberts, L.J., 2nd; Harman, S.M. Tart cherry juice decreases oxidative stress in healthy older men and women. *J. Nutr.* **2009**, *139*, 1896–1900. [CrossRef] [PubMed]
75. Mulabagal, V.; Lang, G.A.; DeWitt, D.L.; Dalavoy, S.S.; Nair, M.G. Anthocyanin content, lipid peroxidation and cyclooxygenase enzyme inhibitory activities of sweet and sour cherries. *J. Agric. Food Chem.* **2009**, *57*, 1239–1246. [CrossRef] [PubMed]

76. Sarić, A.; Sobocanec, S.; Balog, T.; Kusić, B.; Sverko, V.; Dragović-Uzelac, V.; Levaj, B.; Cosić, Z.; Macak Safranko, Z.; Marotti, T. Improved antioxidant and anti-inflammatory potential in mice consuming sour cherry juice (*Prunus Cerasus* cv. *Maraska). Plant Foods Hum. Nutr.* **2009**, *64*, 231–237. [CrossRef]
77. Ou, B.; Bosak, K.N.; Brickner, P.R.; Iezzoni, D.G.; Seymour, E.M. Processed tart cherry products—Comparative phytochemical content, in vitro antioxidant capacity and in vitro anti-inflammatory activity. *J. Food Sci.* **2012**, *77*, H105–H112. [CrossRef]
78. Schumacher, H.R.; Pullman-Mooar, S.; Gupta, S.R.; Dinnella, J.E.; Kim, R.; McHugh, M.P. Randomized double-blind crossover study of the efficacy of a tart cherry juice blend in treatment of osteoarthritis (OA) of the knee. *Osteoarthr. Cartil.* **2013**, *21*, 1035–1041. [CrossRef]
79. Martin, K.R.; Burrell, L.; Bopp, J. Authentic tart cherry juice reduces markers of inflammation in overweight and obese subjects: A randomized, crossover pilot study. *Food Funct.* **2018**, *9*, 5290–5300. [CrossRef]

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Heart alterations in spontaneously hypertensive rats: immunochemical and immunohistochemical assessment of the activity of dextrorotatory form of thioctic acid

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Hypertension represents a risk factor for cardiovascular diseases [1]. Increasing evidence attributed the main role of oxidative stress in cardiovascular damage, promoting endothelial dysfunction, vascular remodeling, and inflammation. Excess bioavailability of reactive oxygen species (ROS), often is accompanied by structural mitochondria abnormalities in the cardiomyocyte [2,3]. The most effective treatment in the management of hypertension seems to be the administration of anti-hypertensive drugs with antioxidant properties [4].

Thioctic acid (TIO) is an antioxidant existing in nature and expressed in two optical isomers. The dextrorotatory form is the naturally occurring eutomer, whereas the most used formulation of the compound in clinical practice is the mixture of (+) and (-) enantiomers. Previously, we demonstrated that TIO-(+) is one of the more appropriate antioxidant molecules to prevent cardiac and renal alterations associated with hypertension [5].

The present study was designed to investigate the effect of 4 weeks of treatment with TIO-(+) on the heart of spontaneously hypertensive rats (SHR), using immunochemical and immunohistochemical techniques. 125 mmol/Kg/day of TIO-(+) was administered intraperitoneally in 24-weeks-old SHR. Hypertensive rats were compared to age-matched normotensive Wistar Kyoto (WKY) rats.

Blood pressure values were significantly decreased in treated SHR compared to the control one. This is probably related to the effects at the levels of the endothelial vessels that determine vasodilation. Left ventricular cardiomyocytes' hypertrophy deposition of reticulin, collagen fibers, proteins, and nucleic acid oxidation accompanied by an increased expression of interleukins, such as IL-1 beta, IL-6 and tumor necrosis factor-alpha were found in SHR. These alterations were reduced in TIO-(+) treated animals. The effects observed after treatment with TIO-(+) nominate this molecule as one of the more appropriate antioxidants to prevent heart injury associated with hypertension, opening the opportunity to further evaluations in clinical trials.

References

[1] Messerli FH, Rimoldi SF, Bangalore S. The Transition from Hypertension to Heart Failure: Contemporary Update. JACC Heart Fail. 2017; 5: 543-551.
[2] Eirin A, Lerman A, Lerman LO. Mitochondrial Injury and Dysfunction in Hypertension-Induced Cardiac Damage. Eur Heart J. 2014; 35: 3258-3266.
[3] Rubattu S, Pagliaro B, Pierelli G, Santolamazza C, Di Castro S, Mennuni S, Volpe M. Pathogenesis of Target Organ Damage in Hypertension: Role of Mitochondrial Oxidative Stress. Int J Mol Sci. 2014; 16: 823-839.
[4] Sorriento, D.; De Luca, N.; Trimarco, B.; Iaccarino, G. The Antioxidant Therapy: New Insights in the Treatment of Hypertension. Front. Physiol. **2018**, 9, 258.
[5] Martinelli I, Tomassoni D, Roy P, Di Cesare Mannelli L, Amenta F, Tayebati SK. Antioxidant Properties of Alpha-Lipoic (Thioctic) Acid Treatment on Renal and Heart Parenchyma in a Rat Model of Hypertension. Antioxidants (Basel). 2021;10(7):1006.

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BMC Complementary Medicine and Therapies

Dear Editor-in-Chief,

It is my pleasure to submit a manuscript entitled: “Protective Effects of the (+)-Thioctic Acid Treatment: Possible Anti-Inflammatory Activity on Heart of Hypertensive Rats.” by P. Roy, D. Tomassoni, I. Martinelli, V. Bellitto, G. Nittari, F. Amenta, and myself.

The manuscript contains 29 pages (7 Figures and a Table). The authors have no Conflict of Interest.

Please feel free to contact me with any questions or requests for clarification.

Thank you in advance for your attention.

Yours sincerely,

S.K. Tayebati, PharmD, PhD

PROTECTIVE EFFECTS OF THE (+)-THIOCTIC ACID TREATMENT: POSSIBLE ANTI-INFLAMMATORY ACTIVITY ON HEART OF HYPERTENSIVE RATS

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Abstract

Background: In cardiovascular disease, high blood pressure is associated with oxidative stress, promoting endothelial dysfunction, vascular remodeling, and inflammation. Clinical trials are discordant that the most effective treatment in the management of hypertension seems to be the administration of anti-hypertensive drugs with antioxidant properties. The study aims to evaluate the effects of the eutomer of thioctic acid on oxidative stress and inflammation in the heart of Spontaneously Hypertensive rats compared to normotensive Wistar Kyoto rats. **Methods**: To study the oxidative status, the malondialdehyde and 4-hydroxynonenal concentration, protein oxidation was measured in the heart. Morphological analyses were performed. Immunohistochemistry and Western blot were performed for alpha-smooth muscle actin and transforming growth factor beta to assess fibrosis; cytokines and nuclear factor kappaB to assess inflammatory process. **Results:** Spontaneously hypertensive rats were characterized by hypertension which increased malondialdehyde levels in the heart. OxyBlot in the heart in spontaneously hypertensive rats showed an increase of proteins's oxidative status. Cardiomyocytes hypertrophy and fibrosis in the ventricles were associated with an increased expression of alpha-smooth muscle actin and pro-inflammatory cytokines, reduced by the eutomer of thioctic acid supplementation. **Conclusions:** Based on this evidence, eutomer of thioctic acid could represent an appropriate antioxidant molecule to reduce oxidative stress and prevent inflammatory processes on the cardiomyocytes and cardiac vascular endothelium.

Keywords: hypertension, oxidative stress, inflammation, heart, thioctic acid

1. Introduction

Hypertension or high blood pressure (HBP), a chronic medical condition in which the arterial blood pressure is persistently elevated [1], represents an important public health problem [2]. HBP constitutes the major risk factor for coronary and peripheral arterial disease, heart failure, stroke, atrial fibrillation, vision loss, chronic kidney disease, and cerebrovascular alterations associated with dementia [3]. Increasing evidence attributed the main role of oxidative stress in cardiovascular damage, promoting vascular remodeling, endothelial dysfunction, and inflammation [4]. The increase of reactive oxygen species (ROS) in the cardiomyocytes often is accompanied by structural and functional mitochondria abnormalities [5,6]. The pathogenesis and progression associated with cardiovascular diseases consist in the inflammation of the endothelial lining tunica intima, the initiation of oxidative stress in the arterial wall, the thickening of the blood vessel and subsequently plaque formation in the arteries [7-9].

Complex interacting mechanisms that direct vascular smooth muscle activity includes the renin-angiotensin-aldosterone system, sympathetic nervous innervation, immunological action, and oxidative stress [10]. The upregulation of the renin-angiotensin-aldosterone system, the sympathetic nervous system activation, the impairment of the G protein-coupled receptor signaling, the altered T-cell activity and inflammation have all been linked to the pathogenesis of hypertension [11]. Alterations of cardiomyocytes and other resident cells of the myocardium (fibroblasts, pericytes, endothelial and immune cells) and recruitment of immune and inflammatory cells and progenitor cells from the circulation lead to a complex process known as myocardial remodeling [12-14]. Increased bioavailability of ROS (also known as oxidative stress) is a common feature of this process [15], as are increased nitric oxide (NO) levels and impaired antioxidant capacity in the cardiovascular, renal, and neurological systems [16]. Oxidative stress is fundamental in the establishment of hypertension. The biomarkers of oxidative stress are increased in patients with hypertension, and oxidative damage is correlated with the molecular mechanisms of cardiovascular injury in hypertension [17,18]. Several clinical trials disagree that

the most effective treatment in managing hypertension is the administration of anti-hypertensive drugs with antioxidant properties [19].

Oxidative stress activates different transcription factors, which lead to the differential expression of genes involved in inflammatory pathways [20]. The inflammation triggered by oxidative stress with the activation of the pro-inflammatory molecules like tumor necrosis factor-alpha (TNF-alpha), interleukin-1 beta (IL-1 beta) and inter-leukin-6 (IL-6), and cell adhesion molecules such as E-selectin has an important role in the pathogenesis of vascular remodeling. Consequently, remodeling causes vascular stiffness and rises the blood pressure [21-23].

Thioctic acid (TIO) is a naturally occurring antioxidant compound that comes in two optical isomers [24]. The naturally occurring R-enantiomer (+)-(TIO) is the dextrorotatory form, whereas the most commonly utilized formulation in clinical practice is a combination of (+) and (-) enantiomers [25,26]. (+)-TIO shows the following cells' functions: acts as a cofactor for several important mitochondrial multienzyme complexes; enhances the uptake of glucose; modulates the transcription factors and activates various signaling pathways. It was shown that TIO and its reduced form have a direct antioxidant effect due to the neutralization of ROS that is destructive to DNA, proteins, and lipids of cells. (+)-TIO is synthesized in the human body and is contained in foods in a form covalently associated with lysine (lipoyllysine) [27].

Even if TIO demonstrated a powerful antioxidant activity in vitro, a short half-life and a modest oral bioavailability were found *in vivo* [28]. Indeed, therapeutic efficacy is relatively low due to its pharmacokinetic limitation (hepatic degradation, reduced solubility, and gastric instability). However, TIO’s liquid preparations, new amphiphilic matrices formulations have significantly enhanced TIO bioavailability and, consequently, therapeutic efficacy [25]. Better pharmacokinetic parameters were found in the (+)-TIO [26]. The advantage in using (+)-TIO conjugated with lysine salt compared to the racemic form may be linked to an amplified bioavailability and biological activity of this enantiomer that enhanced the antioxidant activity as shown both *in vitro* [29] and *in vivo* studies [30-33].

Previously, we demonstrated that (+)-(TIO) represents an appropriate antioxidant molecule to reduce oxidative stress and cardiac alterations [30] and adhesion molecule expression in the vascular endothelium of spontaneously hypertensive rats (SHR) [31]. SHR is a rat strain used in assessing hypertensive-related end-organ damage and its possible treatment. This is possibly related to the effects at the levels of the endothelial walls of vessels that determine vasodilation [34].

Left ventricular cardiomyocyte hypertrophy, the deposition of reticulin and collagen fibers, proteins oxidation, and the inflammatory markers were analyzed on the heart of SHR treated or not with (+)-(TIO), detecting the expression of IL-1 beta, IL-6, and TNF-alpha. The hypertensive rats were compared to the age matched normotensive Wistar Kyoto rats (WKY). The results aim to suggest this molecule as a possible antioxidant to prevent heart injury associated with hypertension.

2. Methods

2.1 Animals handling, Treatment and Tissue Processing

SHR developed genetically arterial hypertension [35], and it is used as an animal model of heart failure because its progression of cardiac remodeling toward heart failure is reportedly like that seen in humans [36]. Male SHR aged 20 weeks (n=16) and age-matched WKY rats (n=8) were used. SHRs were treated (n=8) or not (n=8) for 4 weeks with 125 µmol/kg/day of (+)-(TIO) acid lysine salt (Sintactica, Servizi Chimico Farmaceutici, Lotto RALA. L 1911261) solubilized in physiologic solution and intraperitoneally administered [32]. The (+)-(TIO) is the enantiomer R that represents the active form of the racemic compound [30-32]. Control WKY and SHR rats received the same amounts of vehicles. Rats were housed 1 per cage under constant temperature (22-24 °C), and a 12 h light/dark cycle (light on at 07:00), food, and water were available *at libitum.* Rats were handled according to internationally accepted principles for the care of laboratory animals (European Community Council Directive 86/609, O.J. n° L358, Dec. 18, 1986)

and approved by the Ministry of Health based on the D.lgs 26/2014 (Authorization n°163/2019-PR February 25, 2019) after the acceptance of the Committee “Organismo Preposto al Benessere degli Animali” of University of Camerino. Food and water intake were monitored daily. Once a week, conscious rats' systolic and diastolic blood pressures were measured by a tail cuff equipped with a photoelectric pulse detector. Besides, body weight was also revealed. Animals were anesthetized and then perfused. The heart was dissected out, weighed, divided in two parts. One was fixed for 72 h in 4% paraformaldehyde, and processed for the paraffin wax embedding after alcohol dehydration and clarification in xylene. The second part was immediately freeze at -80°C for biochemical analysis.

2.2 Assay of Thiobarbituric Acid Reactive Substances

Lipid peroxidation was quantified in heart homogenates by measuring the accumulation of thiobarbituric acid reactive substances (TBARS) (Cayman, Chemical Company, Ann Arbor, MI, USA, Cat. No. 10009055) and expressed as malondialdehyde (MDA) content [31,37]. The content of MDA was measured spectrophotometrically at 532 nm, using the method described by the company.

2.3 Western Blot

The tissue was homogenized with lysis buffer to obtain proteins as previously described [30,37]. After the protein concentration measurement by Bradford assay (Bio-Rad, Hercules, CA, USA), 40 µg of proteins were separated through SDS-PAGE gel and transferred to nitrocellulose membranes [30,37]. The membranes were blocked for 1 h using 5% Bovine serum albumin and Non-fat dry milk in phosphate-buffered saline (PBS) containing Tween 20 and were then probed overnight at 4°C using one of the primary antibodies as detailed in **Table 1**. Then, the membranes were transferred at room temperature and blotted for 1 h at room temperature with HRP-conjugated secondary antibodies (Bethyl Laboratories, Inc., Montgomery, TX, USA, dilution 1:5000). The detection of band intensities was performed using the Lite blot Plus or Turbo kits

(Euro Clone, Milan, Italy). The optical density was determined through BioRad image analysis (Bio-Rad, Hercules, CA, USA). For quantification, β-actin was used as a protein loading control. Also, the protein oxidation status was investigated in the heart using the OxyBlot Protein Oxidation Detection kit (Merck Millipore, Burlington, MA, USA, Cat. No. S7150), following the company's instruction.

2.4 Morphological analysis and Immunohistochemistry

Longitudinal consecutive heart sections were cut using a rotary microtome (Leica RM 2145) and processed for morphological and immunohistochemistry (IHC) analysis as described previously [30,31,38]. Sections of the heart were stained with Masson's trichrome to evaluate cardiomyocytes' hypertrophy and fibrosis [30,38]. Silver impregnation (Diapath S.p.A., Martinengo, BG, Italy, Ref. 010211) and Sirius red (Direct Red 80, Sigma Aldrich 365548) staining were performed to highlight the reticulin and collagen fibers deposition, respectively. In addition, sections were processed for IHC analysis as previously described [30,31,38] using different antibodies at various dilutions in PBS + TritonX-100 0.3% (PBS-T), as detailed in **Table 1**. Optimal working concentrations for the antibodies were established through preliminary experiments. After incubation overnight with primary antibodies, slides were exposed for 30 min at 25 °C to the specific biotinylated secondary antibodies (Bethyl Laboratories, Inc., Montgomery, TX, USA) diluted 1:200 in PBS-T. The immunoreaction was revealed after the incubation with an avidin-biotin complex (Vector Laboratories, Inc., Burlingame, CA, USA) and consequently using 3,3′-diamonobenzidine tetrahydrochloride (DAB) solution as a substrate (Vector Laboratories, Inc., Burlingame, CA, USA). The sections were observed using a microscope Leica DMR connected by a DS-Ri2 NIKON camera to NIS Elements Nikon image analyzer software (Nikon, Florence, Italy) to record the mean intensity of immune reaction as previously described [30,38]. For confocal microscopy, the sections were incubated with primary antibodies followed by secondary conjugated Alexa Fluor 594, for 1 h at 37 °C, and then counterstained with DAPI. Slides were observed with a Nikon mod. C2 plus Confocal Laser Microscope (Nikon,

Corporation, Japan). Representative pictures were captured at 40× magnification. Mean fluorescence intensity was measured with the Nikon NIS Element software [38].

2.5 Statistical analysis

Data from the WKY, SHR, and SHR (+)-(TIO) groups were compared using one-way ANOVA to examine groups' differences. Tukey's multiple-sample comparison test was used to identify appropriate differences. Data are expressed as means ± S.D. A p-value less than 0.05 was taken as a minimum level of significance between groups.

3. Results

3.1 Blood pressure

The systolic blood pressure was significantly higher in SHR groups compared to the normotensive WKY starting to increase at the 23rd week of age (data not shown). After four weeks of treatment with (+)-TIO starting with the 24th week of age, the systolic measured on the day of sacrifice decreased in the SHR-treated group compared to the control (**Figure 1**). The body weight of hypertensive rats did not change compared to the age-matched WKY, while the heart weight was significantly higher in SHR compared to WKY (**Additional file 1**). Moreover, the heart weight did not change in the (+)-TIO treated group (**Additional file 1**).

3.2 Oxidative stress

The results of the Oxyblot kit showed an increase of oxidized proteins both in the in the heart parenchyma (**Figure 2**) of SHR compared to normotensive WKY rats suggesting that protein oxidation was related to hypertensive status. Treatment with antioxidant (+)-TIO slightly decreased the level of oxidized proteins, in the heart parenchyma (**Figure 2**). Associated with the increase in the oxidative status of proteins the heart showed an increase in the levels of the 4-hydroxynonenal (4-HNE) (**Figures 2**). The supplementation of (+)-TIO did not modify the level of 4-HNE in the heart parenchyma (**Figures 2**). The TBARS kit revealed in the heart of SHR an

increase in the level of MDA, indicating an increase of lipid peroxidation that was decreased by (+)-TIO supplementation (**Figure 2**). The data of increased pro-oxidative elements revealed in SHR an oxidative stress condition related to hypertension, that was counteracted by the (+)-TIO supplementation.

3.3 Morphological aspects

Analyses of the myocardium focused at the subendocardial level showed a clear connective tissue accumulation between the cardiomyocytes in SHR rats (**Figure 3 A-C**), particularly of the reticulin fibers highlighted by the silver impregnation staining techniques (**Figure 3C**). Moreover, an increase in cardiomyocytes area was reported in SHR rats compared to WKY rats (**Additional file 2**). These phenomena were reduced by treatment of (+)-TIO, which significantly decreased the cardiomyocytes area (**Additional file 2**) and the left ventricular fibrosis (**Figure 3 A-C**).

The ventricular fibrosis was related to an increase of the alpha-smooth muscle actin (alpha-SMA) expression (**Figure 4A**) in the heart of SHR compared to age-matched WKY, but it was not associated with the modulation of latent form of the transforming growth factor-beta 1 (TGF-beta 1) (**Figure 4B**). (+)-TIO was able to decrease the alpha-SMA expression in the heart of SHR (**Figure 4A**).

3.4 Inflammation

The expression of the cytokine IL-1beta with a band at 31 kDa was increased in the SHR rats in comparison to the age-matched WKY rats as showed by western blot analysis (**Figure 5A**). A decreased expression was induced by the treatment with (+)-TIO (**Figure 5A**). IL-6 was revealed with a 21 kDa band (**Figure 5B**); its expression was higher in SHR compared to the WKY (**Figure 5B**) without significant modification in the (+)-TIO treated SHR (Figure 5B). TNF-alpha, that is revealed with a band at 28 kDa (**Figure 5C**) showed a similar trend of the IL-1 beta. The results of western blot analysis were confirmed by the immunohistochemical analysis for IL-1 beta, IL-

6, and TNF-alpha that showed an increase in the immunofluorescence intensities of these cytokines in the SHR's heart. This phenomenon was reverted by TIO-treatment (**Figure 6A-C**).

Immunochemical analysis performed on samples of heart for the evaluation of the expression of intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and platelet endothelial cell adhesion molecule-1 (PECAM-1) revealed a band approximately 90 kDa for ICAM-1, 95 kDa for VCAM-1 and 130 kDa for PECAM-1 approximately (**Additional file 3**). The expression of adhesion molecules was significantly increased in heart of SHR (**Additional file 3**). Treatment with (+)-TIO countered VCAM-1 and PECAM-1 but not ICAM-1 expression (**Additional file 3**). Moreover, western blot analysis showed an increase in the expression of endothelial markers E-selectin, with a band of approximately 90 kDa, in the heart of the hypertensive rats compared to the normotensive one and (+)-TIO decreases the expression of this endothelial marker (**Additional file 3**).

The increased expression of cytokines could be related to the expression of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) p50. As shown in **figure 7**, western blot analysis revealed the elevation of NF-kB levels in the heart of SHR compared to the WKY, modulated by the (+)-TIO treatment (**Figure 7A**). This evidence was confirmed by the immunohistochemical analysis (**Figure 7B**). The elevated NF-kB immunoreactivity in cardiomyocyte of SHR was reduced in (+)-TIO group (**Figure 7B**). Collectively, these results indicated as thioctic acid could reduce the inflammatory process due to hypertension at the cardiovascular level.

4. Discussion

Hypertension is a global health problem and is considered the most common risk factor for cardiovascular diseases (CVD). The recent studies describe a link between HBP and inflammation and demonstrate the involvement of oxidative stress in endothelial dysfunction: two of the key processes in the development of hypertension [39].

CVD are complex events with heterogenous pathophysiologic mechanisms in which increased oxidative stress has been viewed as one of the potential common causes. A balance between the presence of ROS and antioxidants is essential for the proper normal functioning of the cells. A variety of CVD is associated, at least partially, with increased production of ROS [40]. ROS constitute both oxygen free radicals, hydroxyl radicals, peroxyl radicals, and non-radicals such as hydrogen peroxide and hypochlorous acid. Endothelial dysfunction caused by oxidative stress and inflammation represents the main factor that causes different CVD [41].

Oxidative stress is implicated, as a major contributing factor, to hypertension development [42]. One hallmark of this damage is endothelial dysfunction, an impairment characterized by a shift in the endothelium with increased vasoconstriction, oxidation, inflammation, thrombosis, and proliferation [43]. However, whether hypertension is the cause of endothelial cell damage is still not clear, although many vascular beds show endothelial damage during hypertension [44]. There are several oxidative-stress-mediated mechanisms involved in the pathogenesis of programmed hypertension, including increased ROS producing enzyme expression, decreased antioxidant capabilities, impaired asymmetric dimethylarginine-NO pathway, increased peroxynitrite, and increased oxidative damage [45].

In the present study, we investigated in SHR the effects of treatment for four weeks with (+)-TIO to analyze the possible protective role of cardiac alterations due to hypertension. Starting from 24 weeks of age, in which the systolic blood pressure is higher in SHR compared to the normotensive WKY, after four weeks of treatment with (+)-TIO, the values of blood pressure were significantly decreased in SHR. This is possibly related to the effects at the levels of the endothelial cells in the vessel. In fact, TIO appears to improve endothelial function through increasing the bioavailability of endothelium-derived NO, decreasing oxidative stress and inflammation [46]. Endothelial cells are important constituents of blood vessels that play key roles in cardiovascular homeostasis [47]. Endothelial dysfunction implicated in the pathophysiology of hypertension is characterized by a reduction of vasodilation, a pro-thrombotic setting, and a pro-inflammatory

state. Excessive ROS formation by vascular walls can mediate these events in the vessels [48]. The vasculature is a major source of NADPH-oxidase-derived ROS, which has a prominent role in vascular damage under pathological conditions [48]. Additionally, endothelium-derived vasoconstricting factors, such as endothelin, urotensin II, vasoconstrictor prostaglandins, angiotensin II, and thromboxane A2, can be released by endothelial cells and contribute to the vasoconstrictor effects. Conversely, reduced NO bioavailability, a well-known endothelium-derived, relaxing factor, is considered a hallmark of endothelial dysfunction [49]. Additionally, endothelium-derived vasoconstricting factors, such as endothelin, urotensin II, vasoconstrictor prostaglandins, angiotensin II, and thromboxane A2, can be released by endothelial cells and contribute to the vasoconstrictor effects. Conversely, reduced NO bioavailability, a well-known endothelium-derived, relaxing factor, is considered a hallmark of endothelial dysfunction [49]. In contrast with our previous results [30,32], data of the present study, showed that (+)-TIO treatment can reduce systolic blood pressure. This effect could be due to the lower basal blood pressure of the SHR strain used in this study. This evidence may represent an important indication of how a therapy with antioxidants can show antihypertensive properties in the early stages of disease development as suggested by Kizhakekuttu et al. [50]. In an animal model of obesity associated with other diseases, including hypertension, TIO decreased the blood pressure at the standard levels [51]. As was previously reported, the antihypertensive role of TIO depends on initial hypertension, and in the case of early hypertension or non-dramatic hypertension, it is effective as a hypotensive drug [51]. Furthermore, as previously demonstrated, dietary TIO acid supplementation in SHRs lowered the systolic blood pressure, cytosolic [Ca^{2+}], blood glucose and insulin levels, tissue aldehyde conjugates, and attenuated adverse vascular changes [52]. TIO has also effects on vascular relaxation in SHR at the level of the aortic smooth muscle cells [53].

Our data supported the cardiac damage in SHR [54-56]. The attempts of reparative mechanisms lead to end-organ damage, principally due to fibrosis [57]. Concerning this, blood vessel remodeling, excessive matrix deposition, and cardiac hypertrophy become maladaptive responses

to abnormal hemodynamic stress related to hypertension [58-60]. In accordance, we showed in SHR rats left ventricular hypertrophy and increased fibrosis with deposition of collagen and reticulin fibers. Moreover, the inflammatory pathway and oxidative stress can be triggered by hypertension. Previously, it was demonstrated an increase in lipids peroxidation and nucleic acid oxidation in plasma, kidneys, and hearts of SHR rats [30,31]. Not only the presence of oxidative stress but also elevated endothelial adhesion molecules such ICAM-1, VCAM-1 and PECAM-1 expression were found in the heart endothelium of hypertensive rats [31]. The present results confirm in the heart of SHR an increased expression of endothelial adhesion molecules related to oxidative stress.

Myocardial injury in long-term hypertension was associated with activation of NF-kB, increased inflammatory cell infiltrate, and an increase expression of the mediators such as IL-1 beta, TNF-alpha, monocyte chemoattractant protein-1, vascular cell adhesion molecule 1, and angiotensinogen [61,62]. Endothelial cells' response to NF-kB activation and related inflammation is characterized by the production of adhesion molecules that promote leukocyte adherence and transmigration while also boosting their thrombogenic potential [63]. The vascular wall of SHR has shown an increase in the mRNA expressions of IL-6, IL-1 beta and TNF-alpha. Similarly, increased expressions of other markers of inflammation, including ICAM, VCAM, monocyte chemoattractant protein (MCP-1) and IL-6, have also been reported in hypertensive rat [64,65]. In agreement, higher mRNA levels of pro-inflammatory cytokines as well as levels of carbonyl protein were reported in different organs, including the heart, of SHR compared to WKY rats [66].

In the myocardium (+)-TIO showed anti-inflammatory properties, with a reduction of the levels of IL-1 beta TNF-alpha related to a decrease of NF-kB. A previous study demonstrated that TIO improved cardiac and renal functions, and downregulated the expression levels of IL-1β, TNF-alpha, and inducible nitric oxide synthase in the myocardium of septic rats [67]. Furthermore, infection and tissue injury release the pro-inflammatory cytokines, including TNF-alpha, IL-1

beta, and IL-6, which contribute to subsection increased systemic inflammatory responses. TNF-alpha induces an ample range of biological effects, including cell differentiation, apoptosis, and multiple pro-inflammatory effects, which trigger the activation of the NF-B signaling pathway [68]. Like other studies, [69] TIO has been shown to suppress NF-kB activation through direct ROS scavenging or even independent of its antioxidant function [70]. Following these studies, the antioxidant capacity of (+)-TIO is correlated to its anti-inflammatory effect in the animal model of hypertension.

These findings highlighted the importance of antioxidants as supplementary molecules to standard anti-hypertensive therapy. Pre-clinical evidence and clinical randomized studies demonstrated the potential anti-hypertensive effect of antioxidant molecules in the diet both in hypertensive and normotensive subjects [50,51]. Moreover, prenatal use of natural antioxidants may reverse programming progressions and avoid hypertension of developmental origin [71]. Also, similar dietary approaches showed a reduction in cardiovascular morbidity and mortality in hypertensive subjects [71]. Antioxidant molecules used more frequently include flavonoids, vitamins A, C, and E, L-arginine, and mitochondria-targeted agents such as TIO, Coenzyme Q10, and acetyl-L-carnitine [50]. The in vitro and in vivo properties of TIO have been widely revised [25,29,72,73], in particular, its antioxidant potential as a free radical scavenger, its action as metal chelators and its activity on the repair of oxidized injury and regeneration of natural antioxidants defense, such as glutathione, vitamins C and E [24, 25,74]. Further important benefits of (+)-TIO supplementation include contributions to mitochondrial metabolic pathways, cell signaling that may increase endothelial nitric oxide synthase (eNOS) coupling, and anti-inflammatory effects [75,76]. For such reasons, this compound has gained great consideration as an antioxidant in the management of diabetic problems like retinopathy, neuropathy, and other vascular diseases [77]. Moreover, the long-term intermittent treatment with (+)-TIO prevented body weight gain and reduced metabolic and cardiac alterations, corroborating its protective properties on the cardiovascular system [78]. Besides, studies in diabetic rats and other different hypertensive

animal models revealed the potential for TIO supplementation to reduce blood pressure [52,78-80].

5. Conclusions

(+)-TIO may be considered as one of the antioxidant candidate molecules for slowing down cardiac alterations as-sociated with hypertension, not only for the prevention the fibrosis but also for the reduction of inflammatory processes. The effects observed with the treatment of (+)-TIO could open new perspectives for a possible coadjuvant care in association with antihypertensive treatment to counteract heart injury, which represents a common feature in hypertensive patients.

Abbreviation:

4-hydroxynonenal (4-HNE)

Alpha-smooth muscle actin (alpha-SMA)

Cardiovascular diseases (CVD)

Endothelial nitric oxide synthase (eNOS)

High blood pressure (HBP)

Immunohistochemistry (IHC)

Interleukin-1 beta (IL-1 beta)

Interleukin-6 (IL-6)

Intracellular adhesion molecule-1 (ICAM-1)

Malondialdehyde (MDA)

Monocyte chemoattractant protein (MCP-1)

Nitric oxide (NO)

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB

Platelet endothelial cell adhesion molecule-1 (PECAM-1)

Reactive oxygen species (ROS)

Spontaneously hypertensive rats (SHR)

Thiobarbituric-reactive substances (TBARS)

Thioctic acid (TIO)

Transforming growth factor-beta 1 (TGF-beta 1)

Tumor necrosis factor-alpha (TNF-alpha)

Vascular cell adhesion molecule-1 (VCAM-1)

Western blot (WB)

Wistar Kyoto rats (WKY)

Ethics approval and consent to participate: Animals were handled according to internationally accepted principles for the care of laboratory animals (European Community Council Directive 86/609, O.J. n° L358, Dec. 18, 1986) and approved by the Ministry of Health based on the D.lgs 26/2014 (Authorization n°163/2019-PR February 25, 2019) after the acceptance of the Committee "Organismo Preposto al Benessere degli Animali" (OPBA) of University of Camerino.

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References

1. Messerli FH, Rimoldi SF, Bangalore S. The Transition from Hypertension to Heart Failure: Contemporary Update. JACC Heart Fail. 2017;5(8):543-551.

2. Sliwa K, Stewart S, Gersh BJ. Hypertension: a global perspective. Circulation. 2011;123:2892-2896.

3. Lau DH, Nattel S, Kalman JM, Sanders P. Modifiable Risk Factors and Atrial Fibrillation. Circulation. 2017;136(6):583-596.

4. Eirin A, Lerman A, Lerman LO. Mitochondrial Injury and Dysfunction in Hypertension-Induced Cardiac Damage. Eur Heart J. 2014;35:3258-3266.

5. Rubattu S, Pagliaro B, Pierelli G, Santolamazza C, Di Castro S, Mennuni S, et al. Pathogenesis of Target Organ Damage in Hypertension: Role of Mitochondrial Oxidative Stress. Int J Mol Sci. 2014;16:823-839.

6. Touyz RM, Briones AM. Reactive oxygen species and vascular biology: implications in human hypertension. Hypertens Res. 2011;34(1):5-14.

7. Guzik TJ, Touyz RM. Oxidative Stress, Inflammation, and Vascular Aging in Hypertension. Hypertension. 2017;70(4):660-667.

8. Petrie JR, Guzik TJ, Touyz RM. Diabetes, Hypertension, and cardiovascular disease: Clinical Insights and Vascular Mechanisms. Can J Cardiol. 2018;34(5):575-584.

9. Beevers G, Lip GY, O'Brien E. ABC of hypertension: The pathophysiology of hypertension. BMJ. 2001;322(7291):912-916.

10. Touyz RM, Alves LR, Rios FJ, Camargo LL, Anagnostopoulou A, Arner A, Montezano AC. Vascular smooth muscle contraction in hypertension. Cardiovasc Res. 2018;114(4):529-539.

11. Pugliese NR, Masi S, Taddei S. The renin-angiotensin-aldosterone system: a crossroad from arterial hypertension to heart failure. Heart Fail Rev. 2020;25(1): 31-42.

12. González A, Ravassa S, López B, Moreno MU, Beaumont J, San José G, et al. Myocardial Remodeling in Hypertension. Hypertension. 2018;72(3):549-558.

13. Kamo T, Akazawa H, Komuro I. Cardiac nonmyocytes in the hub of cardiac hypertrophy. Circ Res. 2015;117(1):89-98.

14. Psarras S, Beis D, Nikouli S, Tsikitis M, Capetanaki Y. Three in a Box: Understanding Cardiomyocyte, Fibroblast, and Innate Immune Cell Interactions to Orchestrate Cardiac Repair Processes. Front Cardiovasc Med. 2019;6:32.

15. Barrows IR, Ramezani A, Raj DS. Inflammation, Immunity, and Oxidative Stress in Hypertension Partners in Crime? Adv Chronic Kidney Dis. 2019;26(2):122-130.

16. Montezano AC, Touyz RM. Molecular mechanisms of hypertension-reactive oxygen species and antioxidants: a basic science update for the clinician. Can J Cardiol. 2012;28(3):288-295.

17. Togliatto G, Lombardo G, Brizzi MF. The Future Challenge of Reactive Oxygen Species (ROS) in Hypertension: From Bench to Bed Side. Int J Mol Sci. 2017;18(9):1988.

18. Barhoumi T, Briet M, Kasal DA, Fraulob-Aquino JC, Idris-Khodja N, Laurant P, et al. Erythropoietin-induced hypertension and vascular injury in mice overexpressing human endothelin-1: exercise attenuated hypertension, oxidative stress, inflammation and immune response. J Hypertens. 2014;32(4):784-794.

19. Baradaran A, Nasri H, Rafieian-Kopaei M. Oxidative Stress and Hypertension: Possibility of Hypertension Therapy with Antioxidants. J Res Med Sci. 2014;19:358-367.

20. Crowley SD. The cooperative roles of inflammation and oxidative stress in the pathogenesis of hypertension. Antioxid Redox Signal. 2014;20(1):102-120.

21. El Midaoui A, de Champlain J. Prevention of hypertension, insulin resistance, and oxidative stress by alpha-lipoic acid. Hypertension. 2002;39(2):303-307.

22. El Assar M, Angulo J, Rodriguez-Manas L. Oxidative stress and vascular inflammation in aging. Free Radic Biol Med. 2013;65:380-401.

23. Deanfield JE, Halcox JP, Rabelink TJ. Endothelial function and dysfunction: testing and clinical relevance. Circulation 2007;115(10):1285-1295.

24. Biewenga GP, Haenen GR, Bast A. The pharmacology of the antioxidant lipoic acid. Gen Pharmacol. 1997;29(3):315-331.

25. Salehi B, Yılmaz YB, Antika G, Tumer TB, Mahomoodally MF, Lobine D, et al. Insights on the Use of α-Lipoic Acid for Therapeutic Purposes. Biomolecules. 2019;9(8):356.

26. Brufani M, Figliola R. (R)-α-lipoic acid oral liquid formulation: pharmacokinetic parameters and therapeutic efficacy. Acta Biomed. 2014;85(2):108-115.

27. Tutelyan VA, Makhova AA, Pogozheva AV, Shikh EV, Elizarova EV, Khotimchenko SA. Lipoic acid: physiological role and prospects for clinical application. Vopr Pitan. 2019;88(4):6-11.

28. Teichert J, Kern J, Tritschler HJ, Ulrich H, Preiss R. Investigations on the pharmacokinetics of alpha-lipoic acid in healthy volunteers. Int J Clin Pharmacol Ther. 1998;36(12):625-628.

29. Tibullo D, Li Volti G, Giallongo C, Grasso S, Tomassoni D, Anfuso CD, et al. Biochemical and clinical relevance of alpha lipoic acid: antioxidant and anti-inflammatory activity, molecular pathways and therapeutic potential. Inflamm Res. 2017;66(11):947-959.

30. Martinelli I, Tomassoni D, Roy P, Di Cesare Mannelli L, Amenta F, Tayebati SK. Antioxidant Properties of Alpha-Lipoic (Thioctic) Acid Treatment on Renal and Heart Parenchyma in a Rat Model of Hypertension. Antioxidants (Basel). 2021;10(7):1006.

31. Tayebati SK, Tomassoni D, Di Cesare Mannelli L, Amenta F. Effect of Treatment with the Antioxidant Alpha-Lipoic (thioctic) Acid on Heart and Kidney Microvasculature in Spontaneously Hypertensive Rats. Clin Exp Hypertens. 2016;38:30-38.

32. Pacini A, Tomassoni D, Trallori E, Micheli L, Amenta F, Ghelardini C, et al. Comparative Assessment of the Activity of Racemic and Dextrorotatory Forms of Thioctic (Alpha-Lipoic) Acid in Low Back Pain: Preclinical Results and Clinical Evidence From an Open Randomized Trial. Front Pharmacol. 2021;12:607572.

33. Tomassoni D, Amenta F, Di Cesare Mannelli L, Ghelardini C, Nwankwo IE, Pacini A, et al. Neuroprotective activity of thioctic acid in central nervous system lesions consequent to peripheral nerve injury. Biomed Res Int. 2013;2013:985093.

34. Rapp JP. Genetic analysis of inherited hypertension in the rat. Physiol Rev. 2000;80:135-172.

35. Tayebati SK, Tomassoni D, Amenta F. Spontaneously hypertensive rat as a model of vascular brain disorder: Microanatomy, neurochemistry, and behavior. J Neurol Sci. 2012;322(1-2):241-249.

36. Doggrell SA, Brown L. Rat models of hypertension, cardiac hypertrophy, and failure. Cardiovasc Res. 1998;39(1):89-105.

37. Martinelli I, Tomassoni D, Moruzzi M, Roy P, Cifani C, Amenta F, et al. Cardiovascular Changes Related to Metabolic Syndrome: Evidence in Obese Zucker Rats. Int J Mol Sci. 2020;21(6):2035.

38. Martinelli I, Tomassoni D, Bellitto V, Roy P, Micioni Di Bonaventura MV, Amenta F, et al. Anti-Inflammatory and Antioxidant Properties of Tart Cherry Consumption in the Heart of Obese Rats. Biology. 2022;11:646.

39. Barrows IR, Ramezani A, Raj DS. Inflammation, Immunity, and Oxidative Stress in Hypertension-Partners in Crime? Adv Chronic Kidney Dis. 2019;26(2):122-130.

40. Baradaran A, Nasri H, Rafieian-Kopaei M. Oxidative stress and hypertension: Possibility of hypertension therapy with antioxidants. J Res Med Sci. 2014;19(4):358-367.

41. Samman Tahhan A, Sandesara PB, Hayek SS, Alkhoder A, Chivukula K, Hammadah M, et al. Association between oxidative stress and atrial fibrillation. Heart Rhythm. 2017;14(12):1849-1855.

42. Rodrigo R, González J, Paoletto F. The role of oxidative stress in the pathophysiology of hypertension. Hypertension. 2011;34:431-440.

43. Briones AM, Touyz RM. Oxidative stress and hypertension: current concepts. Curr Hypertens Rep. 2010;12:135-142, 487.

44. Park JB, Charbonneau F, Schiffrin EL. Correlation of endothelial function in large and small arteries in human essential 489 hypertension. J Hypertens. 2001;19: 415-420.

45. Tain YL, Hsu CN. Oxidative Stress-Induced Hypertension of Developmental Origins: Preventive Aspects of Antioxidant Therapy. Antioxidants (Basel). 2022;11(3):511.

46. Hajizadeh-Sharafabad F, Sharifi Zahabi E. Role of alpha-lipoic acid in vascular function: A systematic review of human intervention studies. Crit Rev Food Sci Nutr. 2022;62: 2928-2941.

47. Konukoglu D, Uzun H. Endothelial Dysfunction and Hypertension. Adv Exp Med Biol. 2017;956:511–540.

48. Bengtsson SH, Gulluyan LM, Dusting GJ, Drummond GR. Novel isoforms of NADPH oxidase in vascular physiology and pathophysiology. Clin Exp Pharmacol Physiol. 2003;30(11):849-54.

49. Li Q, Youn JY, Cai H. Mechanisms and consequences of endothelial nitric oxide synthase dysfunction in hypertension. J Hypertens. 2015;33:1128–1136.

50. Kizhakekuttu TJ, Widlansky ME. Natural antioxidants and hypertension: promise and challenges. Cardiovasc Ther. 2010;28(4):e20-32.

51. El Midaoui A, Fantus IG, Ait Boughrous A, Couture R. Beneficial Effects of Alpha-Lipoic Acid on Hypertension, Visceral Obesity, UCP-1 Expression and Oxidative Stress in Zucker Diabetic Fatty Rats. Antioxidants (Basel). 2019;8(12):648.

52. Vasdev S, Ford CA, Parai S, Longerich L, Gadag V. Dietary alpha-lipoic acid supplementation lowers blood pressure in spontaneously hypertensive rats. J Hypertens. 2000;18(5): 567-73.

53. Cheng PY, Lee YM, Chung MT, Shih YC, Yen MH. Role of AMP-activated protein kinase in α-lipoic acid-induced vasodilatation in spontaneously hypertensive rats. Am J Hypertens. 2012;25(2):152-8.

54. Tomassoni D, Sabbatini M, Amenta F. Effect of different dihydropyridine-type Ca2+ antagonists on left ventricle hypertrophy and coronary changes in spontaneously hypertensive rats. J Cardiovasc Pharmacol. 2003;41(4):544-552.

55. Amenta F, Peleg E, Tomassoni D, Sabbatini M, Rosenthal T. Effect of treatment with lercanidipine on heart of Cohen-Rosenthal diabetic hypertensive rats. Hypertension. 2003;41(6):1330-5.

56. Zhang J, Fallahzadeh MK, McCullough PA. Aging Male Spontaneously Hypertensive Rat as an Animal Model for the Evaluation of the Interplay between Contrast-Induced Acute Kidney Injury and Cardiorenal Syndrome in Humans. Cardiorenal Med. 2016;7(1):1-10.

57. Muñoz-Durango N, Fuentes CA, Castillo AE, González-Gómez LM, Vecchiola A, Fardella CE, Kalergis AM. Role of the Renin-Angiotensin-Aldosterone System beyond Blood Pressure Regulation: Molecular and Cellular Mechanisms Involved in End-Organ Damage during Arterial Hypertension. Int J Mol Sci. 2016;17:797.

58. Santos M, Shah AM. Alterations in cardiac structure and function in hypertension. Curr Hypertens Rep. 2014;16(5):428.

59. Shimiz I, Minamino T. Physiological and pathological cardiac hypertrophy. J Mol Cell Cardiol. 2016;97:245-62.

60. Iliev A, Kotov G, Dimitrova IN, Landzhov B. Hypertension-induced changes in the rat myocardium during the development of cardiac hypertrophy - a comparison between the left and the right ventricle. Acta Histochem. 2019;121(1):16-28.

61. Ares-Carrasco S, Picatoste B, Benito-Martín A, Zubiri I, Sanz AB, Sánchez-Niño MD, et al. Myocardial fibrosis and apoptosis, but not inflammation, are present in long-term experimental diabetes. Am J Physiol Heart Circ Physiol. 2009;297(6):H2109-19.

62. Agarwal D, Haque M, Sriramula S, Mariappan N, Pariaut R, Francis J. Role of proinflammatory cytokines and redox homeostasis in exercise-induced delayed progression of hypertension in spontaneously hypertensive rats. Hypertension. 2009;54:1393-1400.

63. Mussbacher M, Salzmann M, Brostjan C, Hoesel B, Schoergenhofer C, Datler H, et al. Cell Type-Specific Roles of NF-κB Linking Inflammation and Thrombosis. Front Immunol. 2019;10:85.

64. Diep QN, El Mabrouk M, Cohn JS, Endemann D, Amiri F, Virdis A, et al. Structure, endothelial function, cell growth, and inflammation in blood vessels of angiotensin II infused rats: role of peroxisome proliferator-activated receptor gamma. Circulation 2002;105:2296-2302.

65. Tham DM, Martin-McNulty B, Wang YX, Wilson DW, Vergona R, Sullivan ME, et al. Angiotensin II is associated with activation of NF-kappa B-mediated genes and downregulation of PPARs. Physiol Genomics 2002;11:21- 30.

66. Sun L, Gao YH, Tian DK, Zheng JP, Zhu CY, Ke Y, Bian K. Inflammation of different tissues in spontaneously hypertensive rats. Sheng Li Xue Bao. 2006;58:318-23.

67. Jia J, Gong X, Zhao Y, Yang Z, Ji K, Luan T, et al. Autophagy Enhancing Contributes to the Organ Protective Effect of Alpha-Lipoic Acid in Septic Rats. Front Immunol. 2019;10:1491.

68. Ramesh V, Selvarasu K, Pandian J, Myilsamy S, Shanmugasundaram C, Ganesan K. NFκB activation demarcates a subset of hepatocellular carcinoma patients for targeted therapy. Cell Oncol (Dordr). 2016;39(6):523-536.

69. Li Y, Yan H, Zhang Z, Zhang G, Sun Y, Yu P, et al. Andrographolide derivative AL-1 improves insulin resistance through down-regulation of NF-κB signalling pathway. Br J Pharmacol. 2015;172(12):3151-8.

70. Ying Z, Kampfrath T, Sun Q, Parthasarathy S, Rajagopalan S. Evidence that α-lipoic acid inhibits NF-κB activation independent of its antioxidant function. Inflamm Res. 2011;60(3):219-25.

71. Hsu CN, Tain L. Early Origins of Hypertension: Should Prevention Start Before Birth Using Natural Antioxidants? Antioxidants (Basel). 2020;9(11):1034.

72. Smith AR, Shenvi SV, Widlansky M, Suh JH, Hagen TM. Lipoic acid as a potential therapy for chronic diseases associated with oxidative stress. Curr Med Chem. 2004;11(9):1135-46.

73. Shay KP, Moreau RF, Smith EJ, Smith AR, Hagen TM. Alpha-lipoic acid as a dietary supplement: molecular mechanisms and therapeutic potential. Biochim Biophys Acta. 2009;1790(10):1149-60.

74. Camiolo G, Tibullo D, Giallongo C, Romano A, Parrinello NL, Musumeci G, et al. α-Lipoic Acid Reduces Iron-induced Toxicity and Oxidative Stress in a Model of Iron Overload. Int J Mol Sci. 2019;20(3):609.

75. Zhang WJ, Frei B. Alpha-lipoic acid inhibits TNF-alpha-induced NF-kappaB activation and adhesion molecule expression in human aortic endothelial cells. FASEB J. 2001;15(13):2423-32.

76. Rochette L, Ghibu S, Muresan A, Vergely C. Alpha-lipoic acid: molecular mechanisms and therapeutic potential in diabetes. Can J Physiol Pharmacol.2015;93(12):1021-7.

77. Pop RM, Trifa AP, Popolo A, Chedea VS, Militaru C, Bocsan IC, Buzoianu AD. Nigella, sativa. Valuable perspective in the management of chronic diseases. Iran J Basic Med Sci. 2020; 23(6):699-713.

78. Koçak G, Aktan F, Canbolat O, Ozoğul C, Elbeğ S, Yildizoglu-Ari N, Karasu C. ADIC Study Group--Antioxidants in Diabetes-Induced Complications. Alpha-lipoic acid treatment ameliorates metabolic parameters, blood pressure, vascular reactivity and morphology of vessels already damaged by streptozotocin-diabetes. Diabetes Nutr Metab. 2000;13(6):308-18.

79. Takaoka M, Kobayashi Y, Yuba M, Ohkita M, Matsumura Y. Effects of alpha-lipoic acid on deoxycorticosterone acetate-salt-induced hypertension in rats. Eur J Pharmacol. 2001;424(2):121-9.

80. Dudek M, Razny K, Bilska-Wilkosz A, Iciek M, Sapa J, Wlodek L, Filipek B. Hypotensive effect of alpha-lipoic acid after a single administration in rats. Anatol J Cardiol. 2016;16(5):306-9.

Figure Legends

Figure 1. Blood pressure modulation. Systolic blood pressure values in normotensive Wistar Kyoto rats (WKY), spontaneously hypertensive rats (SHR), and SHR treated with (+)-thioctic acid lysine salt [SHR (+)-TIO]. Data, expressed in mmHg, are the mean ± S.D. (n=8/group). *= $p<0.05$ vs WKY: #= $p<0.05$ vs SHR.

Figure 2. Parameters of oxidative stress in the heart parenchyma. Samples of the heart of normotensive Wistar Kyoto rats (WKY), spontaneously hypertensive rats (SHR), and SHR treated with (+)-thioctic acid lysine salt [SHR (+)-TIO] were immunoblotted with OxyBlot (A) and with specific anti-4-hydroxynonenal (4-HNE) antibody (B). For the Oxyblot analysis, the bar graph reports the values of optical density measured in the optical density unit (ODU). 4-HNE bar graph indicates the ratio of densitometric analysis of bands to β-actin levels used as the reference loading control. (C) Concentration of malondialdehyde (MDA) expressed in pmol/mg of tissue. Data are the mean ± S.D. = $p<0.05$ vs WKY: #= $p<0.05$ vs SHR.

Figure 3. Fibrosis in the heart parenchyma. Cardiac sub-endocardium parenchyma in heart tissue of normotensive Wistar Kyoto rats (WKY), spontaneously hypertensive rats (SHR), and SHR treated with (+)-thioctic acid lysine salt [SHR(+)-TIO] were staining with Masson's Trichrome technique for connective tissue, with the Sirius red technique to reveal the collagen fibers and with silver impregnation histochemistry to highlighted reticulin fibers. The accumulation of connective tissue fibers was indicated with the black arrowhead. Magnification 20×. Scale bar: 50 μm.

Figure 4. Fibrosis was related to the increase of the alpha-smooth muscle actin (alpha-SMA). Lysates of the heart from normotensive Wistar Kyoto rats (WKY), spontaneously hypertensive rats (SHR), and SHR treated with (+)-thioctic acid lysine salt [SHR(+)-TIO] were immunoblotted using specific antibodies against alpha-SMA (A) and transforming growth factor-beta 1 (TGF-beta 1) (B). Values indicate the ratio of densitometric analysis of bands and β-actin levels used as

the reference loading control. Blots are representative of each experimental group. Data are mean ± S.D. *= $p<0.05$ vs WKY; #=$p<0.05$ vs SHR.

Figure 5. Modulation of inflammatory markers. Lysates of the heart from normotensive Wistar Kyoto rats (WKY), spontaneously hypertensive rats (SHR), and SHR treated with (+)-thioctic acid lysine salt [SHR (+)-TIO] were immunoblotted with specific anti-bodies against (A) interleukin 1 beta (IL-1 beta), (B) interleukin 6 (IL-6); (C) tumor necrosis factor alpha (TNF-alpha). Graphs values indicate the ratio of densitometric analysis of bands to β-actin levels used as the reference loading control. Data are mean ± S.D. *= $p<0.05$ vs WKY; #=$p<0.05$ vs SHR. Blots are representative of each experimental group.

Figure 6. Up-regulation of inflammatory cytokines. Sections of the heart of normotensive Wistar Kyoto rats (WKY), spontaneously hypertensive rats (SHR), and SHR treated with thioctic acid lysine salt [SHR (+)-TIO] processed for the confocal immunofluorescence of (A) interleukin-1 beta (IL-beta); (B) interleukin 6 (IL-6); (C) tumor necrosis factor-alpha (TNF-alpha). The graphs showed the values of mean fluorescence intensity (MFI). The immunoreactive cardiomyocytes are indicated with the arrowheads. Data are mean ± S.D. *= $p<0.05$ vs WKY; #=$p<0.05$ vs SHR. Magnification 40×, zoom 2. Scale bar: 10 μm. NC, negative control.

Figure 7. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) p50 modulates the inflammatory process. Lysates of the heart from normotensive Wistar Kyoto rats (WKY), spontaneously hypertensive rats (SHR), and SHR treated with thioctic acid lysine salt [SHR(+)-TIO] were immunoblotted with specific antibodies (A) NF-kB; Values indicate the ratio of densitometric analysis of bands to β actin levels used as the loading control. Data are mean ± S.D. *= $p<0.05$ vs WKY; #=$p<0.05$ vs SHR. Blots are representative of each experimental group. Sections of the heart (B) of WKY, SHR, [SHR (+)-TIO] processed for the immuno-histochemistry of NF-kB p50. The immunoreactive cardiomyocytes are indicated with the head arrows. Magnification 20×. Scale bar: 50 μm.

Table 1. Antibodies' dilution for Immunohistochemistry (IHC) and Western Blot (WB) analysis.

Antibodies	Company	Dilution IHC	Dilution WB
4-Hydroxynonenal (4-HNE)	Santa Cruz Biotechnology	/	1:500
Alpha-smooth muscle actin (alpha-SMA)	Sigma Aldrich Cod. A2547	1:100	1:300
Transforming growth factor-beta 1(TGF-beta 1)	Merck-Millipore SAB4502954	/	1:500
E-Selectin	Santa Cruz Biotechnology sc-14011	1:50	1:500
Intercellular Adhesion Molecule-1 (ICAM-1)	Santa Cruz Biotechnology Sc-7891	/	1:500
Vascular Cell Adhesion Molecule-1 (VCAM-1)	Santa Cruz Biotechnology Sc-8304	/	1:500
Platelet endothelial cell adhesion molecule-1 (PECAM-1)	Santa Cruz Biotechnology sc-1506	/	1:500
Tumor necrosis factor-alpha (TNF-alpha)	Bio-Rad AAR33	1:750	1:5000
Interleukin-1 beta (IL-1 beta)	Bio-Rad AAR15G	1:750	1:5000
Interleukin-6 (IL-6)	GeneTex GTX110527	1:200	1:2000
Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB)	Santa Cruz p50 Sc-114	1:50	1:100
Beta-actin (β-actin)	Merck-Millipore A2228	/	1:3000

Additional Files Figures Caption:

Additional File 1. Body weight (A) and heart weight (B) in normotensive Wistar Kyoto rats (WKY), spontaneously hypertensive rats (SHR), and SHR treated with (+)-thioctic acid lysine salt [SHR (+)-TIO]. Data, expressed in grams, are the mean ± S.D. (n=8/group). *= $p<0.05$ *vs* WKY: #= $p<0.05$ *vs* SHR.

Additional File 2. Cardiomyocytes area in normotensive Wistar Kyoto rats (WKY), spontaneously hypertensive rats (SHR), and SHR treated with (+)-thioctic acid lysine salt [SHR (+)-TIO]. Data, expressed in μm2, are the mean ± S.D (n=8/group). *= $p<0.05$ *vs* WKY: #= $p<0.05$ *vs* SHR.

Additional File 3: Modulation of vascular adhesion molecules. Lysates of the heart from normotensive Wistar Kyoto rats (WKY), spontaneously hypertensive rats (SHR), and SHR treated with (+)-thioctic acid lysine salt [SHR (+)-TIO] were immunoblotted with specific antibodies against (A) Intercellular Adhesion Molecule-1 (ICAM-1), (B) Vascular Cell Adhesion Molecule-1 (VCAM-1), (C) Platelet endothelial cell adhesion molecule-1 (PECAM-1), (D) E-selctin. Graphs values indicate the ratio of densitometric analysis of bands to β-actin levels used as the reference loading control. Data are mean ± S.E.M. (n=6/group) *= $p<0.05$ vs WKY; #=$p<0.05$ vs SHR. Blots are representative of each experimental group.

FIGURE 1

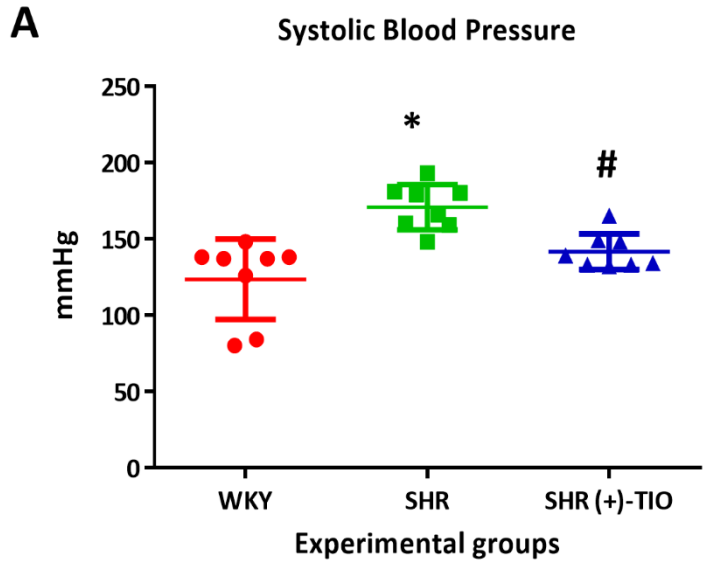

A
Systolic Blood Pressure
250
200
150
100
50
0
mmHg
*
#
WKY
SHR
SHR (+)-TIO
Experimental groups

FIGURE 2

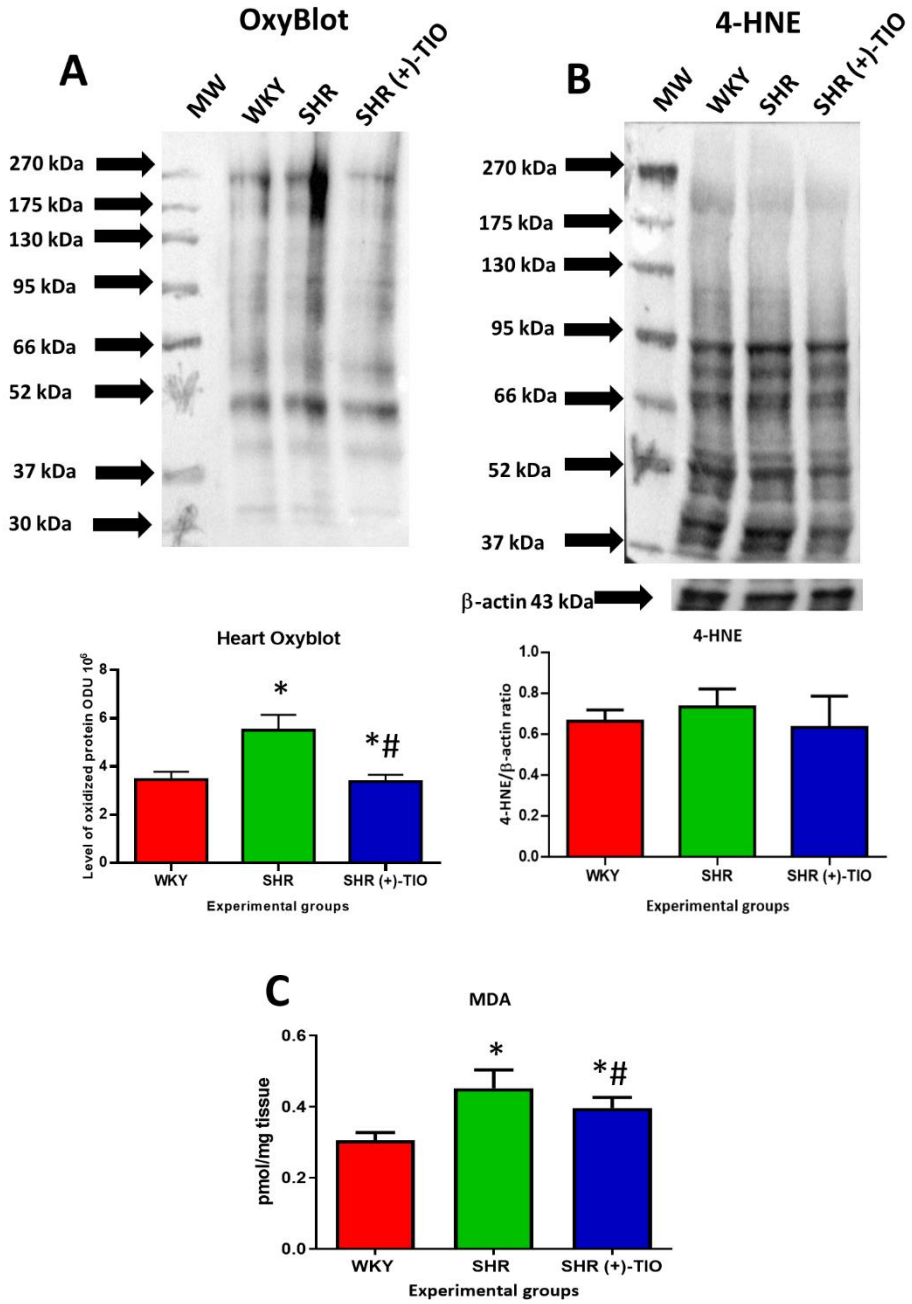

A
OxyBlot
MW
WKY
SHR
SHR (+)-TIO
270 kDa
175 kDa
130 kDa
95 kDa
66 kDa
52 kDa
37 kDa
30 kDa
B
4-HNE
MW
WKY
SHR
SHR (+)-TIO
270 kDa
175 kDa
130 kDa
95 kDa
66 kDa
52 kDa
37 kDa
β-actin 43 kDa
Heart Oxyblot
Level of oxidized protein ODU 10⁶
*
*#
WKY
SHR
SHR (+)-TIO
Experimental groups
4-HNE
4-HNE/β-actin ratio
WKY
SHR
SHR (+)-TIO
Experimental groups
C
MDA
pmol/mg tissue
*
*#
WKY
SHR
SHR (+)-TIO
Experimental groups

FIGURE 3

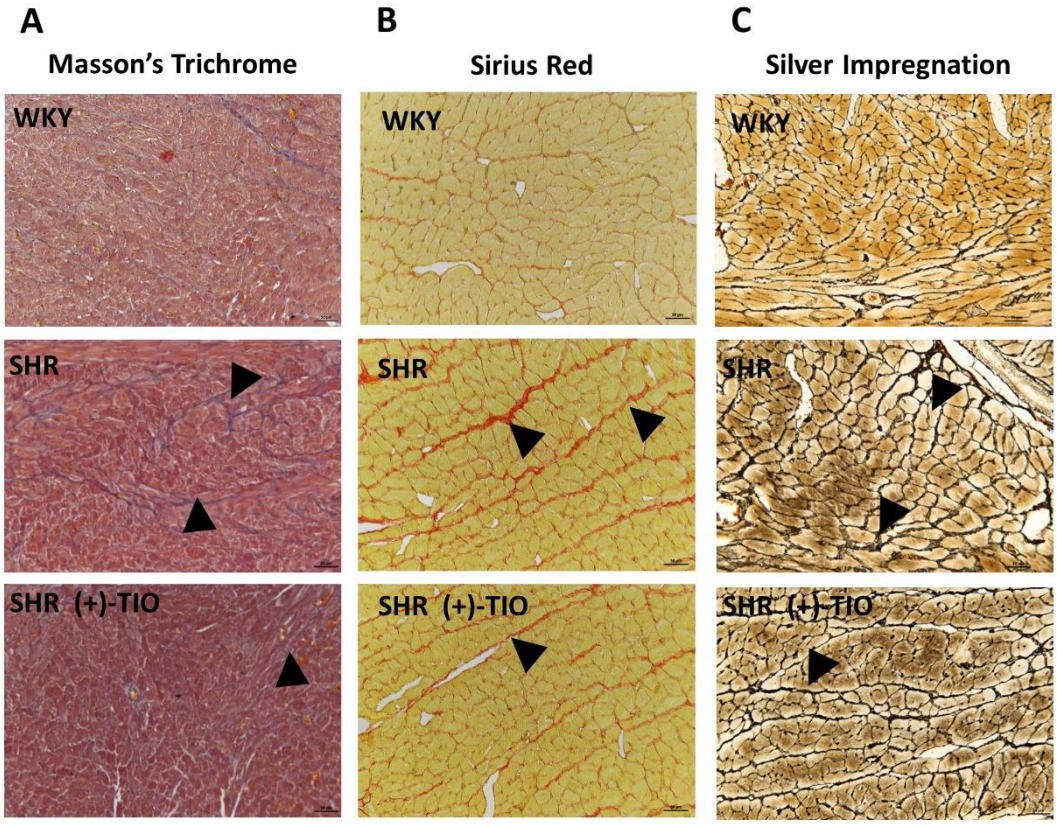

A
Masson's Trichrome
B
Sirius Red
C
Silver Impregnation
WKY
WKY
WKY
SHR
SHR
SHR
SHR (+)-TIO
SHR (+)-TIO
SHR (+)-TIO

FIGURE 4

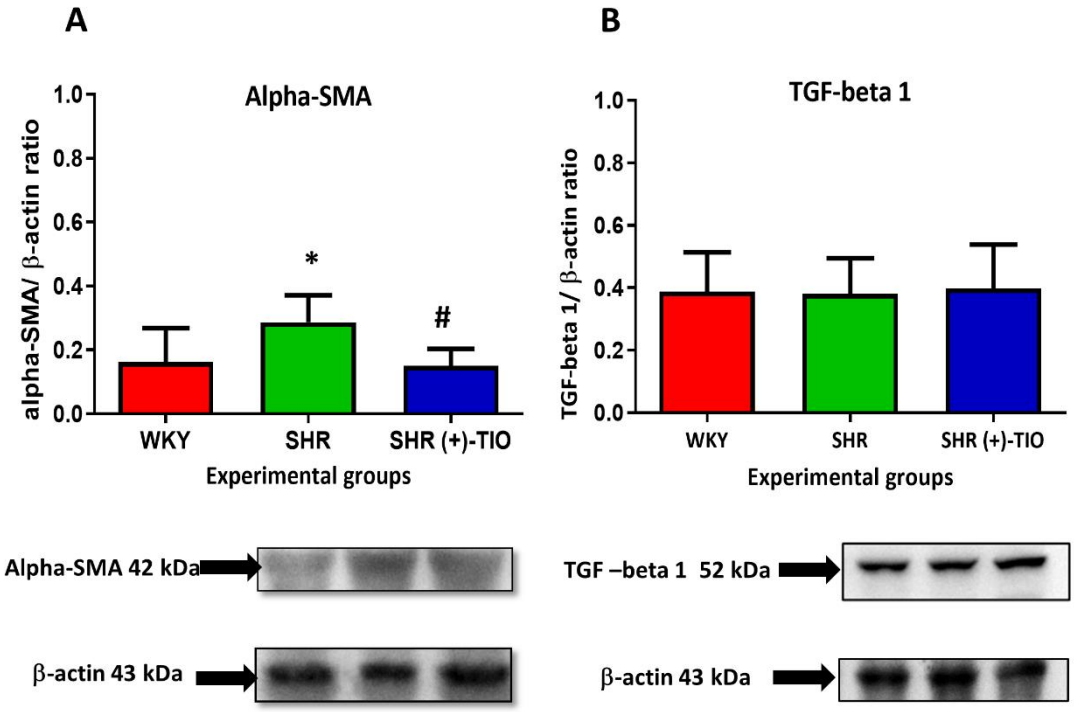

A
Alpha-SMA
alpha-SMA/ β-actin ratio
1.0
0.8
0.6
0.4
0.2
0.0
*
#
WKY
SHR
SHR (+)-TIO
Experimental groups
Alpha-SMA 42 kDa
β-actin 43 kDa
B
TGF-beta 1
TGF-beta 1/ β-actin ratio
1.0
0.8
0.6
0.4
0.2
0.0
WKY
SHR
SHR (+)-TIO
Experimental groups
TGF –beta 1 52 kDa
β-actin 43 kDa

FIGURE 5

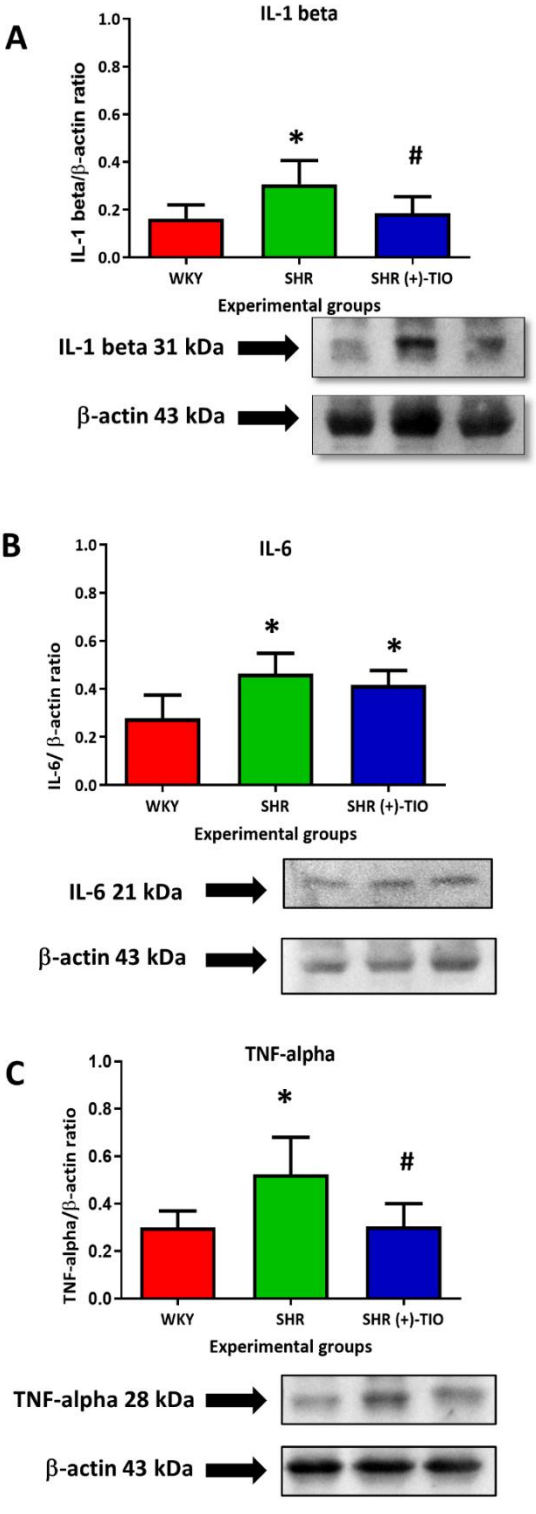

A
IL-1 beta
IL-1 beta/β-actin ratio
1.0
0.8
0.6
0.4
0.2
0.0
*
#
WKY
SHR
SHR (+)-TIO
Experimental groups
IL-1 beta 31 kDa
β-actin 43 kDa
B
IL-6
IL-6/ β-actin ratio
1.0
0.8
0.6
0.4
0.2
0.0
*
*
WKY
SHR
SHR (+)-TIO
Experimental groups
IL-6 21 kDa
β-actin 43 kDa
C
TNF-alpha
TNF-alpha/β-actin ratio
1.0
0.8
0.6
0.4
0.2
0.0
*
#
WKY
SHR
SHR (+)-TIO
Experimental groups
TNF-alpha 28 kDa
β-actin 43 kDa

FIGURE 6

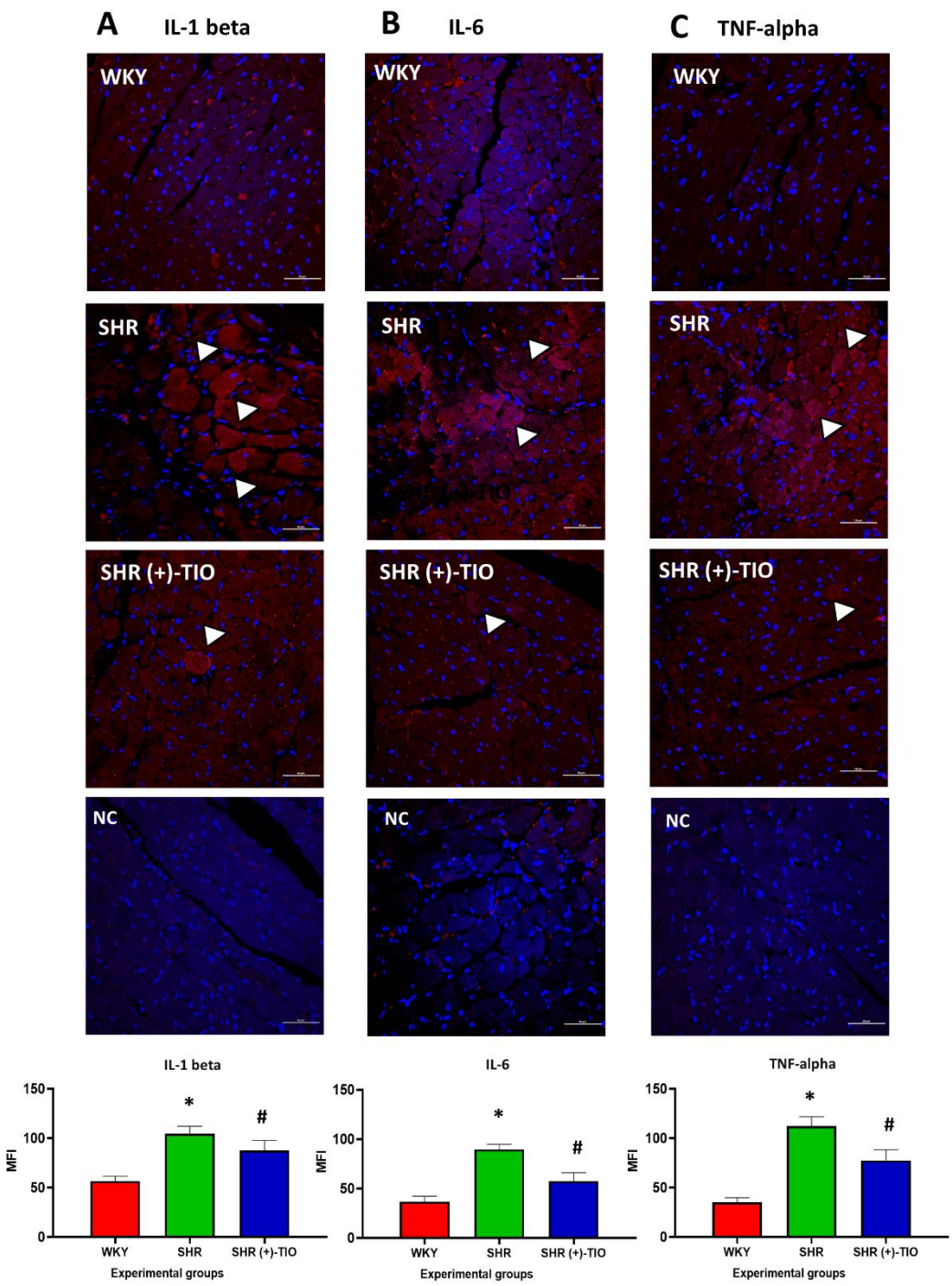
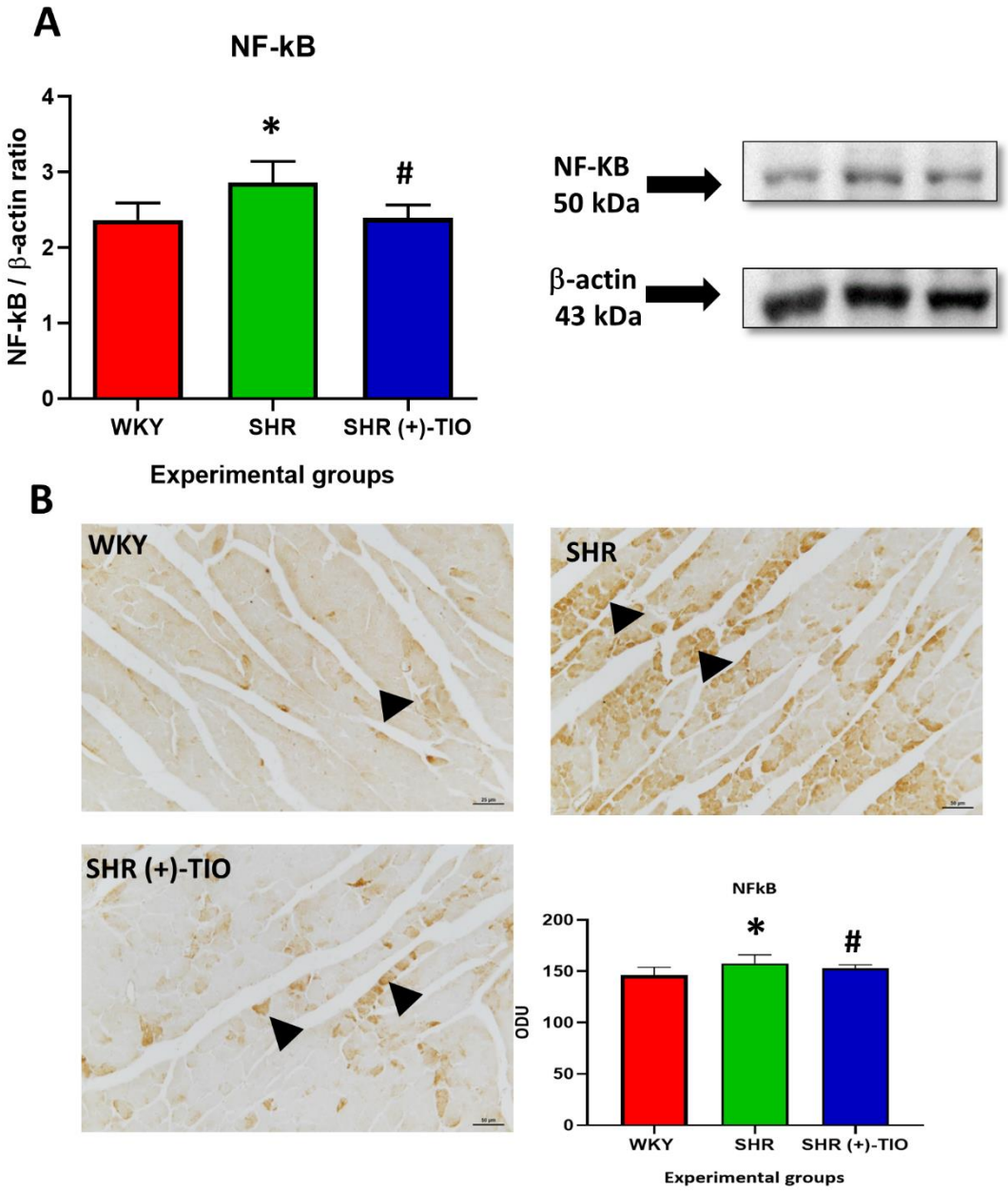
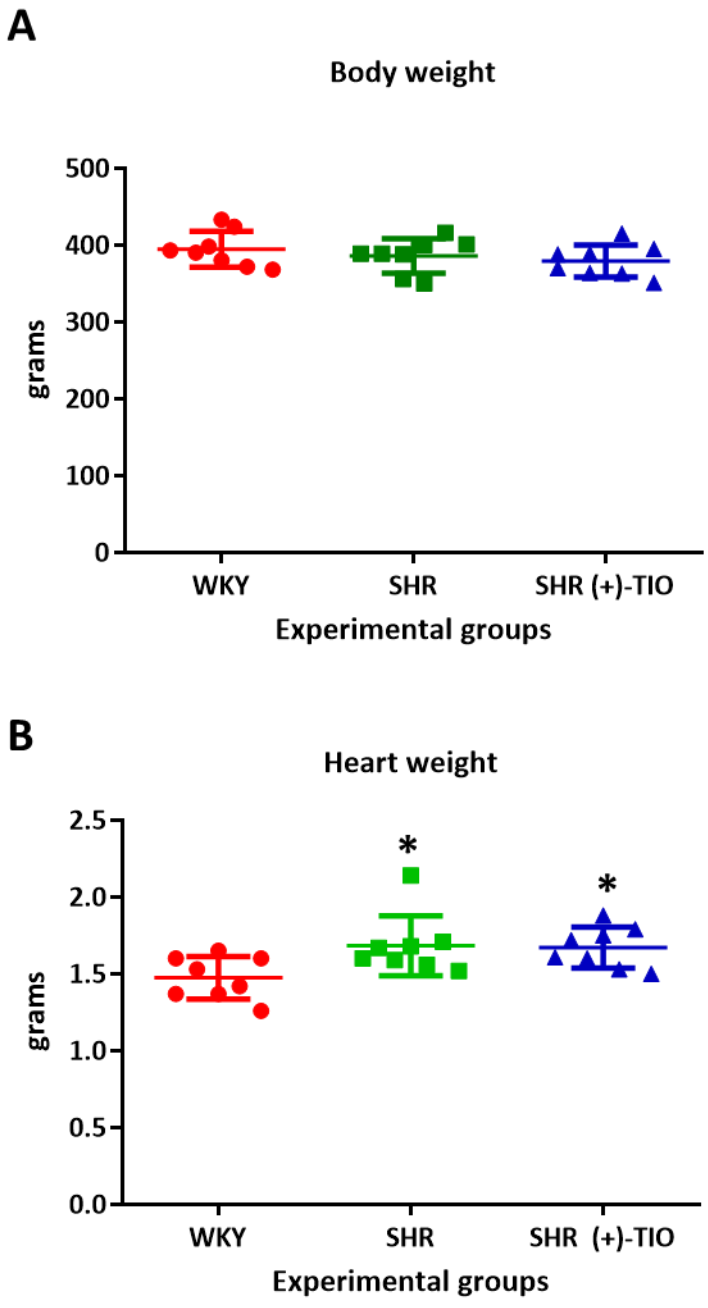

A
IL-1 beta
B
IL-6
C
TNF-alpha
WKY
SHR
SHR (+)-TIO
NC
IL-1 beta
IL-6
TNF-alpha
MFI
150
100
50
0
*
#
WKY
SHR
SHR (+)-TIO
Experimental groups

FIGURE 7

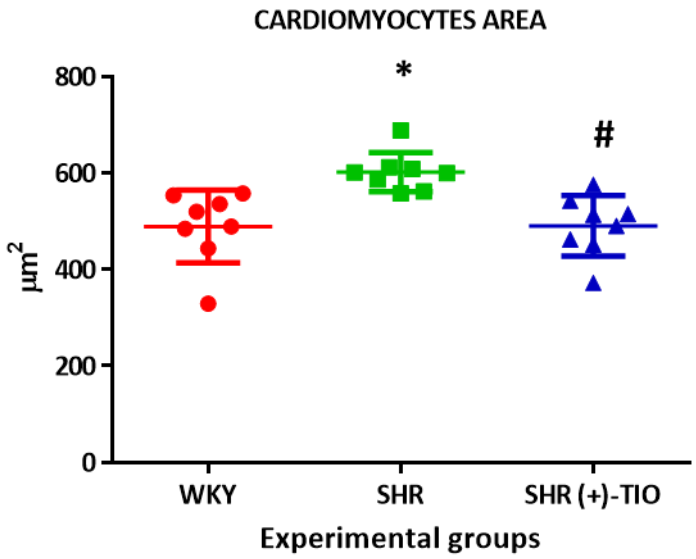

A
NF-kB
NF-kB / β-actin ratio
4
3
2
1
0
*
#
WKY
SHR
SHR (+)-TIO
Experimental groups
NF-KB
50 kDa
β-actin
43 kDa
B
WKY
SHR
SHR (+)-TIO
NFkB
ODU
200
150
100
50
0
*
#
WKY
SHR
SHR (+)-TIO
Experimental groups

ADDITIONAL FILES

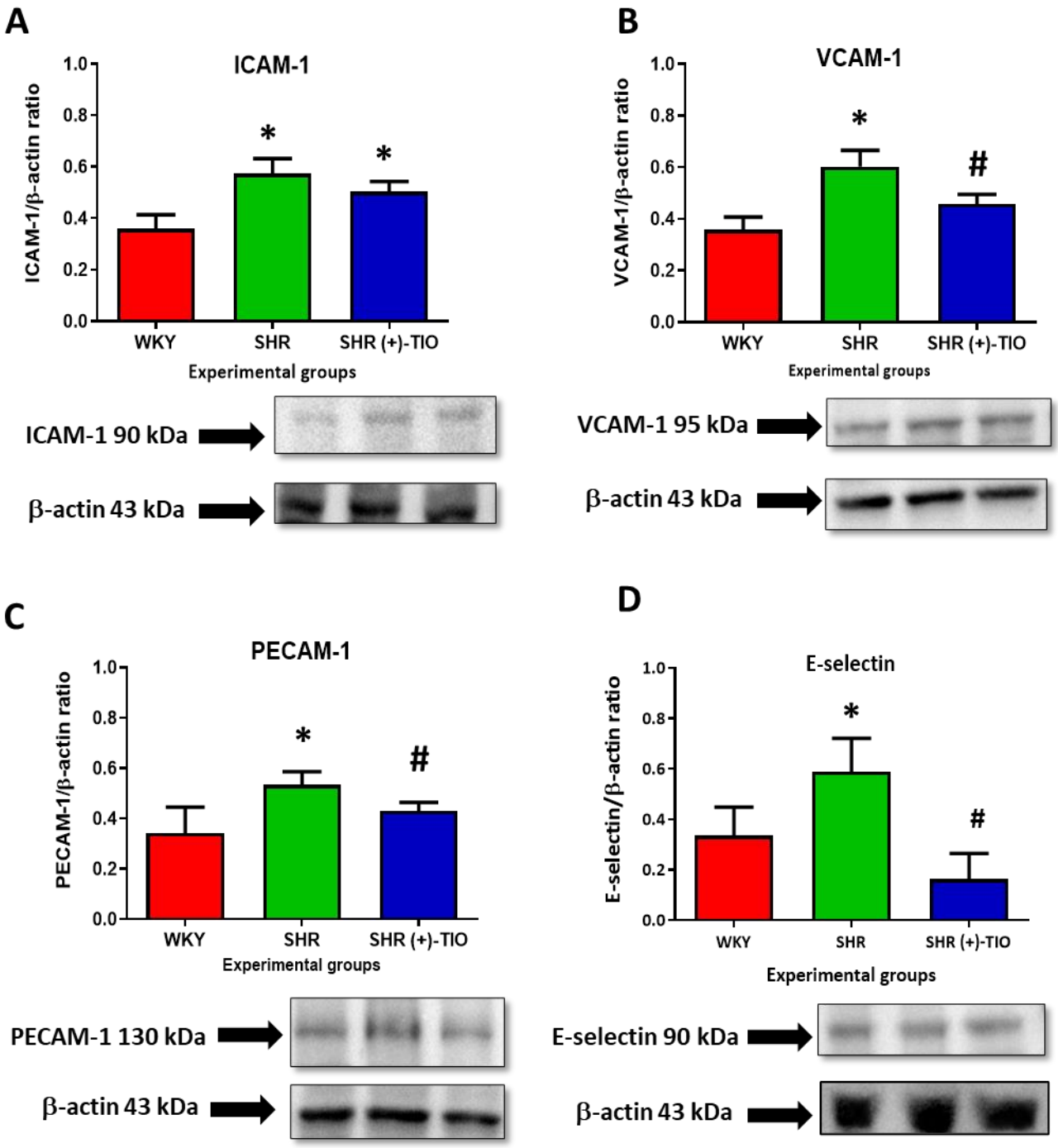
Additional file 1



Additional file 2



Additional File 3



cells

MDPI

Article

Altered Brain Cholinergic and Synaptic Markers in Obese Zucker Rats

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Abstract: The association between obesity and loss of cognitive performance has been recognized. Although there are data regarding the metabolic alterations in obese conditions and the development of neuroinflammation, no clear evidence concerning obesity-related cholinergic and synaptic impairments in the frontal cortex and hippocampus has been reported yet. Here, we investigate different cholinergic and synaptic markers in 12-, 16-, and 20-week-old obese Zucker rats (OZRs) compared with lean littermate rats (LZRs), using immunochemical and immunohistochemical analysis. Consequently, OZRs showed body weight gain, hypertension, and dysmetabolism. In 20-week-old OZRs, the reduction of vesicular acetylcholine transporter (VAChT) and alpha7 nicotinic acetylcholine receptors (α7nAChR) occurred both in the frontal cortex and in the hippocampus, suggesting a cognitive dysfunction due to obesity and aging. Among the muscarinic receptors analyzed, the level of expression of type 1 (mAChR1) was lower in the hippocampus of the older OZRs. Finally, we showed synaptic dysfunctions in OZRs, with a reduction of synaptophysin (SYP) and synaptic vesicle glycoprotein 2B (SV2B) in 20-week-old OZRs, both in the frontal cortex and in the hippocampus. Taken together, our data suggest specific alterations of cholinergic and synaptic markers that can be targeted to prevent cognitive deficits related to obesity and aging.

Keywords: brain; cholinergic system; synaptic transmission; metabolic syndrome; obesity

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1. Introduction

Nowadays, obesity and obesity-related disorders have become widespread conditions. Obesity and the closely related metabolic syndrome (MetS) cause a considerable risk of developing type 2 diabetes mellitus (T2DM), cardiovascular disease (CVD), and other complications [1–3]. The major driver of the increasing obesity and diabetes epidemic is the current obesogenic environment, proffering high-calorie foods and physical inactivity. However, not everyone exposed to this environment gains weight or develops T2DM. Indeed, a genetic predisposition or heritability is reported to contribute to obesity and T2DM [4].

Studies have explored biological mechanisms to explain the negative effects of a high-fat diet (HFD) on cognitive performance. Among them, insulin resistance, inflammation, oxidative stress, altered membrane functioning, and vascularization represent the most documented [5–7]. An HFD is commonly used to study obesity in mice, and neural inflammation can be assessed even before substantial weight gain [8,9]. In these murine models of diet-induced obesity (DIO), the increased fatty acid (FA) intake increases the activation of immune cells and the inflammatory response in different organs [10]. Briefly, the binding of FAs to Toll-like receptor 4 (TLR4) activates two different transcription factors, nuclear factor κB, (NF-κB) and activator protein 1 (AP-1), that in turn upregulate

the expression of proinflammatory mediators, such as cytokines and chemokines [11]. In DIO animals, we have confirmed the presence of hyperglycemia, insulin resistance, and hypertension, accompanied by astrogliosis, microglial activation, and endothelial inflammation in the frontal cortex and in the hippocampus [12]. Among various animal models that have been developed and routinely used to study the pathogenesis and mechanisms of obesity/T2DM, the non-leptin-deficient DIO mice or rats and genetically obese mice or rats, i.e., leptin-receptor-deficient obese (*fa/fa)* Zucker rats (OZRs), remain the most widely used experimental models.

Preclinical genetic models such as *ob/ob* and *db/db* mice or the OZRs were pivotal in unraveling many signaling pathways involved in obesity. As well as in the DIO model, we have previously reported in OZRs blood–brain barrier (BBB) alterations, neuronal loss, and gliosis both in the frontal cortex and in the hippocampus [13]. The behavioral tests revealed cognitive alterations in older OZRs as well as in DIO rats [12,14].

The cholinergic system has been revealed to be involved in the regulation of food intake and energy expenditure. Moreover, physical exercise promotes a reduction of fat pads and body mass by increasing energy expenditure but also influences the cholinergic system and synaptic markers [15,16]. Indeed, the beneficial effects of physical exercise on cognitive functions have been well documented in the studies of both rodents and humans [17]. The brain's cholinergic signaling and the *vagus* nerve have a crucial role in the regulation of metabolic homeostasis and the immune function. Studies supported the therapeutic efficacy of cholinergic stimulation in alleviating obesity-associated metabolic derangements and neuroinflammation [18–22]. The mechanisms of the inflammatory reflex include alpha7 nicotinic acetylcholine receptor (α7nAChR)-mediated signaling in its efferent arm. Cholinergic drugs, including α7nAChR agonists and acetylcholinesterase (AChE) inhibitors, have also been shown to be cognitive enhancers and to reduce inflammation and metabolic imbalances in obesity and in MetS [22,23]. For instance, donepezil reversed obesity-related central inflammation and oxidative damage and improved memory impairments in HFD-fed mice [24]. In addition, galantamine showed anti-inflammatory and beneficial metabolic effects in patients with MetS [25]. The muscarinic acetylcholine receptors (mAChRs) were also found to be strongly influenced by obesity in DIO rats [26].

Not only the cholinergic parameters seem to be altered because of the obese condition but synaptic marker expression in prefrontal and perirhinal cortex also decreased in DIO rats, accompanied by decreased dendritic spine density and finally cognitive deficits [27]. Microglial morphology was also changed in the prefrontal cortex. Synaptic proteins, including vesicle-associated with the pre- and postsynaptic membrane proteins, are closely related to cognitive function. Previous studies have shown that the loss of synapses in the brain tissues of patients with Alzheimer's disease (AD) was associated with cognitive impairment [28,29]. Synaptophysin (SYP), a specific presynaptic marker of vesicles that reflects the density and distribution of synapses, serves a crucial role in neural plasticity, influencing the synaptic structure and mediating neurotransmitter release via phosphorylation [30].

Although there are numerous data regarding the metabolic alterations in obese conditions and the development of neuroinflammation [8,9,12,14,31], no mechanism has been presented concerning obesity-related cholinergic and synaptic impairments in the brain. Therefore, this study was designed to investigate whether the memory and learning impairments in older OZRs [14] were also related to cerebral cholinergic and synaptic alterations, specifically identifying the markers that were implicated. The investigation was carried out in brain areas, in which cholinergic neurotransmission is widely represented: the frontal cortex, especially the motor region, and the hippocampus, which is involved in learning and memory tasks [14]. The OZR is a model of MetS for the concomitant manifestation of obesity, hyperglycemia, hyperinsulinemia, hyperlipidemia, and moderate hypertension [13,14,32], compared to littermate lean Zucker rats (LZRs).

2. Materials and Methods

2.1. Ethical Animal Handling

Experimental procedures were carried out according to the Institutional Guidelines and complied with the Italian Ministry of Health (D. Lgs. 116/92–Art. 7) (Prot. N. 6198/2011) and associated guidelines from European Communities Council Directive (n. 86/609/CEE) governing animal welfare and protection.

2.2. Animals

Male OZRs (n = 18) and their littermate lean Zucker rats (LZRs) (n = 18) were purchased from Harlan (Italy). They were grouped into six animals for each strain based on the age at sacrifice, performed at 12, 16, and 20 weeks of age, as previously described [14,32]. Based on previous studies [13,33–35], the age at sacrifice and the number of animals for each experimental group was identified. Starting from the 10th week of age, the rats were housed in one for cage and maintained on a 12 h light/dark cycle (lights on at 7 a.m.). They were fed with standard diet (Mucedola 4RF18 mice and rats long-term maintenance, containing 16% protein, 2.5% fat, and 7.5% max fiber and other nutritional additives) with *ad libitum* access to food and water. Body weights were measured daily. Values of systolic blood pressure were recorded once a week by tail-cuff methods using an electronic sphygmomanometer (B3Plus, GIMA, Italy) on conscious rats [14,32].

2.3. Biochemical Analysis

Before sacrifice, blood withdrawals were performed from the tail vein in fasted rats. Blood samples were collected into tubes with L-heparin. Serum was separated by centrifugation of samples at 3000 rpm for 10 min to measure the blood glucose, insulin, triglycerides, and total cholesterol, as previously described [14,32].

2.4. Tissue Handling

The brains were carefully removed and divided into two hemispheres. In the right hemisphere, the frontal cortex and hippocampus were collected and frozen at −80 °C for Western blot analysis, while the left one was fixed in 4% paraformaldehyde in 0.1 M pH 7.4 phosphate-buffered saline (PBS) and embedded in paraffin wax for immunohistochemical analysis [14].

2.5. Western Blot (WB) and Quantification

Protein lysate was obtained by homogenizing brain areas (100 ± 2 mg) in a Mixer Mill MM300 (Qiagen, Hilden, Germany) for 10 min, using lysis buffer. Next, 40 µg of proteins were separated on SDS polyacrylamide gels, transferred onto nitrocellulose membranes, and blotted with the specific antibodies as previously described [14,32]. After incubation with blocking solution (5% BSA in PBS 0.1% Tween-20), membranes were incubated at 4 °C overnight with the primary antibodies as detailed in Table 1. The specificity of immune reaction was assessed using antibodies pre-adsorbed with peptides employed for generating them [36,37]. The blots were then incubated for 1 h at room temperature with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (BETHYL Laboratories, Inc., Montgomery, TX, USA, dilution 1:5000). LiteAblot PLUS or Turbo kits (EuroClone, Milan, Italy) were used as the detection system followed by densitometric analysis carried out by Quantity One software of the ChemiDoc apparatus (Bio-Rad, Hercules, CA, USA), using GAPDH as the loading control. Blots are representative of one of three experimental sessions.

2.6. Immunohistochemistry (IHC) and Image Analysis

The paraffin-embedded tissue from each rat was sectioned at 10 µm with a microtome. Five groups of ten consecutive sagittal sections were attached to poly-l-lysine-coated slides. As previously described [14] the first of each group of ten consecutive sections was stained with a 0.5% cresyl violet to highlight the possible morphological alterations. The others

were processed independently for immunohistochemistry using different antibodies at various dilutions in PBS + TritonX-100 0.3% (PBS-T), as detailed in Table 1. Optimal antibody concentration and specificity of the antibodies were established in a series of preliminary experiments in which parallel control slides were exposed to the same antibody absorbed with the blocking peptide for 3 h at 4 °C [36,37]. The immune reaction was revealed by exposing slides for 30 min at 25 °C (dilution 1:200 in PBS-T) to the specific biotinylated secondary antibodies (BETHYL Laboratories, Inc., Montgomery, TX, USA). The sections were incubated with an avidin–biotin kit (Vector Laboratories, Inc., Burlingame, CA, USA, dilution 1:100) using as substrate a 3,3′-diamonobenzidine tetrahydrochloride (DAB) solution (Vector Laboratories, Inc., Burlingame, CA, USA). The sections were observed with a microscope Leica DMR connected by a DS-Ri2 NIKON camera to NIS Elements Nikon image analyzer software (Nikon, Florence, Italy) to record the mean intensities of immune reaction as previously described [14].

Table 1. Primary antibodies used in Western blot (WB) and immunohistochemistry (IHC).

Antibodies	Company and Cat. No	Dilution WB	Dilution IHC
Vesicular acetylcholine transporter (VAChT)	Santa Cruz Biotechnology Cat. sc7717	1:500	1:100
Alpha7 nicotinic acetylcholine receptor (α7nAChR)	Santa Cruz Biotechnology Cat. sc5544	1:500	1:50
Muscarinic acetylcholine receptor subtype 1 (mAChR1)	Santa Cruz Biotechnology Cat. sc9106	1:500	1:50
Muscarinic acetylcholine receptor subtype 3 (mAChR3)	Santa Cruz Biotechnology Cat. sc7474	1:500	1:50
Muscarinic acetylcholine receptor subtype 5 (mAChR5)	Santa Cruz Biotechnology Cat. sc7479	1:500	1:50
Synaptophysin (SYP)	EMD Millipore Cat. MAB368	1:500	1:200
Synaptic vesicle glycoprotein 2A (SV2A)	Santa Cruz Biotechnology Cat. sc11939	1:200	1:50
Synaptic vesicle glycoprotein 2B (SV2B)	Santa Cruz Biotechnology Cat. sc11943	1:200	1:50
Synaptic vesicle glycoprotein 2C (SV2C)	Santa Cruz Biotechnology Cat. sc11946	1:200	1:50
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Sigma Aldrich Cat. G9295	1:5000	/

2.7. Immunofluorescence

For confocal laser microscopy, slides were incubated with vesicular acetylcholine transporter (VAChT) primary antibody (Table 1), followed by incubation with donkey anti-goat Alexa Fluor 488 secondary antibody (1 h at 37 °C) and then counterstained with DAPI (1:100 in PBS-T). Sections were viewed using a Nikon mod. C2 plus Confocal Laser Microscope (Nikon, Corporation, Japan). Representative pictures were captured at 60× magnification, zoom 2×.

2.8. Statistical Analysis

Means of different parameters investigated were calculated from single-animal data, and expressed as the means ± S.E.M. The significance of differences between means was estimated by analysis of variance (ANOVA) followed by the Bonferroni multiple range tests, setting $p < 0.05$ value as a significant difference.

3. Results

3.1. General and Blood Analysis

The value of body weight, as well as the food intake, were significantly higher in OZRs than in LZRs, starting from 10 weeks of age until 20 weeks of age. Serum analyses showed that glucose and insulin were higher in OZRs than in LZRs in all weeks. Furthermore, triglycerides levels were higher, and total, LDL, and HDL cholesterol increased proportionally to age in the obese animals, indicating a condition of dysmetabolism [14,32]. Moreover, the values of systolic blood pressure were significantly higher starting from

16 weeks of age in OZRs (140.8 ± 5.6 mmHg at 16 weeks, $p = 0.002$ and 137.3 ± 4.2 mmHg at 20 weeks of age, $p = 0.007$ vs. age-matched LZRs).

3.2. Cholinergic Marker: Vesicular Acetylcholine Transporter

Western blot analyses, performed in the frontal cortex (Figure 1a) and the hippocampus (Figure 1c), showed a decrease in the expression of VAChT with a band around 80 kDa, corresponding to its mature glycosylated form [36], in OZRs compared to control LZRs at 20 weeks of age. In the 16-week-old obese phenotype, the expression of VAChT was significantly reduced in the hippocampus (Figure 1c) but not in the frontal cortex (Figure 1a). In line with the Western blot was the immunohistochemistry analysis in which the average intensity values of VAChT were remarkably reduced in 20-week-old OZRs, both in the frontal cortex (Figure 1b) and in the hippocampus (Figure 1d). Immunofluorescent procedures revealed that VAChT labeled the neuronal soma of the pyramidal neurons and along the cholinergic fibers in the frontal cortex and in the CA1 subfield of the hippocampus, as showed by representative pictures (Figure 1e).

3.3. Synaptic Markers

3.3.1. Cholinergic Receptors

Among the nicotinic receptors, the alpha7 subunit (α7nAChR) constitutes one of the predominant nAChR subtypes in the mammalian brain [38] and is widely expressed pre- and postsynaptically also in the hippocampus [39]. Immunochemical analyses for the α7nAChR showed a band around 55 kDa both in the frontal cortex (Figure 2a) and in the hippocampus (Figure 2c). In both these areas, protein quantification demonstrated a reduction of α7nAChR expression in OZRs, in particular at 20 weeks, compared with age-matched lean rats (Figure 2a,c). Immunoreactivity for the nicotinic receptor α7nAChR was localized in the pyramidal neurons in the fifth (V) layer of the frontal cortex (Figure 2b). At the level of the hippocampus, pyramidal neurons were reactive both in the CA1 subfield (Figure 2d) and in subfield CA3. The immunoreaction of α7nAChR was reported to be significantly reduced in older obese rats, both in the frontal cortex (Figure 2b) and in the hippocampus (Figure 2d).

We decided to explore the expressions of the following muscarinic receptors: mAChR1, mAChR3, and mAChR5, based on the most abundant localization both in the cerebral cortex and hippocampus [40,41], and the fact that all they are expressed predominantly postsynaptically, G_q coupled, and stimulated by the phospholipase C (PLC) and inositol trisphosphate (IP3) signal transduction pathways to increase cytosolic calcium levels [42]. The results showed that, among the muscarinic receptors analyzed (Figures 3 and 4), only mAChR1 was reduced in obese conditions (Figure 3a–d).

Western blot results for the mAChR1 showed a band around 50 kDa in the frontal cortex (Figure 3a) and the hippocampus (Figure 3c). In 20-week-old OZRs, the expression of mAChR1 was significantly reduced compared to that in age-matched LZRs in the hippocampus (Figure 3c) but not in the frontal cortex (Figure 3a). In addition, the immunohistochemistry analysis confirmed a lower mAChR1 immunoreaction at 20 weeks of age in obese conditions compare to that in control lean rats in both the areas investigated (Figure 3b,d).

mAChR3 and mAChR5 receptors were expressed at around 80 and 55 kDa, respectively (Figure 4a,c). Neither the levels of mAChR3 and mAChR5 (Figure 4a,c) nor their immunoreaction (Figure 4b,d) were significantly different among the animals. Indeed, similar values were reported between the age-matched opposite groups (Figure 4a–d). Both these receptor subtypes were present in the fifth layer of the frontal cortex, and in the CA1 and CA2 subfields of the hippocampus.

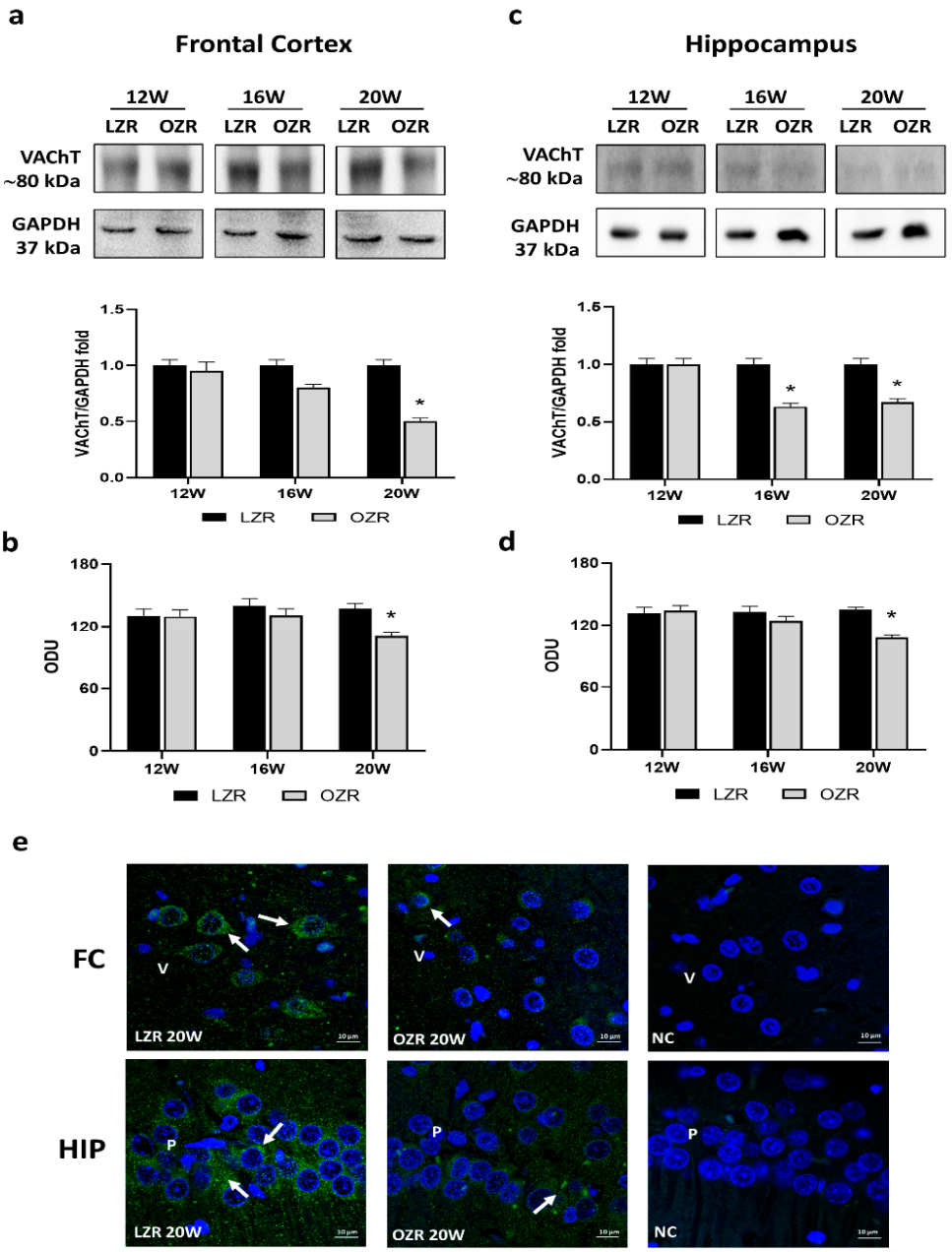

Figure 1. Immunoblotting and immunofluorescence of vesicular acetylcholine transporter (VAChT). Lysates of the frontal cortex (**a**) and hippocampus (**c**) from lean Zucker rats (LZRs) and obese Zucker rats (OZRs) at the age of 12, 16, and 20 weeks were immunoblotted using specific anti-VAChT. Bar graphs indicate the densitometric analysis using LZRs as control, and GAPDH levels were used as a loading control. Blots are representative of one of three separate experiments; intensity values of VAChT immunostaining in the frontal cortex (**b**) and hippocampus (**d**) from LZRs and OZRs at the age of 12, 16, and 20 weeks measured in optical density units (ODUs). Data are mean ± S.E.M. * $p < 0.05$ vs. age-matched LZRs. (**e**) Representative immunofluorescence pictures of 20-week-old LZRs and OZRs in the frontal cortex (FC) and hippocampus (HIP). Arrows indicate VAChT, labeling. V: the fifth layer of the frontal cortex. NC: negative control. P: pyramidal neurons of the hippocampus. 60× magnification zoom 2×. Calibration bar: 10 μm.

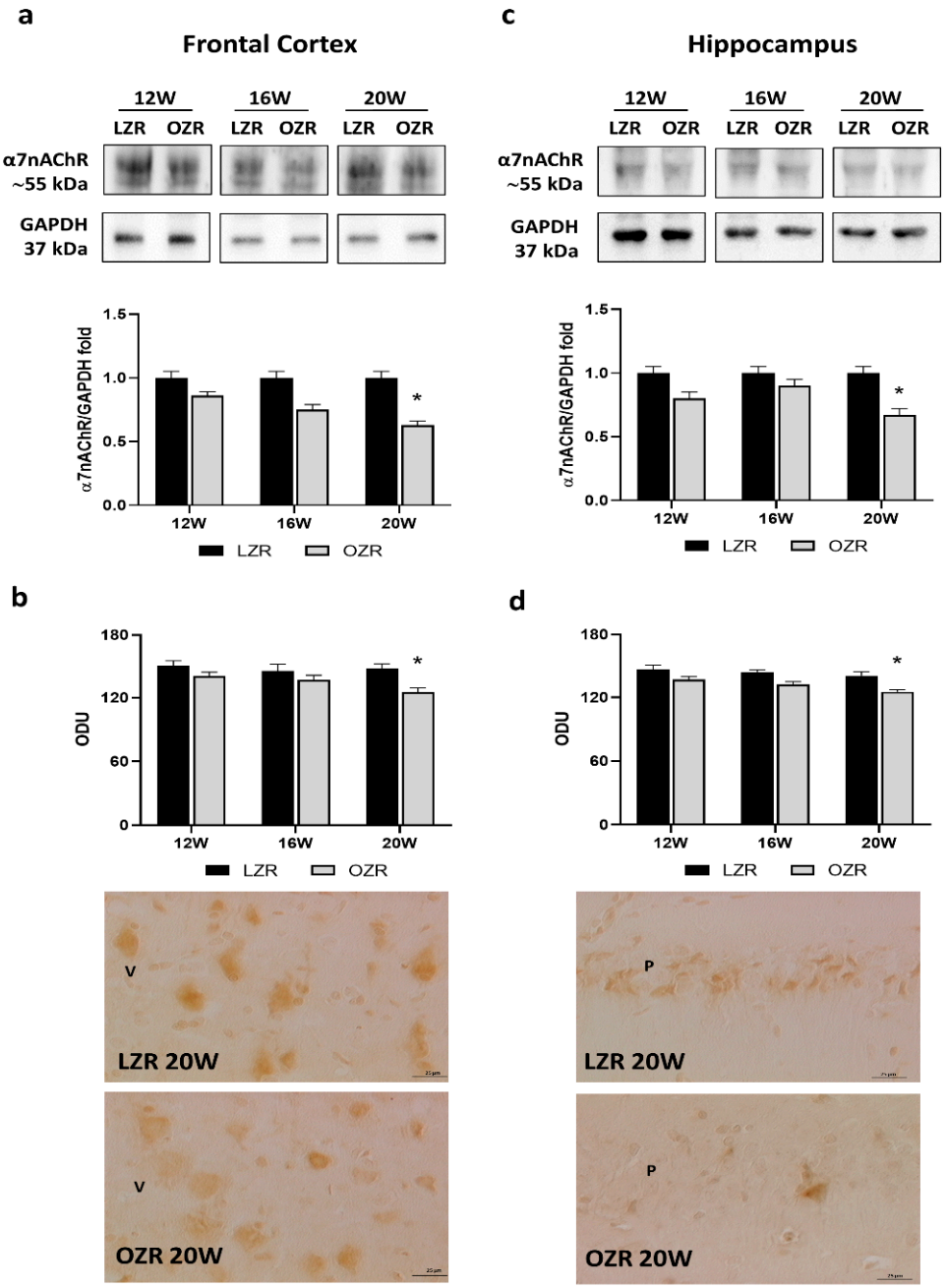

Figure 2. Immunoblotting and immunoreaction of alpha7 nicotinic acetylcholine receptor (α7nAChR). Lysates of frontal cortex (**a**) and hippocampus (**c**) from lean Zucker rats (LZRs) and obese Zucker rats (OZRs) at the age of 12, 16, and 20 weeks were immunoblotted using specific anti-α7nAChR. Bar graphs indicate the densitometric analysis using LZRs as control, and GAPDH levels were used as loading control. Blots are representative of one of three separate experiments; intensity values of α7nAChR immunostaining in the frontal cortex (**b**) and the hippocampus (**d**) from LZRs and OZRs at the age of 12, 16, and 20 weeks measured in optical density units (ODUs). Data are mean ± S.E.M. * $p < 0.05$ vs. age-matched LZR. Representative pictures of 20 weeks old LZR and OZR in frontal cortex (**b**) and hippocampus (**d**). V: the fifth layer of the frontal cortex. P: pyramidal neurons of the hippocampus. 40× magnification. Calibration bar: 25 μm.

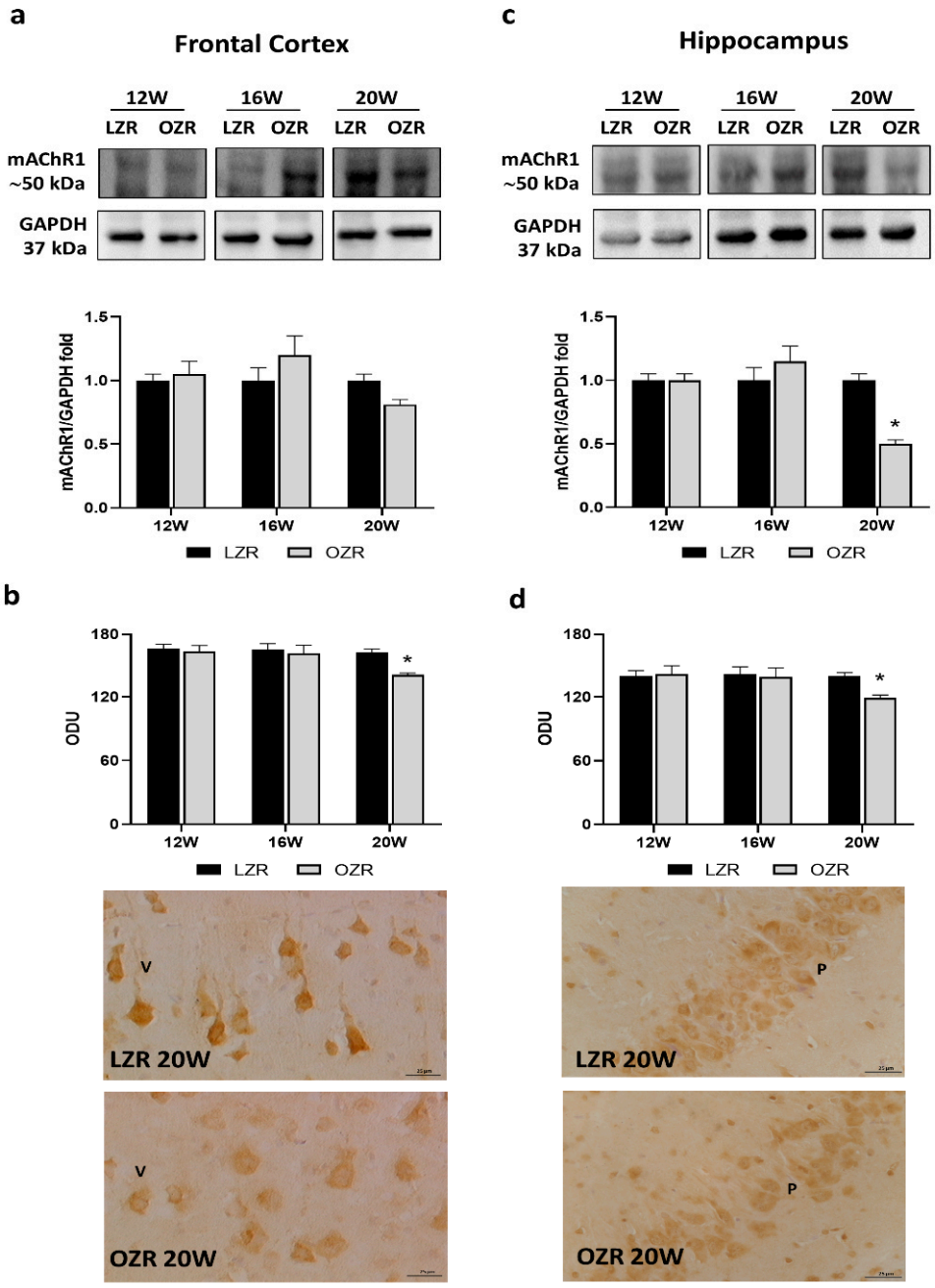

Figure 3. Immunoblotting and immunoreaction of muscarinic acetylcholine receptor subtype 1 (mAChR1). Lysates of the frontal cortex (**a**) and hippocampus (**c**) from lean Zucker rats (LZRs) and obese Zucker rats (OZRs) at the age of 12, 16, and 20 weeks were immunoblotted using specific anti-mAChR1. Bar graphs indicate the densitometric analysis using LZRs as control, and GAPDH levels were used as a loading control. Blots are representative of one of three separate experiments; intensity values of mAChR1 immunostaining in the frontal cortex (**b**) and hippocampus (**d**) from LZRs and OZRs at the age of 12, 16, and 20 weeks measured in optical density units (ODUs). Data are mean ± S.E.M. * $p < 0.05$ vs. age-matched LZR. Representative pictures of 20-week-old LZRs and OZRs in the frontal cortex (**b**) and hippocampus (**d**). V: the fifth layer of the frontal cortex. P: pyramidal neurons of the hippocampus. 40× magnification. Calibration bar: 25 μm.

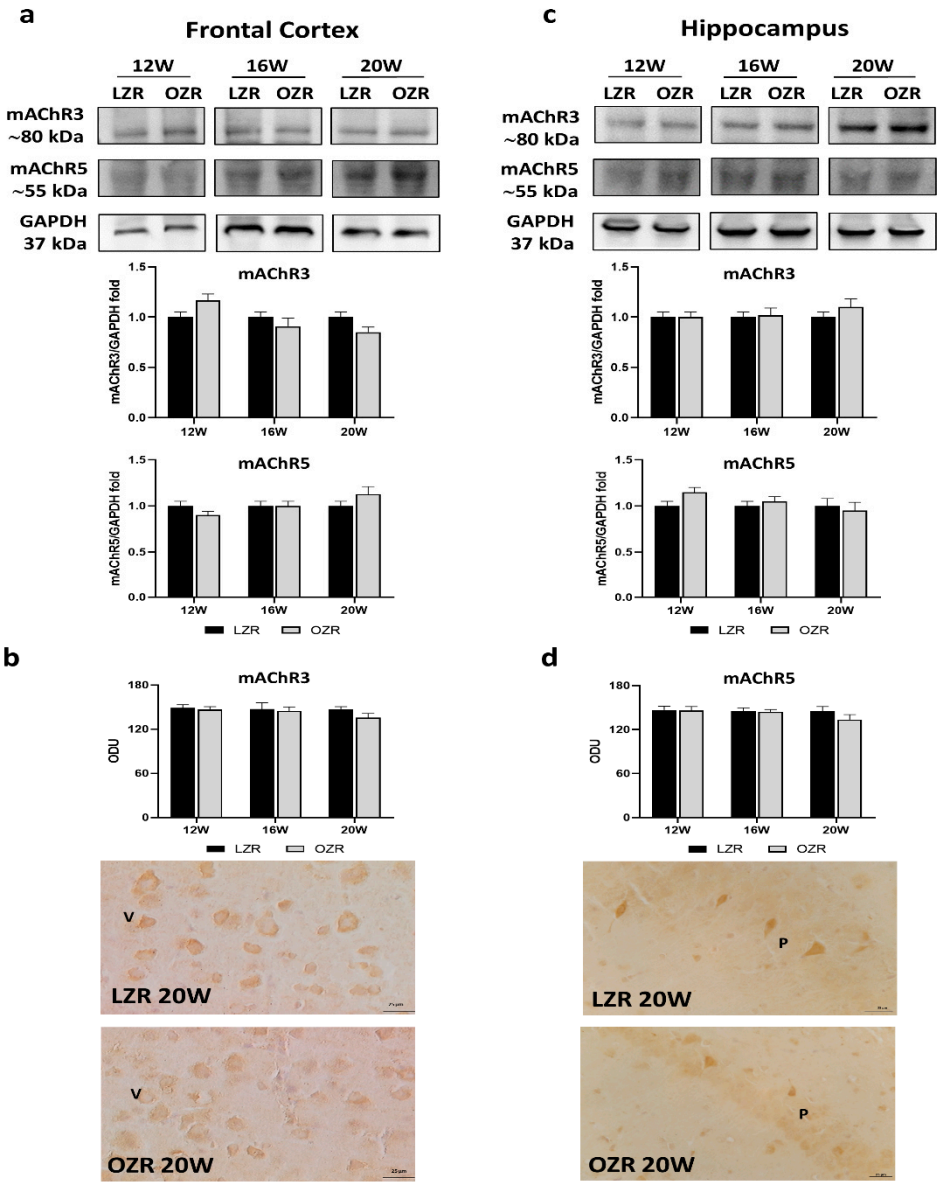

Figure 4. Immunoblotting and immunoreaction of muscarinic acetylcholine receptor subtype 3 (mAChR3) and muscarinic acetylcholine receptor subtype 5 (mAChR5). Lysates of the frontal cortex (**a**) and hippocampus (**c**) from lean Zucker rats (LZRs) and obese Zucker rats (OZRs) at the age of 12, 16, and 20 weeks were immunoblotted using specific anti-mAChR3 and anti-mAChR5. Bar graphs indicate the densitometric analysis using LZRs as control, and GAPDH levels were used as a loading control. Blots are representative of one of three separate experiments; intensity values of mAChR3 immunostaining in the frontal cortex (**b**) and mAChR5 in the hippocampus (**d**) from LZRs and OZRs at the age of 12, 16, and 20 weeks measured in optical density units (ODUs). Data are mean ± S.E.M. Representative pictures of 20-week-old LZRs and OZRs in frontal cortex for mAChR3 (**b**) and hippocampus for mAChR5 (**d**). V: the fifth layer of the frontal cortex. P: pyramidal neurons of the hippocampus. 40× magnification. Calibration bar: 25 μm.

3.3.2. Synaptic Vesicle Glycoproteins

As an abundant synaptic marker, SYP was explored (Figure 5). The Western blot results did not show statistical differences in SYP levels in the obese condition compared to the lean one in the frontal cortex (Figure 5a). Instead, the quantification of SYP immunoreaction was significantly reduced only in 20-week-old OZRs in comparison with the age-matched controls as demonstrated by representative pictures (Figure 5b). Moreover, in

the hippocampus, the SYP expression (Figure 5c), as well as the immunoreaction localized in CA1 and CA3 (Figure 5d), was remarkably reduced in the older OZRs compared to that in controls.

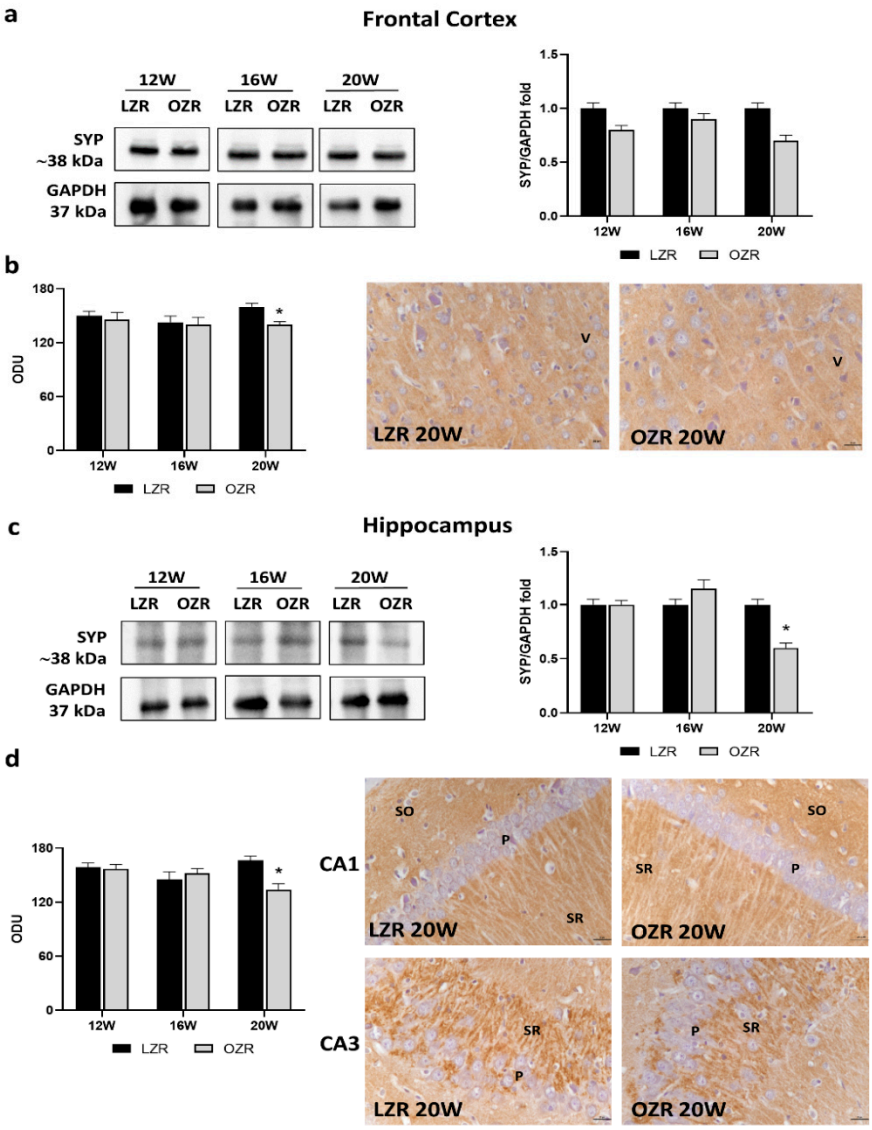

Figure 5. Immunoblotting and immunoreaction of synaptophysin (SYP). Lysates of the frontal cortex (**a**) and hippocampus (**c**) from lean Zucker rats (LZRs) and obese Zucker rats (OZRs) at the age of 12, 16, and 20 weeks were immunoblotted using specific anti-SYP. Bar graphs indicate the densitometric analysis using LZRs as control, and GAPDH levels were used as the loading control. Blots are representative of one of three separate experiments; intensity values of SYP immunostaining in the frontal cortex (**b**) and hippocampus (**d**) from LZRs and OZRs at the age of 12, 16, and 20 weeks measured in optical density units (ODUs). Data are mean ± S.E.M. * $p < 0.05$ vs. age-matched LZRs. Representative pictures of 20-week-old LZRs and OZRs in frontal cortex (**b**) and hippocampus (**d**). V: the fifth layer of the frontal cortex. P: pyramidal neurons in the CA1 and CA3 subfields of the hippocampus. SO: *stratum oriens*. SR: *stratum radiatum*. 40× magnification. Calibration bar: 25 µm.

Among the presynaptic vesicle proteins analyzed, i.e., synaptic vesicle glycoproteins 2A and 2C (SVA and SVC, respectively) (Figure S1), only synaptic vesicle glycoprotein 2B (SV2B) showed alterations related to obesity and age (Figure 6). Indeed, results from Western blot and immunohistochemistry showed significantly reduced SV2B levels in the frontal cortex in 20-week-old OZRs compared to that in the lean ones (Figure 6a,b). More-

over, in the hippocampus, the SV2B expression (Figure 6c), as well as the immunoreaction localized in CA1 (Figure 6d), was remarkably reduced in the older OZRs compared to that in controls.

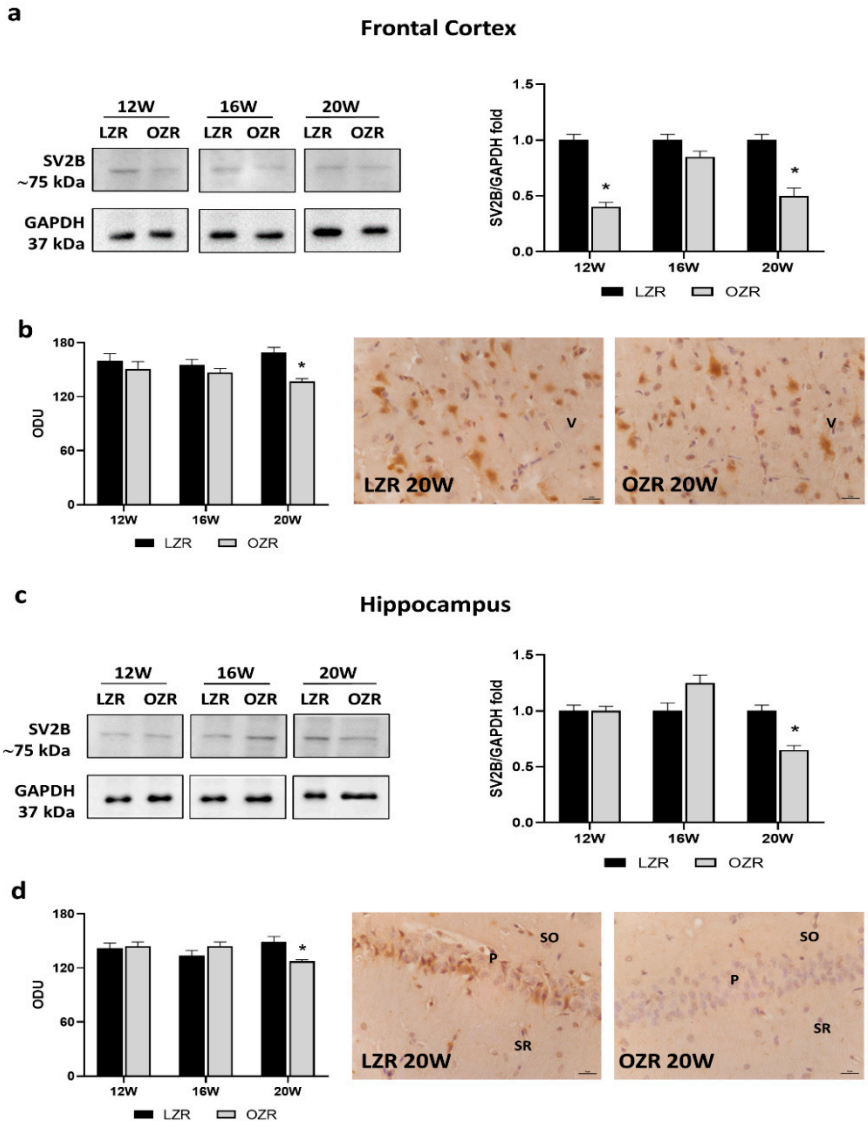

Figure 6. Immunoblotting and immunoreaction of synaptic vesicle glycoprotein 2B (SV2B). Lysates of the frontal cortex (**a**) and hippocampus (**c**) from lean Zucker rats (LZRs) and obese Zucker rats (OZRs) at the age of 12, 16, and 20 weeks were immunoblotted using specific anti-SV2B. Bar graphs indicate the densitometric analysis using LZRs as control, and GAPDH levels were used as the loading control. Blots are representative of one of three separate experiments; intensity values of SV2B immunostaining in the frontal cortex (**b**) and hippocampus (**d**) from LZRs and OZRs at the age of 12, 16, and 20 weeks measured in optical density units (ODUs). Data are mean ± S.E.M. * $p < 0.05$ vs. age-matched LZRs. Representative pictures of 20-week-old LZRs and OZRs in the frontal cortex (**b**) and hippocampus (**d**). V: the fifth layer of the frontal cortex. P: pyramidal neurons in the CA1 subfield of the hippocampus. SO: *stratum oriens*. SR: *stratum radiatum*. 40× magnification. Calibration bar: 25 μm.

4. Discussion

Obesity is a complex disorder connected with several physiological abnormalities that arise from excessive fat tissue accumulation [43]. Different studies showed that obesity related to an HFD impaired learning and memory in rodents, suggesting a strong correlation between obesity and cognitive dysfunction [12,27,44–50].

Several neurotransmitters including acetylcholine (ACh) have been implicated in the regulation of food intake and obesity [51,52]. In the brain region of Zucker fatty rats analyzed by [53], ACh content showed a lower level than that of the lean rats. In these animals, the activities of AChE were found to be significantly lower than those in the lean rats in all the brain areas, except in the striatum and medulla oblongata, where it was significantly reduced [53]. On the contrary, significantly higher AChE activity was seen in the cerebral cortex, cerebellum, midbrain, thalamus, and hypothalamus of 14-week-old OZRs than in their lean littermates [54]. Meanwhile, choline acetyltransferase (ChAT) activity was lower in the cerebellum, pons, and cerebral cortex, while a significant increase in ChAT activity was found in the thalamus and hypothalamus [54]. Thus, the diencephalon of the OZRs showed a significant increase in both ChAT and AChE activities, which may reflect an increase in the ACh turnover rate. It was postulated that the increase in the turnover rate of ACh was probably a cause of obesity rather than a consequence of obesity [54]. From these controversial and complex data, it was concluded that obesity could be associated with changes in the enzymes activities of the brain cholinergic system also depending on the brain regions [54]. To date, no data have been published yet regarding cholinergic transporter and receptors and synaptic markers in OZRs. The availability of genetically obese rats with known changes in the brain neurochemistry provided an excellent model to study obesity and cholinergic as well as synaptic function in the frontal cortex and hippocampus.

The obese rats (*fa*/*fa*) present dysfunctions in the CNS [14,55]. The current study shows a reduction of VAChT and α7nAChR expressions both in the frontal cortex and in the hippocampus of 20-week-old OZRs. Indeed, VAChT and α7nAChR are considered pro-cognitive elements, directly involved in learning and memory, as well as in the pathology of neurodegenerative and cerebrovascular diseases [56–59]. This may justify previous behavioral tests that revealed, in 20-week-old OZRs, anxiety-like behavior compared to age-matched LZRs. In addition, the reduced retention latency time in the emotional learning task also confirmed cognitive impairment in OZRs [14].

Nowadays, it is well recognized that nAChRs are expressed not only on neurons but also in microglia [60] and astrocytes [61]. Moreover, their responses are often mediated specifically by α7nAChRs. Among the responses, its regulation of the cholinergic anti-inflammatory pathway [62,63] is gaining great attention. Studies have revealed that the activation of α7nAChR in astrocytes and microglia can induce anti-inflammatory effects through the downregulation of pro-inflammatory cytokine production [59,64,65]. Thus, we can speculate that the astrogliosis, reactive microglia, and vascular inflammation, characterized by the increase of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) expressions in the brain of older OZRs [14,66], may be related to the low expression of α7nAChR. Among the mechanisms, neuroinflammation with the increase of cytokines and changes in membrane fluidity and, above all, the disruption of the BBB is the most accredited [44,46]. Local and systemic inflammation, induced by obesity or T2DM, has been linked to central disorders, such as depression, and neurodegenerative diseases such as AD, because of BBB breakdown, decreased removal of waste, and increased infiltration of immune cells [8,9,21,31]. This, in turn, leads to cognitive impairment and disruption of neuronal and glial cells, triggering hormonal dysfunction and amplified immune sensitivity, depending on the affected brain areas (hippocampus, cortex, brainstem, or amygdala) [8,9,67].

Moreover, a high-calorie diet could be involved in the alterations of the cholinergic system with the modulation of mAChRs [26]. Following the results carried out in DIO rats [26], we found that the mAChR1, but not mAChR5, was significantly reduced in the hippocampus of 20-week-old OZRs. Even though downregulation of mAChR3 in the hippocampus of DIO animals has been reported [26], here we did not find any differences either in the hippocampus or in the frontal cortex. Taken together, these results indicate a differential modulation of mAChR1, mAChR3, and mAChR5 subtypes in obese rats compared to that in lean ones. mAChRs mediate a wide range of functions peripherally

and in the CNS. The five mAChR subtypes play a role in learning, memory, attention, and sensory-motor processing and are expressed differently in the brain [68–70]. However, we did not investigate their quantities.

The cholinergic alterations were accompanied by synaptic dysfunctions in the obese phenotype, with a reduction of SYP and synaptic vesicle protein SV2B in 20-week-old OZRs, both in the frontal cortex and in the hippocampus. These changes in the rat were associated with behavioral deficits. Cognitive decline was also reported by Bocarsly and coworkers in 2015 with decreased synaptic marker expression in the prefrontal and perirhinal cortex in DIO rats, accompanied by decreased dendritic spine density and changed microglia morphology [27]. Childhood metabolic disorders can impair cognitive development with abnormal synaptic function [71,72]. In addition, exposure to an HFD during the peak period of brain development can also alter neuroplasticity that links to eating disorders [73]. One study showed that longer periods of HFD feeding impair cognitive tasks associated with the hippocampus and reduce synaptic markers and increase microglia activation in the hippocampus [74]. On the contrary, another study reported no effect of long-term HFD on cognitive behaviors associated with the hippocampus [75]. It could be that obesity alone was not enough to compromise hippocampal structure and function. However, in combination with other complications such as chronic stress, it was sufficiently detrimental to impact hippocampal plasticity [76]. Interestingly, researchers found that restored cholinergic inputs and presynaptic synaptophysin contribute to the protective effects of physical running on spatial memory in aged mice [16].

5. Conclusions

The fact that the frontal cortex and hippocampus of OZRs are functionally compromised in cholinergic and synaptic activities provides new insight into how obesity can influence the cholinergic system and synaptic markers and, thus, the cognitive functions. Furthermore, the positive modulation of certain cholinergic and synaptic markers may be a possible therapeutic strategy for the treatment of obesity- and age-related cognitive dysfunction.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/cells10102528/s1, Figure S1: Immunohistochemistry of synaptic vesicle glycoprotein 2A and synaptic vesicle glycoprotein 2C (SV2A and SV2C, respectively). Representative pictures of 20-week-old LZRs and OZRs in the frontal cortex (a) and hippocampus (b). V, VI: the fifth, sixth layers of the frontal cortex. P: pyramidal neurons in the CA1 and CA3 subfields of the hippocampus. SO: *stratum oriens*. SR: *stratum radiatum*. 40× magnification. Calibration bar: 25 µm.

Author Contributions: Conceptualization, D.T., I.M., F.A., and S.K.T.; methodology, I.M. and D.T.; formal analysis, I.M., D.T., and P.R.; investigation, I.M., D.T., and P.R.; resources, F.A., D.T., and S.K.T.; data curation, I.M., D.T., and S.K.T.; writing—original draft preparation, I.M. and D.T.; writing—review and editing, D.T., F.A., and S.K.T.; visualization, D.T., I.M., S.K.T., and F.A.; supervision, D.T., F.A., and S.K.T.; project administration, F.A., D.T., and S.K.T.; funding acquisition, F.A. and S.K.T. All authors have read and agree to the published version of the manuscript.

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References

1. Grundy, S.M. Metabolic syndrome pandemic. *Arterioscler. Thromb. Vasc. Biol.* **2008**, *28*, 629–636. [CrossRef]
2. Van Dieren, S.; Beulens, J.W.; van der Schouw, Y.T.; Grobbee, D.E.; Neal, B. The global burden of diabetes and its complications: An emerging pandemic. *Eur. J. Cardiovasc. Prev. Rehabil.* **2010**, *17*, S3–S8. [CrossRef]
3. Blüher, M. Adipose tissue dysfunction contributes to obesity related metabolic diseases. *Best Pract. Res. Clin. Endocrinol. Metab.* **2013**, *27*, 163–177. [CrossRef]
4. Pillon, N.J.; Loos, R.J.F.; Marshall, S.M.; Zierath, J.R. Metabolic consequences of obesity and type 2 diabetes: Balancing genes and environment for personalized care. *Cell* **2021**, *184*, 1530–1544. [CrossRef]
5. Stranahan, A.M.; Norman, E.D.; Lee, K.; Cutler, R.G.; Telljohann, R.S.; Egan, J.M.; Mattson, M.P. Diet-induced insulin resistance impairs hippocampal synaptic plasticity and cognition in middle-aged rats. *Hippocampus* **2008**, *18*, 1085–1088. [CrossRef] [PubMed]
6. Morrison, C.D.; Pistell, P.J.; Ingram, D.K.; Johnson, W.D.; Liu, Y.; Fernandez-Kim, S.O.; White, C.L.; Purpera, M.N.; Uranga, R.M.; Bruce-Keller, A.J.; et al. High fat diet increases hippocampal oxidative stress and cognitive impairment in aged mice: Implications for decreased nrf2 signaling. *J. Neurochem.* **2010**, *114*, 1581–1589. [CrossRef] [PubMed]
7. McNeilly, A.D.; Williamson, R.; Balfour, D.J.; Stewart, C.A.; Sutherland, C. A high-fat-diet-induced cognitive deficit in rats that is not prevented by improving insulin sensitivity with metformin. *Diabetologia* **2012**, *55*, 3061–3070. [CrossRef] [PubMed]
8. Guillemot-Legris, O.; Muccioli, G.G. Obesity-Induced Neuroinflammation: Beyond the Hypothalamus. *Trends Neurosci.* **2017**, *40*, 237–253. [CrossRef]
9. Van Dyken, P.; Lacoste, B. Impact of Metabolic Syndrome on Neuroinflammation and the Blood-Brain Barrier. *Front. Neurosci.* **2018**, *12*, 930. [CrossRef]
10. Lumeng, C.N.; Saltiel, A.R. Inflammatory links between obesity and metabolic disease. *J. Clin. Investig.* **2011**, *121*, 2111–2117. [CrossRef]
11. Könner, A.C.; Brüning, J.C. Toll-like receptors: Linking inflammation to metabolism. *Trends Endocrinol. Metab.* **2011**, *22*, 16–23. [CrossRef] [PubMed]
12. Micioni Di Bonaventura, M.V.; Martinelli, I.; Moruzzi, M.; Micioni Di Bonaventura, E.; Giusepponi, M.E.; Polidori, C.; Lupidi, G.; Tayebati, S.K.; Amenta, F.; Cifani, C.; et al. Brain alterations in high fat diet induced obesity: Effects of tart cherry seeds and juice. *Nutrients* **2020**, *12*, 623. [CrossRef]
13. Tomassoni, D.; Nwankwo, I.E.; Gabrielli, M.G.; Bhatt, S.; Muhammad, A.B.; Lokhandwala, M.F.; Tayebati, S.K.; Amenta, F. Astrogliosis in the brain of obese Zucker rat: A model of metabolic syndrome. *Neurosci. Lett.* **2013**, *543*, 136–141. [CrossRef] [PubMed]
14. Tomassoni, D.; Martinelli, I.; Moruzzi, M.; Micioni Di Bonaventura, M.V.; Cifani, C.; Amenta, F.; Tayebati, S.K. Obesity and Age-Related Changes in the Brain of the Zucker Lepr fa/fa Rats. *Nutrients.* **2020**, *12*, 1356. [CrossRef]
15. Sagae, S.C.; Grassiolli, S.; Raineki, C.; Balbo, S.L.; Marques da Silva, A.C. Sex differences in brain cholinergic activity in MSG-obese rats submitted to exercise. *Can. J. Physiol. Pharmacol.* **2011**, *89*, 845–853. [CrossRef] [PubMed]
16. Xu, L.; Long, J.; Su, Z.; Xu, B.; Lin, M.; Chen, Y.; Long, D. Restored presynaptic synaptophysin and cholinergic inputs contribute to the protective effects of physical running on spatial memory in aged mice. *Neurobiol. Dis.* **2019**, *132*, 104586. [CrossRef]
17. Mandolesi, L.; Polverino, A.; Montuori, S.; Foti, F.; Ferraioli, G.; Sorrentino, P.; Sorrentino, G. Effects of Physical Exercise on Cognitive Functioning and Wellbeing: Biological and Psychological Benefits. *Front. Psychol.* **2018**, *9*, 509. [CrossRef] [PubMed]
18. Wang, X.; Yang, Z.; Xue, B.; Shi, H. Activation of the Cholinergic Antiinflammatory Pathway Ameliorates Obesity-Induced Inflammation and Insulin Resistance. *Endocrinology* **2011**, *152*, 836–846. [CrossRef]
19. Chavan, S.S.; Tracey, K.J. Essential neuroscience in immunology. *J. Immunol.* **2017**, *198*, 3389–3397. [CrossRef]
20. Pavlov, V.A.; Tracey, K.J. Neural regulation of immunity: Molecular mechanisms and clinical translation. *Nat. Neurosci.* **2017**, *20*, 156–166. [CrossRef]
21. Martinelli, I.; Tomassoni, D.; Moruzzi, M.; Traini, E.; Amenta, F.; Tayebati, S.K. Obesity and Metabolic Syndrome Affect the Cholinergic Transmission and Cognitive Functions. *CNS Neurol. Disord. Drug Targets* **2017**, *16*, 664–676. [CrossRef]
22. Chang, E.H.; Chavan, S.S.; Pavlov, V.A. Cholinergic Control of Inflammation, Metabolic Dysfunction, and Cognitive Impairment in Obesity-Associated Disorders: Mechanisms and Novel Therapeutic Opportunities. *Front. Neurosci.* **2019**, *13*, 263. [CrossRef]
23. Pavlov, V.A.; Tracey, K.J. The vagus nerve and the inflammatory reflex–linking immunity and metabolism. *Nat. Rev. Endocrinol.* **2012**, *8*, 743–754. [CrossRef]
24. Jiang, L.; Wang, Y.; Su, L.; Ren, H.; Wang, C.; Chen, J.; Fu, X. Donepezil Attenuates Obesity-Associated Oxidative Stress and Central Inflammation and Improves Memory Deficit in Mice Fed a High-Fat Diet. *Dement. Geriatr. Cogn. Disord.* **2019**, *48*, 154–163. [CrossRef]
25. Consolim-Colombo, F.M.; Sangaleti, C.T.; Costa, F.O.; Morais, T.L.; Lopes, H.F.; Motta, J.M.; Irigoyen, M.C.; Bortoloto, L.A.; Rochitte, C.E.; Harris, Y.T.; et al. Galantamine alleviates inflammation and insulin resistance in patients with metabolic syndrome in a randomized trial. *JCI Insight* **2017**, *2*, e93340. [CrossRef] [PubMed]
26. Silva, M.F.P.; Alves, P.L.; Alponti, R.F.; Silveira, P.F.; Abdalla, F.M.F. Effects of obesity induced by high-calorie diet and its treatment with exenatide on muscarinic acetylcholine receptors in rat hippocampus. *Biochem. Pharmacol.* **2019**, *169*, 113630. [CrossRef]

27. Bocarsly, M.E.; Fasolino, M.; Kane, G.A.; LaMarca, E.A.; Kirschen, G.W.; Karatsoreos, I.N.; McEwen, B.S.; Gould, E. Obesity diminishes synaptic markers, alters microglial morphology, and impairs cognitive function. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, 15731–15736. [CrossRef]
28. Marcello, E.; Epis, R.; Saraceno, C.; Di Luca, M. Synaptic dysfunction in Alzheimer's disease. *Adv. Exp. Med. Biol.* **2012**, *970*, 573–601. [CrossRef]
29. Wang, D.B.; Kinoshita, Y.; Kinoshita, C.; Uo, T.; Sopher, B.L.; Cudaback, E.; Keene, C.D.; Bilousova, T.; Gylys, K.; Case, A.; et al. Loss of endophilin-B1 exacerbates Alzheimer's disease pathology. *Brain* **2015**, *138*, 2005–2019. [CrossRef] [PubMed]
30. Sivanesan, S.; Tan, A.; Rajadas, J. Pathogenesis of Abeta oligomers in synaptic failure. *Curr. Alzheimer Res.* **2013**, *10*, 316–323. [CrossRef] [PubMed]
31. Zatterale, F.; Longo, M.; Naderi, J.; Raciti, G.A.; Desiderio, A.; Miele, C.; Beguinot, F. Chronic Adipose Tissue Inflammation Linking Obesity to Insulin Resistance and Type 2 Diabetes. *Front. Physiol.* **2020**, *10*, 1607. [CrossRef]
32. Martinelli, I.; Tomassoni, D.; Moruzzi, M.; Roy, P.; Cifani, C.; Amenta, F.; Tayebati, S.K. Cardiovascular Changes Related to Metabolic Syndrome: Evidence in Obese Zucker Rats. *Int. J. Mol. Sci.* **2020**, *21*, 2035. [CrossRef] [PubMed]
33. Amenta, F.; Peleg, E.; Tomassoni, D.; Sabbatini, M.; Rosenthal, T. Effect of treatment with lercanidipine on heart of Cohen-Rosenthal diabetic hypertensive rats. *Hypertension* **2003**, *41*, 1330–1335. [CrossRef] [PubMed]
34. Murase, T.; Hattori, T.; Ohtake, M.; Abe, M.; Amakusa, Y.; Takatsu, M.; Murohara, T.; Nagata, K. Cardiac remodeling and diastolic dysfunction in DahlS.Z-Lepr(fa)/Lepr(fa) rats: A new animal model of metabolic syndrome. *Hypertens. Res.* **2012**, *35*, 186–193. [CrossRef] [PubMed]
35. Riojas-Hernández, A.; Bernal-Ramírez, J.; Rodríguez-Mier, D.; Morales-Marroquín, F.E.; Domínguez-Barragán, E.M.; Borja-Villa, C.; Rivera-Álvarez, I.; García-Rivas, G.; Altamirano, J.; García, N. Enhanced oxidative stress sensitizes the mitochondrial permeability transition pore to opening in heart from Zucker Fa/fa rats with type 2 diabetes. *Life Sci.* **2015**, *141*, 32–43. [CrossRef] [PubMed]
36. Tayebati, S.K.; Di Tullio, M.A.; Amenta, F. Vesicular acetylcholine transporter (VAChT) in the brain of spontaneously hypertensive rats (SHR): Effect of treatment with an acetylcholinesterase inhibitor. *Clin. Exp. Hypertens.* **2008**, *30*, 732–743. [CrossRef]
37. Tayebati, S.K.; Di Tullio, M.A.; Tomassoni, D.; Amenta, F. Localization of the m5 muscarinic cholinergic receptor in rat circle of Willis and pial arteries. *Neuroscience* **2003**, *122*, 205–211. [CrossRef]
38. Jones, I.W.; Wonnacott, S. Precise localization of alpha7 nicotinic acetylcholine receptors on glutamatergic axon terminals in the rat ventral tegmental area. *J. Neurosci.* **2004**, *24*, 11244–11252. [CrossRef]
39. Fabian-Fine, R.; Skehel, P.; Errington, M.L.; Davies, H.A.; Sher, E.; Stewart, M.G.; Fine, A. Ultrastructural distribution of the alpha7 nicotinic acetylcholine receptor subunit in rat hippocampus. *J. Neurosci.* **2001**, *21*, 7993–8003. [CrossRef]
40. Caulfield, M.P. Muscarinic receptors–characterization, coupling and function. *Pharmacol. Ther.* **1993**, *58*, 319–379. [CrossRef]
41. Tayebati, S.K.; Amenta, F.; El-Assouad, D.; Zaccheo, D. Muscarinic cholinergic receptor subtypes in the hippocampus of aged rats. *Mech. Ageing Dev.* **2002**, *123*, 521–528. [CrossRef]
42. Lebois, E.P.; Thorn, C.; Edgerton, J.R.; Popiolek, M.; Xi, S. Muscarinic receptor subtype distribution in the central nervous system and relevance to aging and Alzheimer's disease. *Neuropharmacology* **2018**, *13*, 362–373. [CrossRef]
43. Longo, M.; Zatterale, F.; Naderi, J.; Parrillo, L.; Formisano, P.; Raciti, G.A.; Beguinot, F.; Miele, C. Adipose Tissue Dysfunction as Determinant of Obesity-Associated Metabolic Complications. *Int. J. Mol. Sci.* **2019**, *20*, 2358. [CrossRef] [PubMed]
44. Farr, S.A.; Yamada, K.A.; Butterfield, D.A.; Abdul, H.M.; Xu, L.; Miller, N.E.; Banks, W.A.; Morley, J.E. Obesity and hypertriglyceridemia produce cognitive impairment. *Endocrinology* **2008**, *149*, 2628–2636. [CrossRef]
45. Kanoski, S.E.; Hayes, M.R.; Greenwald, H.S.; Fortin, S.M.; Gianessi, C.A.; Gilbert, J.R.; Grill, H.J. Hippocampal leptin signaling reduces food intake and modulates food-related memory processing. *Neuropsychopharmacology* **2011**, *36*, 1859–1870. [CrossRef] [PubMed]
46. McNeilly, A.D.; Williamson, R.; Sutherland, C.; Balfour, D.J.; Stewart, C.A. High fat feeding promotes simultaneous decline in insulin sensitivity and cognitive performance in a delayed matching and nonmatching to position task. *Behav. Brain Res.* **2011**, *217*, 134–141. [CrossRef] [PubMed]
47. Zainuddin, M.S.; Thuret, S. Nutrition, adult hippocampal neurogenesis and mental health. *Br. Med. Bull.* **2012**, *103*, 89–114. [CrossRef] [PubMed]
48. Gurung, S.; Agbaga, M.P.; Myers, D.A. Cognitive differences between Sprague-Dawley rats selectively bred for sensitivity or resistance to diet induced obesity. *Behav. Brain Res.* **2016**, *311*, 122–130. [CrossRef]
49. Sánchez-Sarasúa, S.; Moustafa, S.; García-Avilés, Á.; López-Climent, M.F.; Gómez-Cadenas, A.; Olucha-Bordonau, F.E.; Sánchez-Pérez, A.M. The effect of abscisic acid chronic treatment on neuroinflammatory markers and memory in a rat model of high-fat diet induced neuroinflammation. *Nutr. Metab.* **2016**, *13*, 73. [CrossRef]
50. Martins, I.V.; Rivers-Auty, J.; Allan, S.M.; Lawrence, C.B. Mitochondrial Abnormalities and Synaptic Loss Underlie Memory Deficits Seen in Mouse Models of Obesity and Alzheimer's Disease. *J. Alzheimers Dis.* **2017**, *55*, 915–932. [CrossRef]
51. Grossman, S.P. Direct adrenergic and cholinergic stimulation of hypothalamic mechanisms. *Am. J. Physiol.* **1962**, *202*, 872–882. [CrossRef] [PubMed]
52. Chance, W.T.; Lints, C.E. Eating following cholinergic stimulation of the hypothalamus. *Physiol. Psychol.* **1977**, *5*, 440–444. [CrossRef]

53. Ohkuma, S.; Ma, F.H.; Kishi, M.; Kuriyama, K. Alteration of acetylcholine metabolism in the brain of zucker fatty rat. *Neurochem. Int.* **1990**, *16*, 99–103. [CrossRef]
54. Goodman, C.B.; Soliman, K.F. Altered brain cholinergic enzymes activity in the genetically obese rat. *Experientia* **1991**, *47*, 833–835. [CrossRef] [PubMed]
55. Baskin, D.G.; Stein, L.J.; Ikeda, H.; Woods, S.C.; Figlewicz, D.P.; Porte, D.; Greenwood, M.R.; Dorsa, D.M. Genetically obese Zucker rats have abnormally low brain insulin content. *Life Sci.* **1985**, *36*, 627–633. [CrossRef]
56. Prado, V.F.; Martins-Silva, C.; de Castro, B.M.; Lima, R.F.; Barros, D.M.; Amaral, E.; Ramsey, A.J.; Sotnikova, T.D.; Ramirez, M.R.; Kim, H.G.; et al. Mice deficient for the vesicular acetylcholine transporter are myasthenic and have deficits in object and social recognition. *Neuron* **2006**, *51*, 601–612. [CrossRef] [PubMed]
57. De Castro, B.M.; Pereira, G.S.; Magalhães, V.; Rossato, J.I.; De Jaeger, X.; Martins-Silva, C.; Leles, B.; Lima, P.; Gomez, M.V.; Gainetdinov, R.R.; et al. Reduced expression of the vesicular acetylcholine transporter causes learning deficits in mice. *Genes Brain Behav.* **2009**, *8*, 23–35. [CrossRef]
58. Nordberg, A. Nicotinic receptor abnormalities of Alzheimer's disease: Therapeutic implications. *Biol. Psychiatry* **2001**, *49*, 200–210. [CrossRef]
59. Xu, Z.Q.; Zhang, W.J.; Su, D.F.; Zhang, G.Q.; Miao, C.Y. Cellular responses and functions of α7 nicotinic acetylcholine receptor activation in the brain: A narrative review. *Ann. Transl. Med.* **2021**, *9*, 509. [CrossRef]
60. Suzuki, T.; Hide, I.; Matsubara, A.; Hama, C.; Harada, K.; Miyano, K.; Andrä, M.; Matsubayashi, H.; Sakai, N.; Kohsaka, S.; et al. Microglial alpha7 nicotinic acetylcholine receptors drive a phospholipase C/IP3 pathway and modulate the cell activation toward a neuroprotective role. *J. Neurosci. Res.* **2006**, *83*, 1461–1470. [CrossRef]
61. Papouin, T.; Dunphy, J.M.; Tolman, M.; Dineley, K.T.; Haydon, P.G. Septal Cholinergic Neuromodulation Tunes the Astrocyte-Dependent Gating of Hippocampal NMDA Receptors to Wakefulness. *Neuron* **2017**, *94*, 840–854. [CrossRef]
62. Wang, H.; Yu, M.; Ochani, M.; Amella, C.A.; Tanovic, M.; Susarla, S.; Li, J.H.; Wang, H.; Yang, H.; Ulloa, L.; et al. Nicotinic acetylcholine receptor alpha7 subunit is an essential regulator of inflammation. *Nature* **2003**, *421*, 384–388. [CrossRef]
63. Echeverria, V.; Yarkov, A.; Aliev, G. Positive modulators of the α7 nicotinic receptor against neuroinflammation and cognitive impairment in Alzheimer's disease. *Prog. Neurobiol.* **2016**, *144*, 142–157. [CrossRef] [PubMed]
64. Shi, S.; Liang, D.; Bao, M.; Xie, Y.; Xu, W.; Wang, L.; Wang, Z.; Qiao, Z. Gx-50 Inhibits Neuroinflammation via α7 nAChR Activation of the JAK2/STAT3 and PI3K/AKT Pathways. *J. Alzheimers Dis.* **2016**, *50*, 859–871. [CrossRef] [PubMed]
65. Zhang, Q.; Lu, Y.; Bian, H.; Guo, L.; Zhu, H. Activation of the α7 nicotinic receptor promotes lipopolysaccharide-induced conversion of M1 microglia to M2. *Am. J. Transl. Res.* **2017**, *9*, 971–985.
66. Tomassoni, D.; Tayebati, S.K.; Ciriaci, M.; Cognigni, F.; Amenta, F. Obesity-related blood brain barrier changes in obese Zucker rats. *Ital. J. Anat. Embryol.* **2015**, *120*, 192.
67. Hwang, I.K.; Choi, J.H.; Nam, S.M.; Park, O.K.; Yoo, D.Y.; Kim, W.; Yi, S.S.; Won, M.H.; Seong, J.K.; Yoon, Y.S. Activation of microglia and induction of pro-inflammatory cytokines in the hippocampus of type 2 diabetic rats. *Neurol. Res.* **2014**, *36*, 824–832. [CrossRef]
68. Wess, J.; Eglen, R.M.; Gautam, D. Muscarinic acetylcholine receptors: Mutant mice provide new insights for drug development. *Nat. Rev. Drug Discov.* **2007**, *6*, 721–733. [CrossRef] [PubMed]
69. Langmead, C.J.; Watson, J.; Reavill, C. Muscarinic acetylcholine receptors as CNS drug targets. *Pharmacol. Ther.* **2008**, *117*, 232–243. [CrossRef]
70. Conn, P.J.; Jones, C.K.; Lindsley, C.W. Subtype-selective allosteric modulators of muscarinic receptors for the treatment of CNS disorders. *Trends Pharmacol. Sci.* **2009**, *30*, 148–155. [CrossRef]
71. Morin, J.P.; Rodríguez-Durán, L.F.; Guzmán-Ramos, K.; Perez-Cruz, C.; Ferreira, G.; Diaz-Cintra, S.; Pacheco-López, G. Palatable Hyper-Caloric Foods Impact on Neuronal Plasticity. *Front. Behav. Neurosci.* **2017**, *11*, 19. [CrossRef]
72. Edlow, A.G. Maternal obesity and neurodevelopmental and psychiatric disorders in offspring. *Prenat. Diagn.* **2017**, *37*, 95–110. [CrossRef] [PubMed]
73. Sestan-Pesa, M.; Horvath, T.L. Metabolism and Mental Illness. *Trends Mol. Med.* **2016**, *22*, 174–183. [CrossRef] [PubMed]
74. Hao, S.; Dey, A.; Yu, X.; Stranahan, A.M. Dietary obesity reversibly induces synaptic stripping by microglia and impairs hippocampal plasticity. *Brain Behav. Immun.* **2016**, *51*, 230–239. [CrossRef] [PubMed]
75. Kesby, J.P.; Kim, J.J.; Scadeng, M.; Woods, G.; Kado, D.M.; Olefsky, J.M.; Jeste, D.V.; Achim, C.L.; Semenova, S. Spatial cognition in adult and aged mice exposed to high-fat diet. *PLoS ONE* **2015**, *10*, e0140034. [CrossRef]
76. Baran, S.E.; Campbell, A.M.; Kleen, J.K.; Foltz, C.H.; Wright, R.L.; Diamond, D.M.; Conrad, C.D. Combination of high fat diet and chronic stress retracts hippocampal dendrites. *Neuroreport* **2005**, *16*, 39–43. [CrossRef]

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Do choline alphoscerate and thioctic acid prevent the cerebrovascular alterations in spontaneously hypertensive rats?

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Chronic brain vascular injury is a severe risk factor of cerebral dysfunction. Brain hypoperfusion and white matter lesions correlate with the development of cognitive impairment leading to Alzheimer's disease or vascular dementia. Hypertension has deleterious effects on the brain carrying out to cognitive decline and dementia. Concerning the mechanisms, hypertension-induced cerebrovascular alterations are due to increased production of reactive oxygen species and cholinergic pathways impairment [1].

(+)-Thioctic acid or (+)-TIO eutomer is a well-known antioxidant compound showing brain activity, while choline alphoscerate (GPC) improves choline availability and acetylcholine synthesis/release in brain areas [2,3]. Our study aimed to assess if long-term treatment with these two compounds could induce neuroprotection in the brain of spontaneously hypertensive rats (SHR), used as an animal model of cerebrovascular alterations. Male SHR aged 24 weeks and age-matched normotensive Wistar–Kyoto (WKY) rats were treated for 4 weeks with GPC (150 mg/kg/day), (+)-TIO (125mmol/kg/day) alone or in the association. The brains were removed for western blot and immunohistochemical analysis for different neuroinflammatory markers in the hippocampus as it is involved in memory and learning tasks.

Blood pressure (BP) was higher in SHR rats compared to normotensive WKY. The compounds, alone or in the association, were able to reduce systolic BP, while only their association reduced the diastolic BP. Our results showed that GPC, alone, restored the levels of neuronal nuclei protein while injection of (+)-TIO and GPC combination did not prevent the downregulation of microtubule-associated protein-2 on dendritic arborization. (+)-TIO and GPC alone and in association counteracted the astrogliosis and microglial activation and decreased the level of tumor necrosis factor-alpha.

Our findings indicate that treatment with GPC plus (+)-TIO attenuates neural damage and glial reaction in the hippocampus of SHR and thus affords neuroprotection in hypertensive rats. The administration of the two compounds could represent a new potential strategy to prevent the cerebral alterations related to hypertension opening the opportunity to further evaluations in clinical trials.

References

[1] Iadecola C, Gottesman RF. Neurovascular and Cognitive Dysfunction in Hypertension. Circ Res. 2019;124(7):1025-1044.
[2] Tibullo D, Li Volti G, Giallongo C, Grasso S, Tomassoni D, Anfuso CD, Lupo G, Amenta F, Avola R, Bramanti V. Biochemical and clinical relevance of alpha lipoic acid: antioxidant and anti-inflammatory activity, molecular pathways and therapeutic potential. Inflamm Res. 2017;66(11):947-959.
[3] Traini E, Bramanti V, Amenta F. Choline alphoscerate (alpha-glyceryl-phosphoryl-choline) an old choline-containing phospholipid with a still interesting profile as cognition enhancing agent. Curr Alzheimer Res. 2013;10(10):1070-9.

could represent a therapeutical tool against many brain diseases, since it mediates stress-pathways, neuronal death and synaptic injury.

VITAMIN C ADMINISTRATION REDUCES NEUROINFLAMMATION IN A MOUSE MODEL OF NEURODEGENERATION

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Vitamin C (Vit C) is vitamin known anti-oxidant and anti-inflammatory properties. In this research we investigated the neuroprotective effects of vitamin C in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced animal model of PD and its role in the modulation of neuroinflammation. Vit C significantly reduced the MPTP-induced loss of tyrosine hydroxylase (TH)-positive dopaminergic neuronal cells in the substantia nigra, as well as microglial cells activation and astrogliosis. In addition, gait and spontaneous locomotor activity, evaluated by an automated treadmill and the Open Field test respectively, were partially ameliorated by Vit C treatment in MPTP-intoxicated animals. Taken together, these results showed that the reduction of some of the motor symptoms of our model of Parkinson's like disease was accompanied by the diminution of the activation of the cells involved in neuroinflammatory processes. In relation to neuroinflammation, results show that Vit C reduced the protein and mRNA expression of pro-inflammatory cytokines such as IL-6, TLR4, TNF-α, iNOS, CD40 while anti-inflammatory proteins such as IL-10, CD163, TGF-β and IL-4 resulted increased. Interestingly, we have demonstrated for the first time that Vit C reduces neuroinflammation by modulating microglial polarization and astrocyte activation. In conclusion, our study has evidenced that Vit C may represents a new promising diet supplement for the prevention and alleviating the inflammatory cascade in PD, contributing to neuroprotection.

EFFECTS OF CHOLINE-ALPHOSCERATE AND THIOCTIC ACID ON THE BRAIN OF HYPERTENSIVE RATS

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Hypertension, which is caused by an elevation of blood pressure and increased arterial wall thickness, represents a risk factor for the development of cerebrovascular disease and cognitive impairment. Evidence suggests that hypertension leads to neuroinflammation, which significantly contributes to the physiopathology of cerebrovascular alterations due to the increasing production of reactive oxygen species and cholinergic pathways dysfunction. A cholinergic precursors drug, choline alphoscerate or alpha-glycerylphosphorylcholine (α-GPC), causes an increase in acetylcholine levels improving the cholinergic system countering cognitive impairment, associated with cerebrovascular damage, and could targeting neuroinflammation. (+)-Thioctic acid [(+)-TIO] is the naturally occurring eutomer that have been shown to anti-inflammatory and antioxidant effects in the brain.

The study was designed to investigate if treatment with the two compounds, alone or in association, could induce neuroprotection in the brain of spontaneously hypertensive rats (SHR) used as an animal model of cerebrovascular alterations. 24-weeks old SHR were treated with α-GPC (150 mg/kg/day) and (+)-TIO (125 μmol/kg/day), alone or in combination, for 4 weeks. Age-matched normotensive Wistar Kyoto (WKY) rats were used as normotensive control. The frontal cortex and the hippocampus were collected for western blot and immunohistochemistry investigations of neuronal and neuroinflammatory markers. Blood pressure (BP) was higher in SHR rats compared to normotensive WKY. After 4 weeks of treatment with α-GPC and (+)-TIO, alone or in association, they reduced systolic BP while only their association reduced the diastolic BP. The immunochemical and immunohistochemical results showed that α-GPC alone restored the levels of neuronal nuclei proteins. The two compounds, alone or in association, did not prevent the downregulation of synaptophysin and microtubule-associated protein-2. α-GPC and (+)-TIO counteracted the astrogliosis, microglial activation, and decreased the level of tumor necrosis factor-alpha. Our results indicate that treating hypertensive rats with α-GPC and (+)-TIO reduced neuronal damage and glial response in the two brain areas, providing neuroprotection. The administration of the two compounds could represent a new perspective strategy to prevent hypertension-associated brain alterations. Further investigations may allow evaluating the effects on clinical trials in hypertensive patients.

BDNF AND TRKB IN THE ROMAN RAT BRAIN AFTER ACUTE MILD STRESS

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The Roman Low Avoidance (RLA) and Roman High Avoidance (RHA) rats are two divergent phenotypes displaying respectively reactive and proactive coping styles in face of aversive environmental conditions. Thus, when exposed to stressors, RLA rats display depression- and anxiety-related behaviors, while RHA rats are resistant to stress-induced depression. Different forms of stress-induced depression-like symptoms impair the signalling of brain neurotrophins like the Brain Derived Neurotrophic Factor (BDNF) and induce alterations of synaptic plasticity. We have previously reported behavioral and immunochemical data in the hippocampus (HC) of Roman rats exposed to the forced swimming (FS) as acute robust stressor. Here we extend the characterization of Roman rat brain by investigating the effects of tail pinch (TP), a mild stressor, on BDNF/trkB neuronal signalling in two brain areas: the HC, which plays a role in the control of emotions and in the mnemonic consolidation, and the prefrontal cortex (PFC), involved in the process of decision-making, by using western blot and immunohistochemistry assays. Behavioural testing showed that after 40 min of TP, the RLA rats exhibited a reactive coping activity characterized by freezing, grooming, and tail licking, while RHA rats behaved proactively, spending a longer time biting the clamp and trying to remove it from their tails. Immunochemistry showed that in the dorsal and ventral HC (dHC and vHC) of RLA rats the TP induced an increase of the basal BDNF- and trkB-like immunoreactivity (LI) in the dHC, whereas in the vHC TP caused a decrease in trkB-LI but did not modify the BDNF-LI. As for the RHA rats, TP increased the

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Choline alphoscerate and thioctic acid activity on brain injury in spontaneously hypertensive rats

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Spontaneously hypertensive rats (SHR) represent a model of hypertension and vascular brain injury [1]. Several studies have shown that cerebrovascular changes in SHR may mimic brain vascular disorders of hypertensive individuals. Hypertension represents a risk factor for the development of cerebrovascular disease and cognitive impairment [2] and contributes to the physiopathology of cerebrovascular alterations. This leads to an increased production of reactive oxygen species, cholinergic pathways dysfunction, and neuroinflammation. In SHR, significant brain atrophy, a reduction of white matter volumes, and blood-brain barrier (BBB) dysfunction correlated with gliosis and neuroinflammation.

The present study was designed to investigate if treatment with choline alphoscerate (a-GPC) and (+)-thioctic acid [(+)-TIO], alone or in association, could induce neuroprotection in SHR brain. a-GPC is a cholinergic precursor enhancing cholinergic neurotransmission and countering cognitive impairment associated with cerebrovascular damage. (+)-TIO, the natural eutomer of TIO, has been shown to display antioxidant effects in the brain.

SHR 24-weeks old were treated with a-GPC and (+)-TIO alone or in combination. Age-matched Wistar Kyoto (WKY) rats were used as normotensive controls. Different brain areas were collected for Western blot and immunohistochemistry analysis of neuronal, glial, BBB, and inflammatory markers.

After four weeks of treatment a-GPC and (+)-TIO alone or in association slightly reduced systolic blood pressure values. Western blot and immunohistochemistry showed that a-GPC restored the expression of neuronal nuclei protein. The two compounds alone or in association did not prevent the downregulation of synaptic proteins. a-GPC and (+)-TIO countered astrogliosis and decreased the level of tumor necrosis factor-alpha. An increase of the BBB markers, aquaporin-4 and glucose transport-1, partially restored by the two compounds was noticeable in SHR.

Our results indicate that treatment with a-GPC and (+)-TIO elicits a neuroprotective activity. These data may have a pharmacological relevance and suggest that the two compounds, although they are not anti-hypertensive drugs, could represent a new perspective strategy to prevent hypertension-associated cerebrovascular injury.

References

[1] Tayebati SK, Tomassoni D, Amenta F. Spontaneously hypertensive rat as a model of vascular brain disorder: microanatomy, neurochemistry and behavior. J Neurol Sci. 2012 Nov 15;322(1-2):241-9.

[2] Mahinrad S, Sorond FA, Gorelick PB. Hypertension and cognitive dysfunction: a review of mechanisms, life-course observational studies and clinical trial results. Rev Cardiovasc Med. 2021 Dec 22;22(4):1429-1449.

15% glycerol and micropatterned to obtain the well-established gratings (GR) and the improved asymmetric pattern with scalene triangles (SCA), both able to induce directional stimuli to cells. Moreover, the controlled release of phosphodiesterase inhibitors (PDEI) was designed to chemically promote nerve regeneration and functional recovery. The results of *in vitro* and *ex vivo* direct cultures on microstructured chitosan membranes suggest that the substrates are useful for the oriented growth of neurons, a very important step in making regeneration more effective. The *in vitro* protocol for the administration of PDEI (sildenafil-PDE5I and rolipram-PDE4I) was developed and for both stimulations an interesting gene regulation linked to the neuroprotective brain-derived neurotrophic factor (BDNF) and the proangiogenic Vascular endothelial growth factor (VEGF) in immortalized cultures of sensory and motor neurons was observed. In glial cell cultures, the administration of PDEI resulted in up-regulation of the transcription factor Krox20, which can positively influence the expression of myelin genes and in a decrease cell migration. Furthermore, the administration of Rolipram has been shown to induce an increase in neuritic extension in neuronal populations. Further investigations are underway to deepen the study of the effect of PDEI administration on organotypic cultures (dorsal root ganglia and autonomic ganglia), where neuronal and glial cells co-exist, *ex vivo* models more similar to what happens *in vivo*.

THALAMIC MORPHO-FUNCTIONAL CHANGES FOLLOWING ELECTRICAL STIMULATION OF THE AXOTOMIZED TRIGEMINAL NERVE

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To understand the morpho-functional changes of the thalamic nuclei following stimulation of the transected infraorbital branch of trigeminal nerve. Continuous electric stimulation was applied to the proximal stump of axotomized left infraorbital branch of trigeminal nerve, 12h/day for four weeks. Brain sections were immunostained for cytochrome oxidase (CyO), paravalbumin (Pv) and calbindin (Cb) and quantified in the ventral posteromedial o (VPM), posterior o (PO) and reticular (Rt) thalamic nuclei. Intragroup comparisons between left and right sides and intergroup comparisons between control, axotomized and stimulated-axotomized animals were performed. Axotomization of trigeminal nerve reduced the number of positive Pv and Cb cells in the Rt and the CyO density in all the analyzed thalamic nuclei. Electrical stimulation of the proximal nerve stump restored the cellular density in the Rt and the CyO density. Trigeminal nerve transection induces morpho-functional changes in the thalamus that might trigger chronic neuropathic trigeminal pain. These maladaptive changes are rescued by peripheral electric stimulation, that might represent a potential therapeutic strategy.

ACETYLCHOLINE PRECURSORS ATTENUATE NEUROINFLAMMATION IN LPS-STIMULATED BV2 CELLS

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Choline-containing phospholipids, choline alphoscerate (α-GPC), and cytidine 5′-diphosphocholine (CDP-choline) are both acetylcholine precursors crossing the blood-brain barrier. As procholinergic nootropic agents, studies have provided their neuroprotective effects. Currently, there is a limited number of studies concerning whether they have a similar effect in treating cognitive impairment. Indeed, contradictory results have been reported in their mechanisms of action on the neurovascular units. Since microglia play a crucial role in neuronal damage and protection, this study investigated the effects of α-GPC and CDP-choline on the inflammatory response in activated microglia using an immortalized murine microglial cell line (BV-2) stimulated with lipopolysaccharide (LPS). BV2 microglia were treated with or without LPS and were incubated with LPS and different concentrations of both acetylcholine precursors for 24 h. MTT assay, immunocytochemistry, and Western blotting methods were utilized. MTT assay did not show significant changes in cell viability after treatments at different concentrations. Here, we report no differences in untreated cells. On the contrary, morphological changes and an increase in ionized calcium-binding adapter molecule 1 (Iba1) expression were found in LPS-stimulated BV-2 cells. In addition, the nuclear translocation of nuclear factor-kappa B (NF-κB) and the up-regulation of inflammatory interleukin-1β (IL-1β) were accompanied by an increase in oxidative state proteins and lipid peroxidation in LPS-treated BV2 cells. These alterations were reversed after the treatments with both α-GPC and CDP-choline. Our data demonstrate that these compounds attenuate equally LPS-induced neuroinflammatory responses and suggest insights to explain their therapeutic role in brain disorders characterized by vascular impairment.

STRATEGIES TO IMPROVE PROSTATIC NERVE REGENERATION AFTER RADICAL PROSTATECTOMY

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Prostate cancer is the most frequent cancer among males surpassing the lung and the colorectal cancers, representing the second cause of cancer mortality in industrialized countries. The current treatment of localized prostate cancer in patients with a life-expectancy >10 years is radical prostatectomy (RP). Unfortunately, in patients who undergo RP, frequently iatrogenic