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Asinara male donkey (*Equus africanus asinus var. Albina*) and stallion (*Equus ferus caballus*) reproductive characteristics: Correlations between testicular blood supply and sperm production

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ABSTRACT

The Asinara donkey is a rare small albino variety of feral donkey listed as "critical" by the Food and Agriculture Organization of the United Nations (FAO) and by the Domestic Animal Diversity Information System (DAD-IS) in 2022. The objectives of the present study were to evaluate the reproductive characteristics of healthy male Asinara donkeys through andrological evaluation and testicular echo-color-Doppler examination to assess correlations between testicular blood supply and sperm production and to compare it with horse reproductive characteristics. Eight healthy donkeys and stallions were subjected to semen collection and evaluation, ultrasound and Doppler evaluation of the testicles and serum testosterone determination. The testosterone concentrations in donkeys were 1.42 ± 0.69 ng/ml while in horses were 1.90 ± 0.63 ng/ml within the normal values reported in these species. Horses had greater mean and total testicular volume, semen gel-free volume, and total sperm number than donkeys ($P < 0.001$). Blood flow analysis of the testicular artery in both locations was a relatively simple procedure. The waveforms recorded in a convoluted location (-con) of the testicular artery were characteristic of the high-resistance vessel, while in the marginal location (-marg) were of low-resistance. It was also recorded a gradual decrease in peak systolic velocity (PSV), resistive index (RI), pulsatility index (PI), mean velocity (MV) and testicular arterial blood flow (TABF) values along the peripheral course of the testicular artery. Therefore, donkeys had greater PSV-con ($P \leq 0.01$), PSV-marg ($P < 0.01$), MV-con ($P < 0.001$), MV-marg ($P < 0.01$), testicular arterial blood flow rate (TABFr-con) ($P < 0.001$) and TABFr-marg ($P < 0.01$) than horses. Conversely, pulsatility index (PI-con) was higher in horses ($P < 0.05$). The comparative analysis between the Doppler parameters and the reproductive/seminal characteristics showed a positive correlation of TTBF-con with Testosterone ($\rho = 0.976$, $p < 0.01$ and $\rho = 0.905$, $p < 0.01$ in donkeys and horses, respectively), with Total Testicular Volume ($\rho = 0.952$, $p < 0.01$ and $\rho = 1.000$, $p < 0.01$ in donkeys and horses, respectively), and with Total spermatozoa concentration ($\rho = 0.905$, $p < 0.01$ and $\rho = 0.813$, $p < 0.05$ in donkeys and horses, respectively). Additionally, only in the donkey there was a positive correlation with Semen gel free volume ($\rho = 0.881$, $p < 0.01$) and Spermatozoa concentration × ml ($\rho = 0.786$, $p < 0.05$). The testosterone concentration was positively correlated in both species with Total testicular volume ($\rho = 0.976$, $p < 0.01$ and $\rho = 0.905$, $p < 0.01$ in donkeys and horses, respectively) and Total sperm concentration ($\rho = 0.881$, $p < 0.01$ and $\rho = 0.976$, $p < 0.01$ in donkeys and horses, respectively). It was also correlated positively with Semen gel free volume ($\rho = 0.857$, $p < 0.01$) and spermatozoa concentration ($\rho = 0.762$, $p < 0.05$) in donkeys. Finally, the PI-con ($\rho = -0.786$, $p < 0.05$) was negatively correlated with Semen gel free volume in the donkeys. The results of this study show that Total Testicular Blood Flow is the parameter, together with testosterone, most positively correlated with testicular volume and total sperm concentration in both species. Furthermore, differences in both reproductive and testicular hemodynamic characteristics between the Asinara donkey and stallion are highlighted, suggesting that caution should be taken in transferring knowledge from one species to another. New data on reproductive aspects, seminal

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characteristics, testicular blood flow perfusion in healthy Asinara donkeys were reported and these may represent reference value for further research or clinical applications.

1. Introduction

In some parts of the world, donkeys are used for work while in others they are bred to produce mules and hinnies or are popular as pets. The donkey differs from the horse in some anatomical characteristics and external conformation. They have heavy and coarse head with orbital arches and pronounced zygomatic ridges, big lips, and long ears. Recently, the reproductive aspects of the donkey, have received particular attention in order to avoid the risk of extinction, increase the population and preserve the biodiversity of the breed. However, while the reproductive aspects of the equine stallion have been extensively investigated, the relatively few studies on reproductive aspects of donkey stallion have mainly focused on reproductive and seminal characteristics [1–8], seminal storage techniques [9–14] and testicular and accessory sex gland hemodynamic by ultrasound Doppler evaluation [15–17]. In particular, to the best of our knowledge, totally absent are the information about reproductive characteristics of the male Asinara donkey.

The Asinara donkey (alternative names include "white donkey" or "albino donkey") is a rare small albino variety (withers from 80 to 105 cm high) of feral donkey with a white coat, unpigmented skin and mucosa and pinkish-blue eyes (Fig. 1). The latter two features are caused by a mutation in the tyrosinase gene (TYR) [18]. It is given the specific name of "*Equus africanus asinus var. Albina*", but this is not recognized by the taxonomic authorities, even if it is not a breed in the formal sense. The dwelling of this rare variant is limited in the wild to the island of Asinara (where the total population of donkeys is estimated at about 120 individuals), which is located off the north-western coast of Sardinia, in Italy in the province of Sassari. That being said, there are some conservational groups located in Tuscany, Emilia Romagna, and Umbria in the naturalistic park "Città della Domenica -Perugia". In 2019 the total breed population was 334. Its conservation status was listed as "critical" by the Food and Agriculture Organization of the United Nations (FAO) and by the Domestic Animal Diversity Information System (DAD-IS) in 2022.

The assessment of male reproductive capacity is generally based on the assessment of seminal characteristics. The testicles are the site of sperm production and adequate blood perfusion is necessary for its full functionality. The testicle's blood supply originates from a testicular artery with an unusual length [19] and high flow resistance, which causes reduced intra-testicular capillary pressure with values being lower than the ones encountered in any other organ and only slightly higher than venous pressure [20]. The high metabolic demand of seminiferous tubules exposed to these particular conditions of low pressure and low oxygen tension is ensured by vascularization which, under normal circumstances, is able to provide the testis with sufficient quantities of nutrients and oxygen [21]. A malfunction of the testis can be caused by a moderate disturbance of blood flow [22]. The vascular study by angiography is seldom used due to the danger of radiation in this area [23], while technical advances in ultrasound applications and post-processing development have brought new insights into the structure and function of testicular tissue and therefore into male fertility. In human medicine, Doppler ultrasonography in the testicular examination is regularly applied by virtue of its effectiveness and simplicity being one of the most accurate methods for blood flow evaluation. This technique is able to provide information on the presence, direction and characteristics of the blood flow [24]. Furthermore, the correlation between testicular blood flow characteristics and spermatogenesis has been reported in humans [25], bulls [26], dog [27–29] and stallion [30] and it is also useful in differentiating various causes for dyspermia [24,31]. In equine andrology, several studies have been conducted on testicular ultrasound [32–34] and the assessment of testicular blood flow by Doppler examination [30,35–38] while limited information is available regarding testicular ultrasound of the donkey [15–17,39]. Moreover, since donkeys are different from the stallion in many reproductive, behavioral and physiological aspects [10], the transfer of knowledge obtained in the latter remains to be verified.

The objectives of the present study were i) to evaluate the reproductive characteristics of healthy male Asinara donkeys through andrological evaluation and testicular echo-color Doppler examination ii) to assess correlations between testicular blood supply and sperm production and iii) to compare it with horse reproductive characteristics.

Fig. 1. Male Asinara donkey.

2. Material and methods

2.1. Animals

In this study, eight healthy Asinara donkeys of proven fertility, aged between 3 and 8 years, weighing between 103 and 156 kg and with a withers height of 83–91 cm, and eight healthy stallions of different breeds (3 English thoroughbred, 5 Trotters) weighing between 400 and 520 kg, aged between 6 and 15 years, were used. All donkeys were fed with meadow hay, oats, grains, and carrots, while the horses were fed hay and oats three times a day; all subjects had free access to water. Clinical history confirmed that all subjects had previously procreated.

2.2. Clinical procedure

The animals were conducted at Veterinary Teaching Hospital of the Department of Veterinary Medicine, University of Perugia, Italy during the period April-September 2018. All clinical procedures were performed in accordance with the animal welfare committee's ethical guidelines and were carried out according to the European Guidelines on Animal Welfare (Directive 2010/63/EU) and Italian legislation on animal care (DL n. 26, 04 March 2014). Written informed consent was obtained from each owner to submit donkeys and horses to clinical procedures, and to use clinical records for research purposes. All subjects undergoing to general and particular examination of genital tract, semen collection and evaluation, B-Mode ultrasound of the testicles, Doppler evaluation of the testicular artery and venous blood sampling.

2.3. Semen collection and evaluation

The ejaculates were obtained using an estrus jump-mare and estrus jump-jenny and the semen was collected with a Colorado and Missouri

model artificial vagina, respectively. Each subject had previously undergone semen collection in order to eliminate the semen resulting from sexual inactivity. Semen collection was performed on three consecutive day every four days and the values of seminal parameters were calculated on the average of the three samples.

Color, semen gel free volume, and pH were evaluated immediately after collection. Total and progressive motility (%) of spermatozoa from all samples were evaluated by an expert operator in seminal evaluation, using a prewarmed phase-contrast microscope (Nikon TMS) at x 200 by diluting the semen samples 1:3–1:8, depending on the concentration, with non-fat dry skim milk-glucose extender (E–Z Mixin, Animal Reproduction System, Chino, CA, USA) at 37∘C. Sperm concentration was measured with a Burker counting chamber. The percentage of morphologically normal and abnormal live spermatozoa was calculated in a smear of semen stained with eosin-nigrosin [40] counting 200 spermatozoa assessed under bright-field illumination at 1000 × (Optiphot 2, Nikon, Japan).

2.4. Ultrasound study

The ultrasound machine (SONOACE 8800 Full Digital Medison Inc. ® Austria.) equipped with a micro-convex probe (6.5–7.5 MHz) for B mode and Doppler ultrasound scanning was used. All patients were evaluated between 08:00 and 11:00 a.m. to avoid any effect of circadian rhythmicity on blood flow [41]. All subjects were examined in an upright position, held by a halter and without tranquillization. The B-Mode ultrasound scan of the testicles was performed (longitudinal and transverse sections) and the parameters considered to exclude intercurrent diseases were echogenicity, contours, shape, and presence of the cavity; three dimensions of each testicle were measured and the volume (V) was calculated using the formula for the ellipsoid volume [$V = (4/3\pi) \times (\text{length}/2) \times (\text{width}/2) \times (\text{height}/2)$] [32].

Color Doppler (CD) of testicular arteries was displayed and blood flow parameters were determined by pulsed-wave Doppler (PW). The scan was performed in a quiet room and, after the application of the acoustic gel in the scrotal region, the transducer was placed in direct contact with the scrotum. The blood flow of the testicular artery was measured in each testicle in two locations: i) in a convoluted location (-con) in the ring part of the spermatic cord as close as possible to the cranial pole of the testis and ii) in the marginal location (-marg) in the epididymal border on the caudal pole of the testis as previously described in the horse [35]. Briefly, scanning the cranial pole of the testicle, the probe in cross section was moved cranially on the spermatic cord and when the image of the cranial pole disappeared the CD was activated (duplex mode) and the testicular artery was visualized as areas of blue or red spot in the portion closest to the testis (Fig. 1). This alternation of colors was caused by the detection of blood flow towards and away from the probe, due to the tortuosity of this artery within the spermatic cord. In PW mode (triplex mode), the size of the sample volume, which determines the Doppler information, was kept constant at 1 mm and when at least three arterial Doppler waveforms with maximum Doppler shift were displayed, blood flow parameters were automatically calculated using the algorithm package provided with the ultrasound unit. The blood flow parameters calculated in each position were: peak systolic velocity (PSV), end-diastolic velocity (EDV), mean velocity (MV), resistive index [RI = (PSV-EDV)/EDV] [42], pulsatility index [PI = (PSV-EDV)/MV] [43]. The values obtained at three different points at each location were averaged to obtain a single mean value for each measurement. All measurements were obtained with an angle of insonation $< 60^{\circ}$ to which angle correction was applied. To minimize the variation, presets (depth 1–5 cm, wall filter 0.001 m/s, pulsed repetition frequency 2.91 kHz) were used during the examinations. The color gain was adjusted to reduce flash artifacts.

Three images of the testicular artery were used to calculate the average diameter of this variable and the total arterial blood flow [TABF = MV × A; A = cross-sectional area of the blood vessel, calculated from the formula $A = \pi r^2$ (r = arterial diameter/2); units: ml/s] and total arterial blood flow rate (TABFr = TABF/V; V = testicular volume; units = ml/s/cm^3) [44] were calculated. In addition, to evaluate the amount of blood received by both testes in the unit of time (1 min) to compare with seminal parameters the Total Testicular Blood Flow (TTBF = TABF-con dx + TABF-con sx; units = ml/s) was calculated.

2.5. Blood collection and testosterone analysis

A blood sample was collected from a jugular vein in an evacuated EDTA tube and centrifuged at 200 × *g* for 15 min. The plasma was stored at − 20 °C until analysis. Testosterone concentrations were measured using the chemiluminescent enzyme immunoassay method (Immulite 2000 Xpi, Siemens Healthcare GmbH, Erlangen, Germany). Validation for Equine serum samples showed an intra-assay coefficient of variation of 3.42–4.57 % and an inter-assay coefficient of variation (CV) of 2.62–3.12 %.

2.6. Data analysis

Diagnostic graphics and Levene's test were used to check assumptions and outliers. Since the sample size was small, a non-parameter approach was still chosen to analyze the data. First, for each species, a Related-Samples Wilcoxon signed rank test was used to investigate whether there were differences between the parameters evaluated on the right and on the left side. Since these differences were borderline conventional significance or clinically insignificant (Table 1), and in order to simplify the presentation of the results, the mean of right and left side values was used for further analysis. Then, seminal values and Doppler parameters evaluated in donkeys and horses were compared using Mann-Whitney U tests. Data were reported as means and standard deviations (SD), medians and range. Finally, the associations between Doppler parameters and reproductive/seminal characteristics were evaluated using the Spearman's rho coefficient (ρ). The correlation was considered poor if $\rho < |0.3|$, medium if $|0.3| \leq \rho < |0.5|$, and large if $\rho \geq |0.5|$ [45]. Statistical analysis was performed using SPSS Statistics version 25 software (IBM, SPSS Inc., Chicago, IL, USA). Statistical significance occurred when $P < 0.05$.

3. Results

No clinical, sperm quality, or B-mode abnormalities of the testicle were found in any of the subjects, confirming good reproductive health. The measured serum concentrations of testosterone in donkeys were 1.42 ± 0.69 ng/ml (range, 0.84–2.84 ng/ml) while in horses were 1.90 ± 0.63 ng/ml (range, 0.98–2.63 ng/ml) within the normal values reported in these species [46,47]. The reproductive characteristics and seminal parameters of each subject are reported in Table 1.

Table 2 summarizes seminal parameters of Asinara donkeys and horses and the differences between species. Horses had greater mean and total testicular volume, semen gel-free volume, and total spermatozoa number than Asinara jackass ($P < 0.001$). However, the ratio of testicular volume to body weight is higher in the donkey than in the horse ($P < 0.001$). The differences in motility (total and progressive), sperm concentration x ml, and normal sperm were, in contrast, not significant.

The display and recording of blood flow parameters proved possible in all subjects. Blood flow analysis of the testicular artery in both locations took approximately 35 min for the donkeys and 20 min for the horses and it was a relatively simple procedure. The waveforms recorded by PW in both the donkeys and the horses, in a convoluted location of the testicular artery, were characteristic of the high-resistance vessel displaying a biphasic blood flow pattern with a rapid systolic peak followed by a rapid telediastolic decrease, and a diastolic peak followed by a slow mesotelediastolic flow (Fig. 1). Furthermore, in the

Table 1
Reproductive characteristic and seminal parameters in eight Asinara donkeys and horses.

	AGE (year)	BW (kg)	L Tes Vol (cm^3)	R Tes Vol (cm^3)	Tot Tes Vol (cm^3)	S gel free Vol (ml)	Conc spz (ml × 10^6)	Tot spz num (× 10^9)	Tot mot (%)	Prog mot (%)	Nor spz (%)	Tes (ng/ml)
Donkey 1	3	103	78	76	154	25.3	102.36	2.590	80	75	94	1.31
Donkey 2	5	156	71	73	144	23.6	104.56	2.468	75	70	92	0.84
Donkey 3	5	142	102	102	204	27.0	119.07	3.215	80	75	85	1.54
Donkey 4	4	139	126	115	241	26.0	149.04	3.875	70	60	79	1.96
Donkey 5	8	128	79	75	154	24.9	86.14	2.145	85	60	85	0.96
Donkey 6	7	119	73	76	149	25.0	94.62	2.365	65	60	86	0.89
Donkey 7	8	145	90	84	174	26.8	113.47	3.041	70	60	75	1.05
Donkey 8	8	148	134	131	265	32.5	126.92	4.125	80	75	87	2.84
Horse 1	6	485	209	218	427	41.0	155.0	6.354	75	65	78	1.89
Horse 2	15	490	227	212	439	48.0	151.3	7.263	80	70	84	2.63
Horse 3	8	412	181	199	380	49.0	116.3	5.698	85	80	79	1.46
Horse 4	6	458	199	221	420	54.0	121.0	6.532	75	70	90	1.56
Horse 5	9	469	227	194	421	43.0	138.0	5.934	65	55	85	1.54
Horse 6	12	410	170	163	333	43.0	130.8	5.624	80	75	85	0.98
Horse 7	14	463	209	225	434	58.0	121.4	7.041	75	70	86	2.54
Horse 8	12	460	219	209	428	65.0	109.8	7.137	80	70	83	2.62

BW: Body Weight; **L Tes Vol**: Left Testicular Volume; **R Tes Vol**: Right Testicular Volume; **Tot Tes Vol**: Total Testicular Volume; **S Gel Free Vol:** Semen gel free volume; **Conc spz × ml**: Spermatozoa concentration x ml; T**ot spz num**: Total spermatozoa number; **Tot mot**: Total motility; **Prog mot**: Progressive motility; **Norm spz**: spermatozoa morphologically normal; **Tes**: Testosterone.

marginal location, the waveforms were characteristic of low-resistance vessels with a monophasic blood flow pattern defined by a slow systolic flow followed by a long diastolic decrease with a relatively high telediastolic rate (Fig. 2).

The comparison between right and left testicles for each Doppler parameter evaluated showed no statistically significant differences (data not shown). The comparison between the donkeys and horses for each Doppler parameter of the testicular artery recorded in a convoluted and a marginal location (Table 3) showed a gradual decrease in PSV, RI, PI, MV, and TABF values along the peripheral course of the testicular artery. Therefore, donkeys had greater PSV-con ($P < 0.01$), PSV-marg ($P < 0.01$), MV-con ($P < 0.001$), MV-marg ($P < 0.01$), TABFr-con ($P < 0.001$) and TABFr-marg ($P < 0.01$) than horses. Conversely, PI-con was higher in horses ($P < 0.05$).

The comparative analysis between the Doppler parameters and the reproductive/seminal characteristics showed a positive correlation of TTBF-con with Testosterone ($\rho = 0.976$, $p < 0.01$ and $\rho = 0.905$, $p < 0.01$ in donkeys and horses, respectively), with Total Testicular Volume ($\rho = 0.952$, $p < 0.01$ and $\rho = 1.000$, $p < 0.01$ in donkeys and horses, respectively), and with Total spermatozoa c ($\rho = 0.905$, $p < 0.01$ and $\rho = 0.813$, $p < 0.05$ in donkeys and horses, respectively). Additionally, only in the donkey there was a positive correlation with Semen gel free volume ($\rho = 0.881$, $p < 0.01$) and Spermatozoa concentration × ml ($\rho = 0.786$, $p < 0.05$). The testosterone concentration was positively correlated in both species with Total testicular volume ($\rho = 0.976$, $p < 0.01$ and $\rho = 0.905$, $p < 0.01$ in donkeys and horses, respectively) and Total sperm number ($\rho = 0.881$, $p < 0.01$ and $\rho = 0.976$, $p < 0.01$ in donkeys and horses, respectively). It was also correlated positively with Semen gel free volume ($\rho = 0.857$, $p < 0.01$) and spermatozoa concentration × ml ($\rho = 0.762$, $p < 0.05$) in donkeys. Finally, the PI-con ($\rho = -0.786$, $p < 0.05$) was negatively correlated with Semen gel free volume in the donkeys.

4. Discussion

The donkey is very similar to the horse in several reproductive aspects. However, the diversity between the two species requires that the application of reproductive knowledge acquired in the stallion must be verified. In recent decades, the non-use of donkey for agricultural purposes has led to the gradual abandonment of the breeding of most of the Italian donkey populations. Only recently, in order to preserve biodiversity in the livestock sector, has grown interest in this species in order to protect its genetic heritage. Therefore, to increase the number of individuals and to

Table 2
Comparison of reproductive and seminal parameters in Asinara donkeys and horses.

Parameter	Donkeys			Horses			P value
	Median	Mean	SD	Median	Mean	SD	
T V (cm^3)	82.1	92.8	22.3	212.0	205.0	19.8	< **0.001**
T T V (cm^3)	164.2	185.5	45.9	424.0	410.1	36.2	< **0.001**
T V (proportion relative to BW)	.069	.015	.067	.045	.002	.045	< **0.001**
S G F V (ml)	25.7	26.4	2.7	48.5	50.1	8.4	< **0.001**
C spz × ml (× 10^6)	109.0	112.0	19.9	126.1	130.4	16.4	0.050
Tot spz num (× 10^9)	2.8	3.0	0.7	6.4	6.4	0.7	< **0.001**
T M %	77.5	75.6	6.8	77.5	76.9	5.9	0.798
P M %	65.0	66.9	7.5	70.0	69.4	7.3	0.645
N spz %	85.5	85.4	6.2	84.5	83.8	3.8	0.382
T (ng/ml)	1.2	1.4	0.7	1.7	1.9	0.6	0.130

T V: Testicular Volume; **T T V**: Total Testicular Volume; BW = body weight; **S G F V:** Semen gel free volume; **C spz**: Spermatozoa concentration × ml; **Tot spz num**: Total spermatozoa number; **T M**: Total motility; **P M**: Progressive motility; **N spz**: spermatozoa morphologically normal; **T**: Testosterone.
Bold text indicates a statistically significant difference($p < 0.05$).

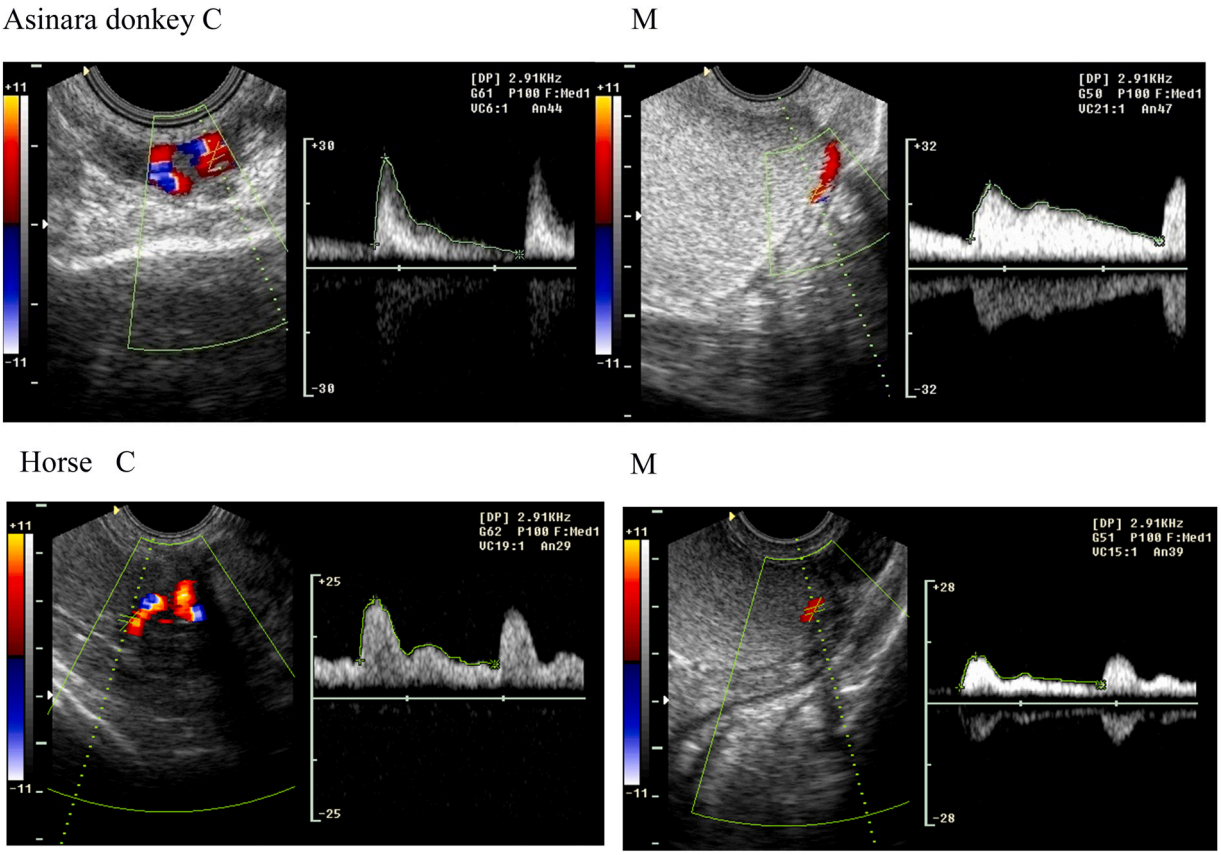

Fig. 2. Waveforms of the testicular artery recorded by PW (Triplex mode) in a convolute (C) and marginal (M) location in Asinara donkeys and horses.

Table 3
Comparison between Asinara donkeys and horses for each Doppler parameter of the testicular artery in a convoluted and marginal location.

Parameter	Donkey				Horse				P value
	Mean	SD	Median	Range	Mean	SD	Median	Range	
PSV-con (cm/s)	23.1	1.9	22.8	21.1–26.4	19.5	1.7	19.2	16.6–21.5	**0.001**
PSV-marg (cm/s)	16.8	4.2	16.5	10.1–23.2	11.7	1.1	11.9	9.9–13.1	**0.007**
EDV-con (cm/s)	4.8	2.2	4.5	2.4–9.4	5.3	1.1	5.7	3.0–6.4	0.382
EDV-marg (cm/s)	5.8	2.3	6.0	3.1–9.9	4.2	1.0	4.2	2.8–5.6	0.161
RI-con	0.79	0.10	0.79	0.62–0.94	0.79	0.06	0.79	0.74-.86	0.878
RI-marg	0.65	0.09	0.66	0.52-.77	0.55	0.09	0.56	0.44-.70	0.065
PI-con	1.50	0.30	1.54	0.97–1.97	1.88	0.22	1.9	1.5–2.1	**0.015**
PI-marg	1.04	0.22	0.97	0.83–1.56	1.22	0.15	1.3	1.0–1.4	0.050
MV-con (cm/s)	12.5	1.6	12.4	10.8–15.8	7.7	1.6	7.3	5.6–10.5	< **0.001**
MV-marg (cm/s)	10.8	2.8	11.4	6.7–15.1	6.3	1.5	5.7	4.5–9.2	**0.003**
TABF-con (ml/s)	0.87	0.2	0.88	0.63–1.16	0.89	0.27	0.87	0.55–1.42	1.000
TABF-marg (ml/s)	0.56	0.13	0.52	0.41–0.79	0.47	0.11	0.44	0.32–0.65	0.195
TABFr-con (ml/s/cm^3)	0.009	0.0003	0.009	0.006–0.016	0.004	0.0001	0.004	0.003–0.006	< **0.001**
TABFr-marg (ml/s/cm^3)	0.006	0.0002	0.007	0.003–0.008	0.002	0.0006	0.002	0.001–0.003	< **0.001**
TTBF-con (ml/s)	1.74	0.4	1.75	1.26–2.33	1.78	0.55	1.74	1.10–2.85	1.000
TTBF-marg (ml/s)	1.12	0.26	1.05	0.82–1.58	0.94	0.21	0.90	0.64–1.30	0.195

PSV-con: peak systolic velocity convolute; **PSV-marg**: peak systolic velocity marginal; **EDV-con**: end diastolic velocity convolute; **EDV-marg**: end diastolic velocity marginal; **RI-con**: resistive index convolute; **RI-marg**: resistive index marginal; **PI-con**: pulsatility index convolute; **PI-marg**: pulsatility index marginal; **MV-con**: mean velocity convolute; **MV-marg**: mean velocity marginal; **TABF-con**: total arterial blood flow convolute; **TABF-marg**: total arterial blood flow marginal; **TABFr-con**: total arterial blood flow rate convolute; **TABFr-marg**: total arterial blood flow rate marginal; **TTBF-con**: total testicular blood flow convolute; **TTBF-marg**: total testicular blood flow marginal. Bold text indicates a statistically significant difference($p < 0.05$).

avoid their extinction, it is necessary to deepen the study reproductive characteristics.

In the present study, we evaluated and compared the reproductive characteristics of the Asinara donkey with those of the stallion, also analyzing the correlations between testicular blood supply and sperm production. To the best of our knowledge, this is the first report that has analyzed testicular blood flow detected by Doppler ultrasonography in healthy male Asinara donkeys.

Testicular blood flow is the major route for the transport of nutrients, regulatory hormones, and secretory products to and from the testis [19]. The testicular artery provides a stable blood supply necessary for metabolic processes and sperm production. The control of blood flow is of extreme importance in every organ, especially in the testis where the concentration of oxygen in the seminiferous tubules is very low [48]. Reduced blood flow results in ischemic damage leading to impaired spermatogenesis. Partial restriction of the testicular artery

has been reported to adversely affect the growth, volume, and histological structure of bull testes, causing the complete or partial arrest of spermatogenesis [49], whilst the total occlusion of the testicular artery caused selective damage to spermatogonia in rats [50,51], and rams [52].

In the present study ultrasound evaluation and analysis of testicular blood flow characteristics were performed in all subjects without any particular difficulties. The longer time required to perform the echo-color-Doppler examination in the donkey was caused by the increased mobility of the Asinara donkeys during the examination due to their being less accustomed to being manipulated.

Testicular artery blood flow characteristics showed no differences between the left and right testis. The waveforms, in the convoluted location in both species were biphasic typical of high resistance vessels, while monophasic non-resistive at the marginal location (Fig. 1). These features were similar to those reported by other authors in the donkey [15,16] and stallion [35] while differing from those reported in humans [53] and dogs [27,54] where they showed a non-resistive, monophasic appearance. The resistive nature of the testicular artery in the convoluted position may be due to the horizontal orientation of the testis associated with a relatively short spermatic cord with a highly convoluted artery. However, although the waveforms recorded by PW of the testicular artery were similar in the two species, some differences were highlighted. The Asinara donkey showed in both locations greater PSV and MV compared to the horse. The donkey showed in both locations greater PSV and MV compared to the horse. The velocity of blood flow within the arterial vessels during the cardiac cycle is directly visualized by the PSV and EDV, and the mean values of these variables in the testicular artery in our group of animals were analogous to those previously reported in donkeys [5,16] and stallions [30,35].

The gradual decrease of the Doppler parameters (PSV, RI, PI, MV) recorded along the course of the testicular artery was the expression of the gradual reduction in peripheral resistance. However, because blood flow velocities represent an angle-dependent technique, careful interpretation is required. This crucial limiting factor is absent when using indices (RI and PI) to assess blood flow, as they are angle-independent. The RI is a parameter that reflects the resistance to blood flow caused by the microvascular bed distal to the measurement site whereas PI is designed to quantify pulsatility or waveform oscillations. These indices are widely used to study testicular perfusion in humans and animals under physiological [27,31,35,54–57] and pathological conditions [54,58,59]. It has also been suggested that RI is significantly correlated with sperm production rate score [31]. In the present study RI and PI values detected in the testicular artery were similar to those previously reported in donkeys [5] and stallions [35] in both locations. However, in our group of animals PI was negatively correlated with Semen gel-free volume differently from what has been reported in other works [15,30].

A recent work [17] measuring RI in convoluted and marginal locations of the testicular arteries, registered higher values for the hipospermic donkeys (0.88 ± 0.01, 0.76 ± 0.01) in comparison with the normospermic donkeys (0.69 ± 0.02, 0.55 ± 0.01). In our study RI in convoluted and marginal locations, was 0.79 ± 0.1 and 0.65 ± 0.1, values similar to the ones registered for hypospermic males from the previously mentioned study.

In a study regarding seasonal effects on testicular perfusion in five mature fertile stallions, were introduced the parameters of TABF and TABF rate [60]. TABF represents the amount of blood conveyed by the artery in the unit of time (ml/s), while TABFr represents the amount of blood conveyed by the artery in the unit of time related to testicular volume (ml/s/cm^3) [44]. These parameters are the best indicators of organ perfusion and their change may serve as an early sign of testicular pathology associated with vascular disorders. In addition, has been shown that tissue perfusion within the testis influences sperm quality and quantity [61].

In the present work, TABF and TABFr were evaluated for the first time in the Asinara donkey. Furthermore, to evaluate the relationship between the amount of blood received by both testicles of each subject and its sperm production, we have for the first time calculated the Total Testicular Blood Flow (TTBF = TABF-con dx + TABF-con sx; units = ml/s). This parameter, together with testosterone, was found to be the most positively correlated with testicular volume and total sperm concentration in both Asinara donkeys and horses. It is interesting to note that, although there are no statistically significant differences regarding the amount of blood carried by the testicular artery in both species in the two locations (see TABF-con and TABF-marg, Table 3), due to the different testicular volume, the amount of blood per cm^3 of testicular parenchyma is significantly greater in the Asinara donkeys compared to the horses. Consequently, greater TABFr values for both convoluted and marginal locations of the donkey's testis were obtained. It is possible to hypothesize that the increased testicular blood supply of these subjects could be the expression of an adaptive process to maintain the physiological conditions necessary for testicular function and therefore for spermatogenesis. In fact, in these albino subjects, to compensate for the absence of the photoprotective action of melanin there are several adaptive protective mechanisms such as the presence of high levels of available retinol (vitamin A) in the bloodstream capable to reach peripheral tissues [62].

The operator's experience and the number of measurements are of great significance for an accurate blood flow analysis [63–65]. They may influence the average values obtained. Two to seven sweeps are generally used in most Doppler studies [36,37,66]. The repeatability and reproducibility of Doppler measurements were not evaluated in this study; however, they were obtained by an experienced operator.

In conclusion, new data on reproductive aspects, seminal characteristics, testicular blood flow perfusion in healthy Asinara donkeys and horses were reported. The results of this study confirm that Doppler ultrasonography is a non-invasive method which can be used to detect and measure testicular blood flow in donkeys and horses and that Total Testicular Blood Flow is the parameter, together with testosterone, most positively correlated with testicular volume and total sperm concentration in both species. Furthermore, differences in both reproductive and testicular hemodynamic characteristics between the stallion and donkey are highlighted, suggesting caution should be taken in transferring knowledge from one species to another. The data provided can ensure reference value for further research or clinical applications.

CRediT authorship contribution statement

Conceptualization, R.Z.; Methodology, G.C.; Software, O.B.; Validation, L.M., and G.B.; Formal analysis, G.C. and S.A.; Investigation, R.Z.; Resources, N.T.C.; Data curation, R.Z.; Writing – original draft, R.Z. and O.B.; Writing – review & editing, R.Z. and N.T.C.; Visualization, O.B. and S.A.; Supervision R.Z. and G.B. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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