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Immunolocalisation of nitric oxide synthase isoforms in the *ductuli efferentes* and epididymis of prepubertal and adult alpaca (*Lama pacos*)

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The present research used immunohistochemistry to analyse the detection and localisation of nitric oxide synthase (NOS) isoforms in the ductuli efferentes and epididymis of prepubertal and adult alpaca. In the ductuli efferentes and epididymis of prepubertal and adult animals, nNOS and eNOS were similarly expressed in epithelial lining cells, conversely differences were observed in the immunopresence of iNOS. Our data provide evidence that NOS isoforms may have roles in reproductive functions and in the developmental processes of the excurrent duct system in the alpaca. (Folia Morphol 2017; 76, 4: 603–607)

Key words: immunohistochemistry, NOS isoforms, genital tracts, alpaca

INTRODUCTION

Nitric oxide (NO) synthase (NOS) is the enzyme that convert L-arginine to NO and L-citrulline; NO acts as an intracellular messenger in many physiological and pathophysiological events, in numerous cell types, including those of the reproductive system [6, 7]. NO functions as an atypical neurotransmitter in the central nervous system and as a non-adrenergic and non-cholinergic mediator in the control of different reproductive tract organs [2]. NOS exists in two forms: constitutive, Ca^{2+}-dependent forms that are rapidly activated by agonists that elevate intracellular free Ca^{2+}, including neuronal NOS (nNOS) and endothelial NOS (eNOS); and a Ca^{2+}-independent inducible form (iNOS) [8]. Both constitutive nNOS and eNOS are normally expressed in cells other than the neuronal and endothelial cells in which they were respectively first detected; additionally, they produce low levels of NO. Conversely, iNOS generates large amounts of NO in various cell types when expression is activated; it can be induced after several hours of immunological stimulation and is detectable in macrophages, neutrophils and endothelial cells [9]. The presence of NOS has been demonstrated by immunohistochemical and enzyme studies in female reproductive tissues, including the ovary, oviduct, and uterus showing the involvement of NO in a number of reproductive processes such as ovulation, implantation and embryo development and uterine contractility [1, 16, 18, 22, 26]. In male, NO regulates the mating behaviour and is involved in sexual performance through its direct involvement in penile erection and contractility of seminal vesicles; additionally, it is implicated in different sperm functions including motility, metabolism, acrosome reaction and biosynthesis and secretion of steroid hormones [22, 23].

The male alpaca (*Lama pacos*), a South American camelid species, has unique reproductive characteristics, which cause their poor breeding performance compared with other domestic species [21]. There are some basic descriptions of the histology, histochemistry and immunohistochemistry of the male

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genital tracts of alpacas [10–12, 14, 19] and also some aspects of the male reproductive physiology have been examined [3, 13, 24].

In order to add new data about the male reproductive biology of this camelid, the present research was designed to evaluate the cellular localisation of NOS isoforms in the *ductuli efferentes* and epididymis of prepubertal and adult alpaca.

MATERIALS AND METHODS

Animals and tissue collection

Five normal male alpacas of the farm "Maridiana" (Umbertide, Umbria, Italy), ranging in age from 4 to 8 years, were used. These animals were castrated, following the description provided by Fowler [5], to prevent aggressive behaviour or to allow housing with non-pregnant females. Immediately after castration at the Veterinary Teaching Hospital of the University Camerino, the testes and epididymides were promptly removed, trimmed of excess tissue, and weighed separately.

Immunohistochemistry of nNOS, eNOS and iNOS

Ductuli efferentes and epididymides (divided into *caput, corpus, cauda*) were processed for immunohistochemical investigation according to procedures previously described [25, 27]. The slides were incubated with the following primary rabbit polyclonal antibodies (Abcam, Cambridge): anti-nNOS (1:250), anti-eNOS (1:10) and anti-iNOS (1:200) [15, 17]. Then, the slides were incubated with biotinylated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, CA, USA), exposed to avidin-biotin complex (ABC kit, Vector Laboratories) and the peroxidase activity sites were visualised using the DAB kit (Vector Laboratories) as chromogen [4, 20]. In some cases, the sections were counterstained with Mayer's haematoxylin. Tissue sections in which the primary antibody was omitted or substituted by rabbit IgG were used as negative controls of non-specific staining.

RESULTS AND DISCUSSION

In this research, we have demonstrated a variable presence of NOS isoforms in the *ductuli efferentes* and in the epididymal tracts of the prepubertal and adult alpaca.

In the *ductuli efferentes* of prepubertal animals, immunosignals for nNOS are localised in the nucleus and cytoplasm of non-ciliated and ciliated cells (Fig. 1A), whereas in adult alpaca, they were observed only in non-ciliated cells (Fig. 1B). Immunoreactivity of eNOS was evidenced in the nucleus and cytoplasm of epithelial lining cells of the *ductuli efferentes* of both prepubertal (Fig. 1C) and adult animals (Fig. 1D), conversely, immunosignals for iNOS were detected in cytoplasm and nucleus of ciliated and non-ciliated cells only in prepubertal alpaca (Fig. 1E). The smooth muscle cells, neuronal fibres, endothelial cells, and fibroblasts were immunostained with nNOS and eNOS in prepubertal and adult animals (Fig. 1A–D), whereas with iNOS only in prepubertal ones (Fig. 1E).

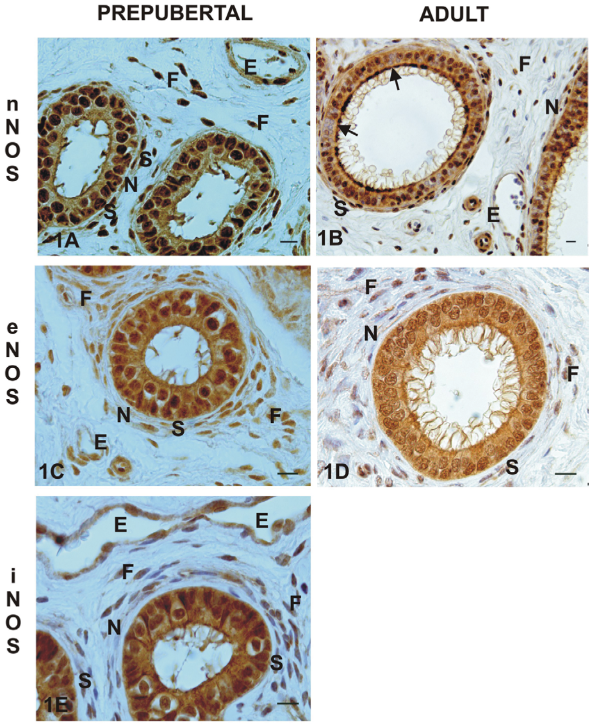

Figure 1. Immunoreactivity of nitric oxide synthase (NOS) isoforms in the *ductuli efferentes* of prepubertal (**A**, **C**, **E**) and adult (**B**, **D**) alpaca; **A.** nNOS; **B.** nNOS (counterstained with haematoxylin): the ciliated cells are unreactive (arrows); **C.** eNOS; **D.** eNOS (counterstained with haematoxylin); **E.** iNOS; S — smooth muscle cells; N — neuronal fibres; E — endothelial cells; F — fibroblasts. Bars = 10 μm.

In all tracts of epididymis, immunostaining for nNOS is localised in the nucleus and cytoplasm of principal and basal cells in prepubertal (Fig. 2A, C, E) and adult (Fig. 2B, D, F) animals. In particular, in the adults, it was evidenced the intense immunoreactivity of the supranuclear cytoplasm (probably corresponding to the Golgi zone) in the principal cells of the epididymal *corpus* (Fig. 2D) and *cauda* (Fig. 2F). The apical mitochondria-rich cells (AMRCs), that we

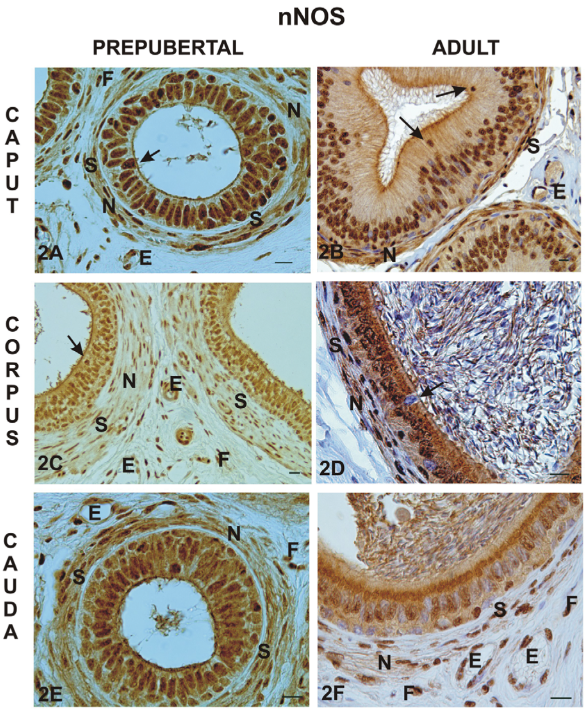

Figure 2. Immunoreactivity of neuronal nitric oxide synthase (nNOS) in the *caput*, *corpus*, and *cauda* epididymis of prepubertal (**A**, **C**, **E**, respectively) and adult (**B**, **D**, **F**, respectively — counterstained with haematoxylin) alpaca; AMRCs — apical mitochondria-rich cells (arrows); S — smooth muscle cells; N — neuronal fibres; E — endothelial cells; F — fibroblasts. Note in panel D the AMRCs (arrow) is unreactive. Bars = 10 μm.

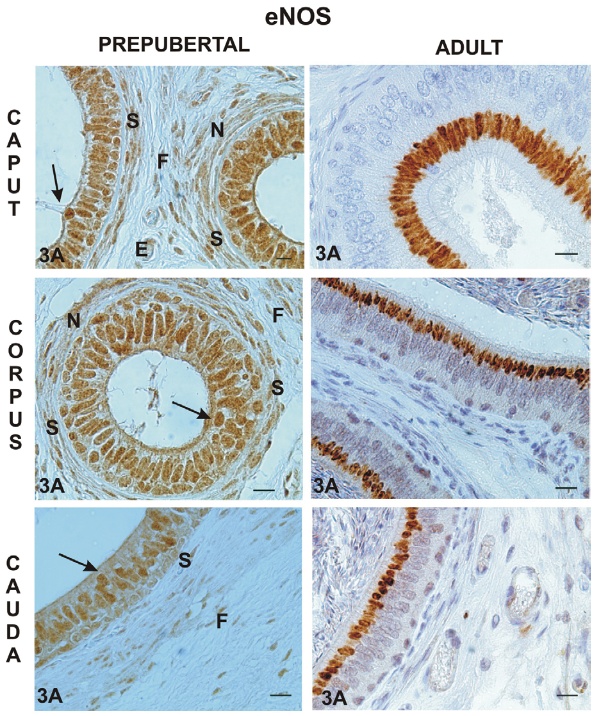

Figure 3. Immunoreactivity of endothelial nitric oxide synthase (eNOS) in the *caput*, *corpus*, and *cauda* epididymis of prepubertal (**A**, **C**, **E**, respectively) and adult (**B**, **D**, **F**, respectively — counterstained with haematoxylin) alpaca; AMRCs — apical mitochondria-rich cells (arrows); S — smooth muscle cells; N — neuronal fibres; E — endothelial cells; F — fibroblasts. Bars = 10 μm.

previously described in the alpaca epididymis [10], expressed nNOS in the *caput* (Fig. 2A) and *corpus* (Fig. 2C) epididymis of prepubertal animals and in the *caput* epididymis of adults (Fig. 2B). Smooth muscle cells, neuronal fibres, endothelial cells and stromal fibroblasts displayed immunosignals for nNOS in all epididymal regions of both prepubertal and adult alpaca (Fig. 2A–F).

The immunopresence of eNOS was observed mainly in the nucleus of epithelial lining cells of epididymal *caput* (Fig. 3A), *corpus* (Fig. 3C) and *cauda* (Fig. 3E) of prepubertal animals, whereas it was detected in the Golgi zone of principal cells of all the epididymal tracts in the adults (Fig. 3B, D, F). The AMRCs expressed eNOS in all epididymal tracts only in prepubertal alpaca (Fig. 3A, C, E); smooth muscle cells, neuronal fibres, endothelial cells and stromal fibroblasts were immunostained with eNOS in all epididymal regions but only in prepubertal animals (Fig. 3A, C, E).

Inducibile NOS immunoreactivity was evidenced in the epithelial cells of the epididymal *corpus* and *cauda* and it was localised in the cytoplasm and nucleus of these cells in prepubertal alpaca (Fig. 4B, D) and in their cytoplasm in adults (Fig. 4C, E). The AMRCs expressed iNOS in all epididymal tracts of prepubertal alpaca (Fig. 4A, B, D). Smooth muscle cells, neuronal fibres, endothelial cells and stromal fibroblasts showed immunosignals for iNOS in prepubertal epididymal *corpus* (Fig. 4B) and *cauda* (Fig. 4D), whereas in adults, they were always unreactive (Fig. 4C, E).

CONCLUSIONS

On the basis of the widespread distribution of NOS isoforms in the genital tracts of the alpaca, we suggest the potential role for NO in the mediation of various reproductive functions such as sperm transit, storage and maturation [6, 22]. Additionally, NO may also act locally within the epithelial lining cells affecting their functions. The greatest immunopresence of iNOS detected in prepubertal *ductuli efferentes* and epididymis respect to the adult animals suggests that this enzyme could be involved in the develop-

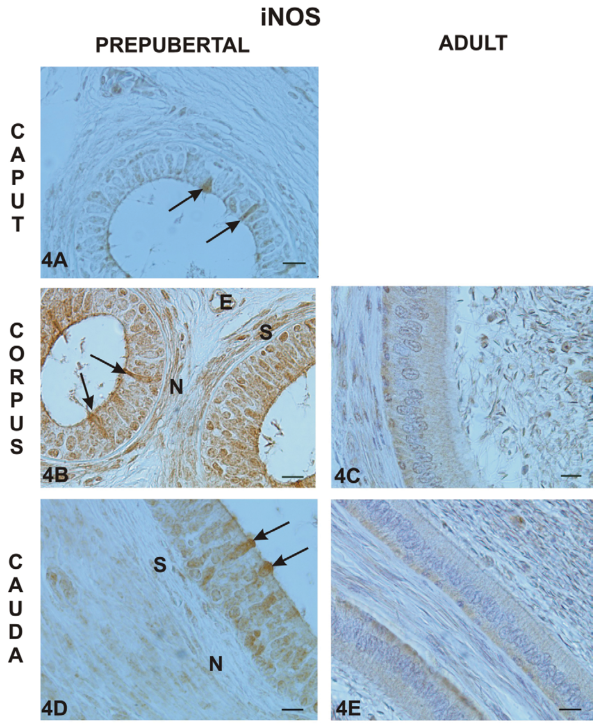

Figure 4. Immunoreactivity of inducible form nitric oxide synthase (iNOS) in the *caput*, *corpus*, and *cauda* epididymis of prepubertal alpaca (**A**, **B**, **D**, respectively) and in the corpus and cauda of adult animals (**C**, **E**, respectively — counterstained with haematoxylin); AMRCs — apical mitochondria-rich cells (arrows); S — smooth muscle cells; N — neuronal fibres; E — endothelial cells. Bars = 10 μm.

mental processes of the excurrent duct system in the alpaca [13].

However, further functional studies are warranted to clarify the exact role of NO in the male reproductive tracts of this species.

REFERENCES

1. Boiti C, Zerani M, Zampini D, et al. Nitric oxide synthase activity and progesterone release by isolated corpora lutea of rabbits in the early and mid-luteal phases of pseudopregnancy are modulated differently by prostaglandin E-2 and prostaglandin F-2alpha via adenylate cyclase and phospholipase C. J Endocrinol. 2000; 164(2): 179–186, indexed in Pubmed: 10657853.
2. Burnett AL, Ricker DD, Chamness SL, et al. Localization of nitric oxide synthase in the reproductive organs of the male rat. Biol Reprod. 1995; 52(1): 1–7, indexed in Pubmed: 7536043.
3. Catone G, Zerani M, Quassinti L, et al. GnRH receptor immunolocalization and *in vitro* GnRH effects in Leydig cells of adult alpaca (Lama pacos) testis. Reprod Dom Anim. 2010; 45(Suppl. 3): 81.
4. Colitti M, Parillo F. Immunolocalization of estrogen and progesterone receptors in ewe mammary glands. Microsc Res Tech. 2013; 76(9): 955–962, doi: 10.1002/jemt.22254, indexed in Pubmed: 23818009.
5. Fowler ME. Medicine and Surgery of South American Camelids: Llama, Alpaca, Vicuňa, Guanaco. Ames I.A. ed. Iowa State University Press 1998.
6. Ha Ty, Kim Hs, Shin T. Expression of constitutive endothelial, neuronal and inducible nitric oxide synthase in the testis and epididymis of horse. J Vet Med Sci. 2004; 66(4): 351–356, indexed in Pubmed: 15133263.
7. Kim HC, Byun JS, Lee TK, et al. Expression of nitric oxide synthase isoforms in the testes of pigs. Anat Histol Embryol. 2007; 36(2): 135–138, doi: 10.1111/j.1439-0264.2006.00739.x, indexed in Pubmed: 17371387.
8. McCann SM, Mastronardi C, de Laurentiis A, et al. The nitric oxide theory of aging revisited. Ann N Y Acad Sci. 2005; 1057: 64–84, doi: 10.1196/annals.1356.064, indexed in Pubmed: 16399888.
9. Meiser H, Schulz R. Detection and localization of two constitutive NOS isoforms in bull spermatozoa. Anat Histol Embryol. 2003; 32(6): 321–325, indexed in Pubmed: 14651478.
10. Parillo F, Verini Supplizi A, Macrì D, et al. The ductus epididymis of the alpaca: immunohistochemical and lectin histochemical study. Reprod Domest Anim. 2009a; 44(2): 284–294, doi: 10.1111/j.1439-0531.2008.01067.x, indexed in Pubmed: 18992100.
11. Parillo F, Magi GE, Diverio S, et al. Immunohistochemical and lectin histochemical analysis of the alpaca efferent ducts. Histol Histopathol. 2009; 24(1): 1–12, doi: 10.14670/HH-24.1, indexed in Pubmed: 19012239.
12. Parillo F, Verini Su, Scrollavezza P, et al. Immunolocalization of ciclooxygenases in the *ductuli efferentes* and epididymis of pre-pubertal and adult alpaca (*Lama pacos*). Reprod Dom Anim. 2009c; 44(Suppl. 3): 119.
13. Parillo F, Catone G, Boiti C, et al. Immunopresence and enzymatic activity of nitric oxide synthases, cyclooxygenases and PGE2-9-ketoreductase and in vitro production of PGF2α, PGE2 and testosterone in the testis of adult and prepubertal alpaca (*Lama pacos*). Gen Comp Endocrinol. 2011; 171(3): 381–388, doi: 10.1016/j.ygcen.2011.03.001, indexed in Pubmed: 21377467.
14. Parillo F, Verini Supplizi A, Mancuso R, et al. Glycomolecule modifications in the seminiferous epithelial cells and in the acrosome of post-testicular spermatozoa in the alpaca. Reprod Domest Anim. 2012; 47(4): 675–686, doi: 10.1111/j.1439-0531.2008.01134.x, indexed in Pubmed: 19192215.
15. Parillo F, Catone G, Maranesi M, et al. Immunolocalization, gene expression, and enzymatic activity of cyclooxygenases, prostaglandin e2-9-ketoreductase, and nitric oxide synthases in Mediterranean buffalo (*Bubalus bubalis*) corpora lutea during diestrus. Microsc Res Tech. 2012; 75(12): 1682–1690, doi: 10.1002/jemt.22116, indexed in Pubmed: 22865504.
16. Parillo F, Catone G, Gobbetti A, et al. Cell localization of ACTH, dopamine, and GnRH receptors and PPARγ in bovine corpora lutea during diestrus. Acta Sci Vet. 2013; 41: e1129.
17. Parillo F, Dall'Aglio C, Brecchia G, et al. Aglepristone (RU534) effects on luteal function of pseudopregnant rabbits: steroid receptors, enzymatic activities, and hor-

mone productions in corpus luteum and uterus. Anim Reprod Sci. 2013; 138(1-2): 118–132, doi: 10.1016/j.anireprosci.2013.02.001, indexed in Pubmed: 23517855.
18. Parillo F, Maranesi M, Brecchia G, et al. Immunohistochemial study on effectors of ovary medulla blood vessel in pseudopregnant rabbit. Acta Sci Vet. 2013; (in press).
19. Parillo F, Catone G, Capezzone C, et al. Presence of immunoreactive cyclooxygenases in the *ductuli efferentes* and epididymis of prepubertal and adult alpaca (*Lama pacos*). Vet Arhiv. 2013; (in press).
20. Parillo F, Zerani M, Maranesi M, et al. Ovarian hormones and fasting differentially regulate pituitary receptors for estrogen and gonadotropin-releasing hormone in rabbit female. Microsc Res Tech. 2014; 77(3): 201–210, doi: 10.1002/jemt.22328, indexed in Pubmed: 24375763.
21. Tibary A, Vaughan J. Reproductive physiology and infertility in male South American camelids: A review and clinical observations. Small Ruminant Research. 2006; 61(2-3): 283–298, doi: 10.1016/j.smallrumres.2005.07.018.
22. Yang JZ, Ajonuma LC, Rowlands DK, et al. The role of inducible nitric oxide synthase in gamete interaction and fertilization: a comparative study on knockout mice of three NOS isoforms. Cell Biol Int. 2005; 29(9): 785–791, doi: 10.1016/j.cellbi.2005.05.005, indexed in Pubmed: 16087361.
23. Zini A, O'Bryan MK, Magid MS, et al. Immunohistochemical localization of endothelial nitric oxide synthase in human testis, epididymis, and vas deferens suggests a possible role for nitric oxide in spermatogenesis, sperm maturation, and programmed cell death. Biol Reprod. 1996; 55(5): 935–941, indexed in Pubmed: 8902202.
24. Zerani M, Catone G, Quassinti L, et al. *In vitro* effects of gonadotropin-releasing hormone (GnRH) on Leydig cells of adult alpaca (*Lama pacos*) testis: GnRH receptor immunolocalization, testosterone and prostaglandin synthesis, and cyclooxygenase activities. Domest Anim Endocrinol. 2011; 40(1): 51–59, doi: 10.1016/j.domaniend.2010.08.006, indexed in Pubmed: 20961724.
25. Zerani M, Catone G, Maranesi M, et al. Gonadotropin-releasing hormone 1 directly affects corpora lutea lifespan in Mediterranean buffalo (*Bubalus bubalis*) during diestrus: presence and *in vitro* effects on enzymatic and hormonal activities. Biol Reprod. 2012; 87(2): 45, doi:10.1095/biolreprod.112.099598, indexed in Pubmed: 22592497.
26. Zerani M, Catone G, Betti G, et al. Immunopresence and functional activity of prostaglandin-endoperoxide synthases and nitric oxide synthases in bovine corpora lutea during diestrus. Folia Morphol. 2013; 72(1): 36–40, indexed in Pubmed: 23749709.
27. Zerani M, Maranesi M, Brecchia G, et al. Evidence for a luteotropic role of peroxisome proliferator-activated receptor gamma: expression and *in vitro* effects on enzymatic and hormonal activities in corpora lutea of pseudopregnant rabbits. Biol Reprod. 2013; 88(3): 62, doi: 10.1095/biolreprod.112.107383, indexed in Pubmed: 23365414.