PAPER

Innervation and immunohistochemical characteristics of epididymis in Alpaca camelid (*Vicugna pacos*)

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Abstract

Alpacas (*Vicugna pacos*) are domesticated camelids indigenous to south America and recently also bred in Europe and Italy for their high quality wool. There is little data available regarding the innervation of the male reproductive tract of this species. In the present study, the distribution of protein gene product 9.5 (PGP 9.5), neuropeptide Y (NPY), tyrosine hydroxilase (TH), calcitonin gene related peptide (CGRP) and substance P (SP) was analyzed in the epididymis by using immunohistochemical methods. Specimens of the caput, corpus and cauda epididymis were fixed in Bouin's fluid and processed for immunohistochemistry analysis with primary antibodies against PGP 9.5, NPY, TH, CGRP and SP. Immunopositivity to PGP 9.5 and TH and NPY was observed in nerve fibre bundles and in single nerve fibres contained into the peritubular connective tissue. Many TH and NPY immunopositive cells were found to innervate blood vessels. Rare CGRP and SP immunopositive nerves were observed. Several PGP 9,5 and NPY immunopositive epithelial cells were observed in the caput epididymis. The results of the present study suggest a role for the innervations in modulate reproductive functions in the alpaca epididymis.

Introduction

Alpacas are domesticated camelids which play an important socio-economic role in high altitude regions like Andean regions of south America from Ecuador to southern Chile. Recently, they have become increasingly popular in the USA, Australia and Europe. In particular, in Italy, alpacas are considered producers of high quality wool. Knowledge about the reproduction functions in male alpaca is limited. In south America, alpacas are seasonal breeders for 2-3 months (from December to March) when the availability of food is better (Fowler, 1998). Outside their natural habitat, alpacas are considered non seasonal breeders (Sumar *et al*., 1999). Alpacas, moreover, are an induced ovulating species (San Martin *et al*., 1968).

In the male of other camelid species, seasonal histological changes were observed in the testis and epididymis (Zayed, 1994; Saleh, 2002). Up until now, however, histological changes in the genital tract of these species were not yet reported. There are several studies describing male genital tract of alpacas. In particular, Parillo *et al.* (2009a, 2009b, 2012) reported a detailed distribution of glycosidic residues of glycoconjugates in the epididymis, testis and spermatozoa. Wang *et al*. (2011) reported that NGF is espressed in the testis of alpaca . In addition, it has been demonstrated that GnRH directly up-regulates testosterone production in Leydig cells with a postreceptorial mechanism that involves phospholipase C, COX1 and PGF2 (Zerani *et al.,* 2011). Recently, moreover, it has been postulated that orexin and its receptor 1 are involved in the regulation of steroidogenesis in the alpaca testis (Liguori *et al*., 2012).

The mammalian epididymis is macroscopically divided in three regions: caput (proximal and distal tracts), corpus and cauda and it plays a pivotal role in the process of sperm maturation. During the transit through the epididymis, the testicular fluid is modified as a result of the absorbptide and secretory function of the epithelial cells lining the epididymal lumen (Dacheux *et al*., 2003; Srivastav *et al*., 2004; Tulsiani, 2006). The autonomic innervation plays a key role in regulation of male genital functions. Anatomical, pharmacological and physiological data (Hib, 1976; Laitinen and Talo, 1981; Pholpramool and Triphon, 1984; Mirabella *et al*., 2006, 2008; Squillacioti *et al*., 2008, 2009) suggest that the role of adrenergic and cholinergic inner-

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Table 1. Primary antibodies used in immunohistochemistry.

Primary antibodies	Species	Code	Dilution	Supplier
PGP9.5	Rabbit	7863-0504	1:500	AbD Serorec, Oxford, UK
TH	Mouse	22941	1:250	DiaSorin Inc., Stillwater, MN, USA
NPY	Rabbit	22940	1:250	Immunostar, Hudson, WI, USA
SP	Rabbit	IHC7451	1:250	Peninsula lab., Belmont, CA, USA
CGRP	Guinea pig	T 5027	1:250	Peninsula lab., Belmont, CA, USA

Table 2. Secondary antibodies used in immunohistochemistry.

Secondary antibodies	Code	Dilution	Supplier
Biotinylated anti-mouse IgG	BA- 9200	1:200	Vector Laboratories Inc., Burlingame, CA, USA
Biotinylated anti-goat IgG	BA- 5000	1:200	Vector Laboratories Inc., Burlingame, CA, USA
Biotinylated anti guinea-pig IgG	BA- 7000	1:200	Vector Laboratories Inc., Burlingame, CA, USA
Biotinylated anti-rabbit IgG	BA- 1000	1:200	Vector Laboratories Inc., Burlingame, CA, USA

vation in male genital tract is to regulate epididymis contraction, sperm transport, emission and ejaculation of secretions from the sex accessory glands (Farrel and Lyman, 1937; Sjostrand, 1965; Mirabella *et al*., 2007) and blood flow (Baumgarten and Holstein, 1968; Kuwahara and Frick, 1974; Damber *et al*., 1982; Billups *et al*., 1990; Santamaria *et al*., 1995). Epithelial exo/endocytic events and ionic exchange between cellular and luminal compartments are other processes that are influenced by autonomic innervations (Mayerhofer *et al*., 1992; Chan *et al*., 1994; Kempinas *et al.*, 1995; Lamano-Carvalho *et al*., 1996; Zhu *et al*., 1998). Immunohistochemical studies on the mammalian epididymis generally reveal the presence of noradrenergic, cholinergic and peptidergic nerves which have a different distribution within the epididymis (Larsson, 1977; Alm *et al*., 1978, 1980; Greenberg *et al*., 1985; Lamano-Carvalho, 1986; Schindermeiser *et al*., 1989; Tainio, 1994; Lakomy, *et al*., 1997; Gürtler, 2001). In literature, however, there exist no reports on the innervation of the alpaca epididymis. The aim of present study was, therefore, to describe the local distribution of protein gene product 9.5 (PGP 9.5), neuropeptide Y (NPY), tyrosine hydroxilase (TH), calcitonin gene related peptide (CGRP) and substance P (SP) in the nerves of the alpaca epididymis in order to clarify the role of these neurotransmitters in the male reproductive functions.

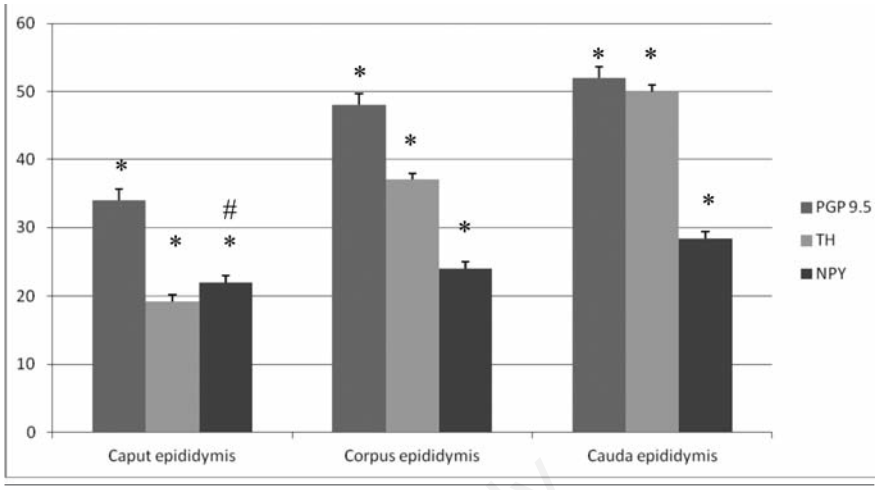

Figure 1. Density of innervation in the different tracts of alpaca epididymis. Immunoreactivity to PGP 9.5, TH and NPY. All data represent media ± SEM, *P<0.01; NPY caput *vs* corpus #P=0.11.

Materials and methods

Animals and tissue processing

Five adult male alpacas of six-seven years old and sexually mature were used in the present study. The subjects utilized for this research were from the farm *Domus Alpaca* (Pratola Peligna, AQ, Italy) and tissues were collected on autumn and spring. The animals were anesthetized and surgically operated in accordance to the ethical animal welfare. The testes were removed and epididymis collected. The specimens were divided in caput (proximal and distal tracts), corpus and cauda and fixed in Bouin's fluid for 24-48 h and successively processed for paraffin embedding. Histological sections of 6 µm thick were obtained.

Immunohistochemistry

The avidin-biotin-peroxidase complex (ABC) method was performed by using the

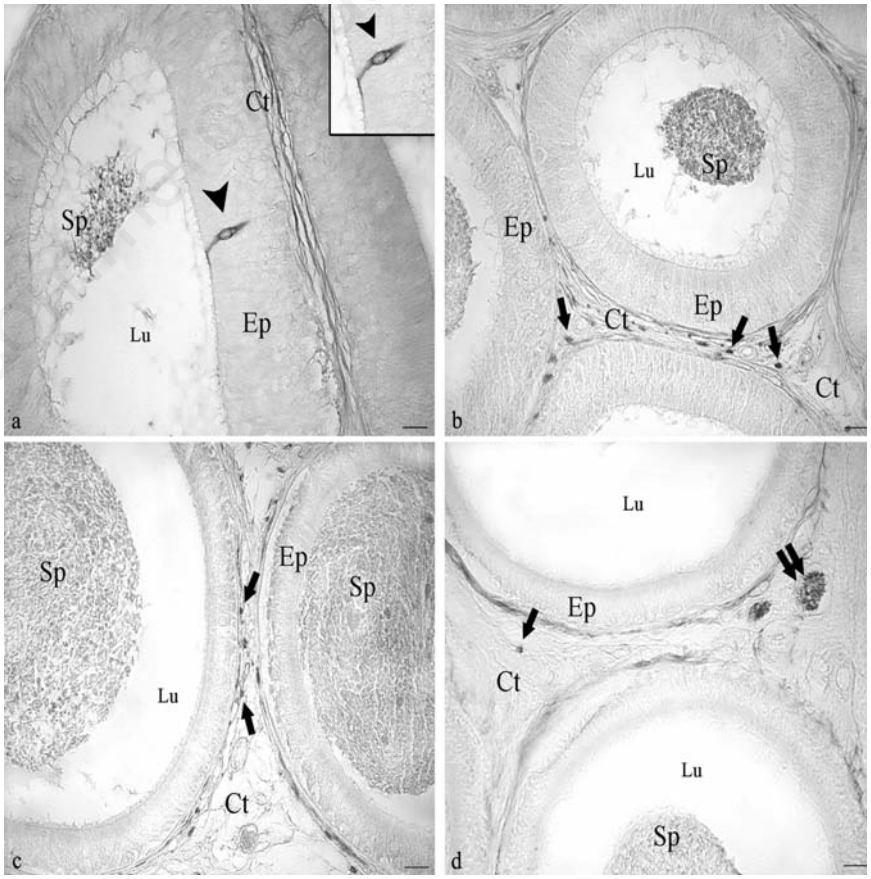

Figure 2. PGP 9.5-immunoreactivity was observed in some principal epithelial cell facing to the tubular lumen (a, arrowhead); many nerve fibers located in the peritubular connective tissue of caput (b, single arrow), corpus (c, single arrow) and cauda (d, single arrow); large nerve bundles in the cauda (d, double arrow). Avidin-biotin immunohistochemical technique. Ep, epithelium; Lu, lumen; Sp, spermatozoa; Ct, connective tissue. Scale bar: 20 µm.

Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) as described more fully elsewhere (Squillacioti *et al*., 2011). Primary antibodies were listed in Table 1. Secondary specific antibodies were listed in Table 2. The specificity of the immunoreactions was tested by omitting the primary antibodies, using PBS and by preadsorption of the primary antibodies with an excess of the antigen.

The numbers of PGP 9.5-, TH- and NPY-positive nerve fibres were counted in 20 randomly chosen microscopic fields per animal by using light microscope Nikon Eclipse E-600 equipped with a Coolpix 8400 digital camera. All data have been expressed as mean ± SEM. The statistical analysis was performed by using ANOVA Tukey's HDS test for independent samples. For this test, $P<0.01$ was regarded as significant. The distribution and density of the nerve fibres in the different epididymal tracts are shown in Figure 1.

Results and discussion

PGP 9.5, a marker of the entire innervation (Figure 2), showed the presence of large nerve fibre bundles in the connective tissue encircling the organ and in that distributed among the tubules. Small nerve fibre bundles and single nerve fibres were found around the tubules and around blood vessels. The density of PGP 9.5- positive nerve fibres was evaluated in the different epididymal tracts and it was found to increase from the caput to the cauda (Figure 1). Moreover, several PGP 9.5 immunopositive scattered epithelial cells were observed in the caput. These cells are slender cells without an apparent connection with the epithelial basal lamina and are similar to mithocondria-rich cells. These cells were reported in the initial epididymal segments in men (Fraile *et al*., 1996) and in other mammalian species (Martìnez-Garcìa *et al.,* 1995). In the alpaca, they were observed to contain lectins and were postulated to regulate epididymal ionic composition and intraluminal pH (Parillo *et al.,* 2009b).

The cathecolaminergic innervation was investigated using TH (Figure 3). TH- positive nerve fibre bundles were found in the connective tissue enveloping the organ and in that distributed among the tubules. TH-positive nerve varicosities were found around the tubules. In addition, TH- immunoreactivity (IR) was observed in the muscle coat of some blood vessels. The number of TH- positive fibres increased from the caput to the cauda (Figure 1). The peptidergic innervations was investigated using NPY (Figure 4), CGRP (Figure 5 c,d) and SP (Figure 5 a,b). NPY- positive nerve fibre bundles were observed in the connective tissue distributed among the epididymal tubules. NPY- positive varicosities were found around the tubules. In addition, NPY- immunopositive nerve fibres were observed around the blood vessels. The number of NPY- positive fibres increased from the caput to the cauda (Figure 1). Moreover, several NPY immunopositive epithelial cells were observed in the caput. CGRP and SP positive nerve fibres were rarely found in nerve fibre bundles located within the connective tissue distributed around the tubules.

In the present study, the density of nerve fibres, as revealed by PGP 9.5-, TH- and NPY-IRs, was found to increase from the caput to the cauda of the alpaca epididymis. These results agree with those reported in the cat, dog, guinea pig and human (El Badawi and Schenk, 1967; Norberg *et al*, 1967; Baumgarten *et al.*, 1968; Hodson *et al*., 1970; Dail *et al*., 1993; Gürtler, 2001) including camelus dromedarius (Saleh *et al*., 2002). The smooth musculature of the caput and corpus epididymis displays spontaneous contraction involving cGMP in the signalling pathway (Risley, 1958; Talo, 1981; Gerendai *et al*., 2001; Mewe *et al*., 2006), while the cauda epididymis is normally quiescent until neural input is received during the ejaculatory process (Hib *et al*., 1982). The localization of

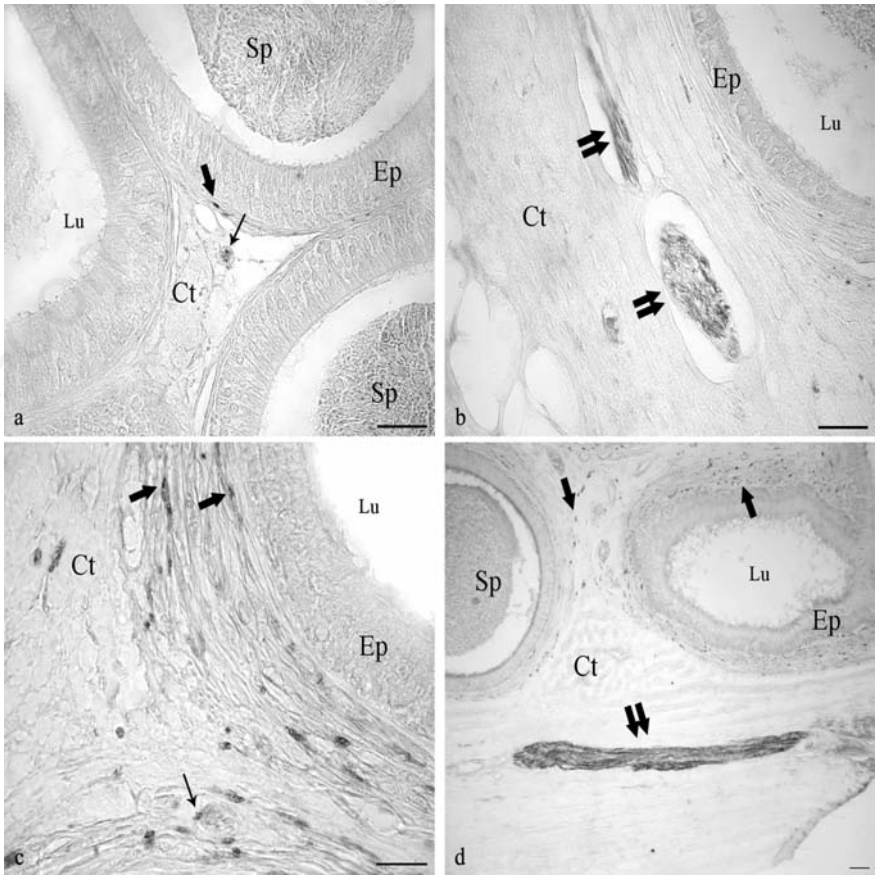

Figure 3. TH-immunopositivity was observed in nerve fibers in the peritubular connective tissue (a,c,d, single arrow); nerve bundles immersed in peritubular connective tissue (b, d, double arrow); muscle coat of blood vessels (a,c, small arrow). Avidin-biotin immunohistochemical technique. Ep, epithelium; Lu, lumen; Sp, spermatozoa; Ct, connective tissue. Scale bar: 20 μm.

cathecolaminergic fibres within the epididymis in peritubular and subepithelial regions suggests that cathecolamines play a role in neuromuscular events required for the transport of spermatozoa through the duct and for seminal emission. Studies using surgical and guanethidine induced denervation have shown, moreover, that the decreased contractility observed in the rat epididymis with the loss of adrenergic innervation, induces a delay in cauda luminal transit with a significant increase in the number of spermatozoa present in the cauda epididymis (Billups *et al*., 1990; Ricker *et al*., 1996; Kempinas *et al*., 1998). The presence of TH-immunopositive fibres around blood vessels suggest a role for the catecholaminergic innervations in regulate epididymal blood flow. These results confirm previous observations in the camel epidydimis (Saleh *et al*., 2002).

Our results showed that the distribution of NPY-IR was similar to that of the cathecolaminergic nerves, thus, the possibility that cathecolamines and NPY are co-stored in the same nerve fibre vesicles cannot be excluded. In camel epididymis NPY-positive fibres were reported to co-localize with dopamine beta hydroxylase (DBH). A colocalization of NPY with DBH has also been described in the man (Tainio *et al*., 1994), hamster, (Schindelmeiser *et al*., 1989), bull, (Rose *et al*., 1992), cat (Gürtler, 2001) and pig (Kaleczyc *et al*., 1997, 1999). For this reason NPY seems to be a modulator of adrenergic neurotransmission, playing a role in the ejaculation and sperm transport (Lamano-Carvalho *et al*., 1986; Mirabella *et al*., 2003;) and regulation of blood flow (Tainio *et al*., 1994). The NPY-containing nerves are also distributed intimately with the epithelial cells of the monkey ejaculatory duct which have few ultrastructural features indicating a positive secretory activity (Yokoyama, 1989). CGRP was considered a marker of sensory nerves (Yamada *et al*., 1977; Ngassapa *et al*., 1998) and had numerous biological functions like relaxing non vascular smooth musculature (Anouar *et al*., 1998; Yousufzai and Abdel-Latif, 1998; Hislop *et al*., 1998) and modulating sensory signals (Häppola and Lakomy, 1989; Csillik *et al*., 1993) and together to SP were involved in the control of the pain sensation (Santicioli *et al*., 1988). Rare SP- and CGRP- positive nerve fibres were found in alpaca, thus confirming the results in the camel epididymis (Saleh *et al*., 2002). Epithelial cells positive to PGP9.5 and NPY were found in the luminal portion of the alpaca caput epididymis.

These cells could have a possible endocrine/paracrine function taking into account the role of endocrine cells in other apparatuses like gastrointestinal tract and urogenital tract of domestic ungulates and their secretory activity (Arrighi and Domeneghini, 1997, 1998; Kaleczyc *et al*., 1999). Their role towards growth and differentiation (regulation of mitosis) especially in accessory gland, enables male urogenital endocrine cells in human to act the pathogenesis of cancer and hyperplasia (Yu *et al*., 2001).

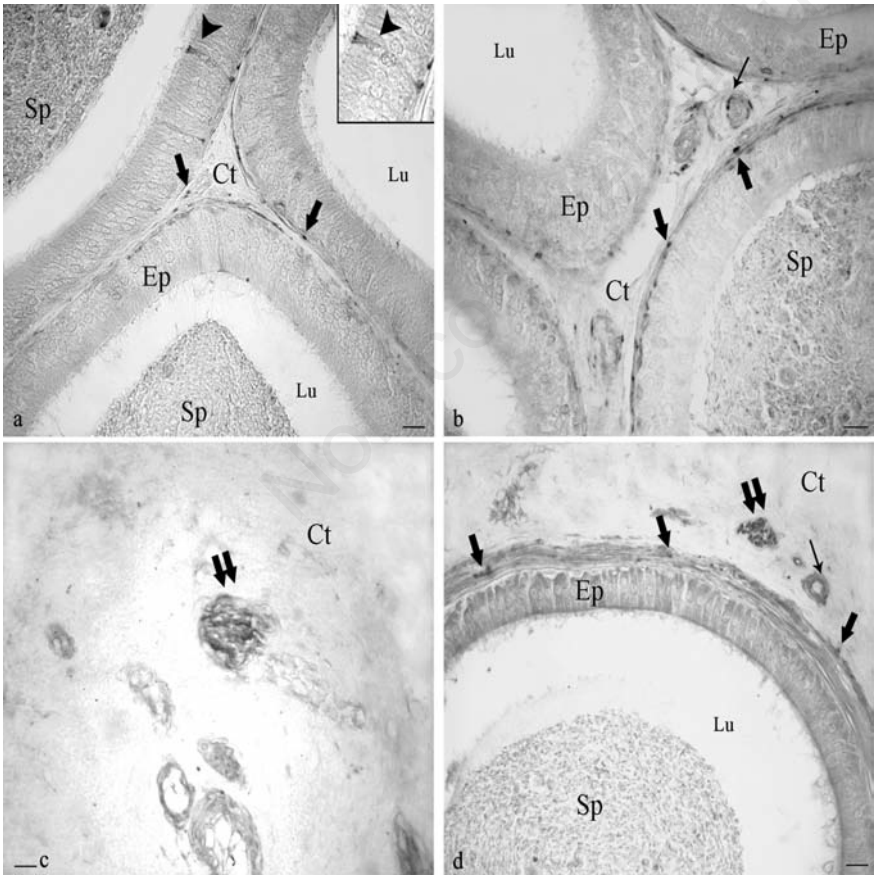

Figure 4. NPY-immunopositivity was observed in some principal epithelial cell in the distal tract of the caput (a, arrow head), nerve fibers, (a, single arrow), corpus (b, single arrow) and (d, single arrow); nerve bundles in peritubular connective tissue (c,d, double arrow); muscle coat of blood vessels (b,d, little arrow). Avidin-biotin immunohistochemical technique. Ep, epithelium; Lu, lumen; Sp, spermatozoa; Ct, connective tissue. Scale bar: 20 μm.

Conclusions

This research describes the presence of some neurotransmitter in the innervation of alpaca epididymis and provides statistical analysis of their distribution in the three tracts of the organ. The results confirmed those observed in other species and suggest that innervation plays an important role expecially in the caudal portion of the organ. Moreover, the presence of epithelial cells containing neuropeptides suggests a specific role for these cells in the regulation of some epididymal epithelial functions.

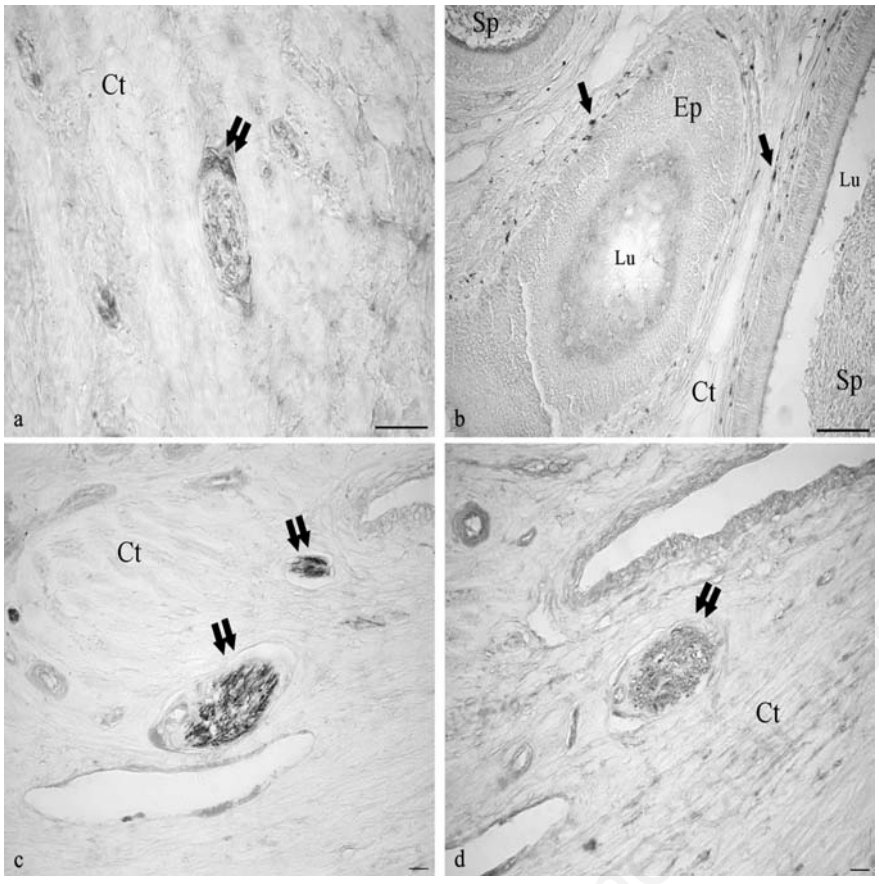

Figure 5. SP-immunopositivity was observed in rare nerve fibers (b, single arrow) and in rare nerve bundle immersed in the connective tissue (a, double arrow). CGRP immunopositivity was observed in rare nerve bundle (c,d, double arrow). Avidin-biotin immunohistochemical technique. Ep, epithelium; Lu, lumen; Sp, spermatozoa; Ct, connective tissue. Scale bar: 20 μm.

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