

## The autonomous innervation of the buffalo (*Bubalus bubalis*) testis. An immunohistochemical study

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The innervation pattern in the buffalo testis was determined by using histochemical and immunohistochemical methods. Nerves were concentrated in the tunica albuginea and septula testis, and did not show an uniform distribution. The tunica albuginea at the lateral and medial sides and at the free border of the testis is most densely innervated than at the epididymal border. At the cranial pole thick nerve bundles were observed between albugineal vessels and muscle bundles. Rare parenchymal nerves were found in perivascular position between seminiferous tubules and their occurrence is confined to lobules at the cranial and caudal testicular poles. An intense NPY immunoreactivity occurred in nerve bundles and in solitary varicose fibres. Nerves were concentrated in the tunica albuginea at the lateral and medial side and at the free border of the testis, and in the lobules at the cranial and caudal testicular poles. Sub P immunoreactivity was occasionally detected in some thicker nerve bundles and solitary fibers, in the tunica albuginea and in the wall of blood vessels, showing a similar distribution but less intensity and density than NPY immunoreactivity. TH immunoreactivity stained nerve fibers in the buffalo testis with a distribution pattern similar to that obtained with general neuronal markers. The histochemical reaction for AchE was negative, so cholinergic fibers cannot be detected in the buffalo testis. The histochemical NADPHd reaction stained rare nitrgic nerve bundles and solitary fibers. The majority of NADPHd activity was confined to the vascular endothelium, and rarely to the interstitial Leydig cells, whereas the Sertoli and germ cells did not show any reaction.

**Key words:** autonomous innervation, testis, buffalo, immunohistochemistry.

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The autonomic nervous system has an important role in the regulation of the mammalian male reproductive functions (for review, see Dail 1993). It is a general acknowledge that the intrinsic testicular innervation is mainly associated with blood vessels and involved in the control of testicular blood flow and temperature (Hodson, 1970). In species with smooth muscle in the testicular tunica albuginea and visceral serosa the sympathetic nervous system may participate in the extrusion of spermatozoa from the testis (Davies and Langford, 1971; Bell and McLean, 1973).

The organization and the chemical coding of testicular nerve fibers of mammals have been the subject of many morphological and physiological studies, mostly focused on laboratory mammals (Greenberg et al., 1985; Campos et al., 1990; Properi et al., 1992) and man (Vaalasti et al., 1986; Taino, 1994). Other mammalian species including large, breeding mammals have largely been ignored, although there is some evidence suggesting the existence of distinct species-dependent variations in the neurochemical coding of nerve fibers innervating the testis (Wrobel and Abu-Ghali, 1997; Wrobel and Brandl, 1998; Wrobel and Moustafa, 2000). Particularly, in the testis of bull, pig and donkey local differences in the innervation quality and quantity have been reported. The porcine testis was completely devoid of intrinsic innervation in adult subject, while displays evident age-related changes (Wrobel and Brandl, 1998). No cholinergic and myelinated fibers have been detected in this species, and NPY was the dominating neuropeptide in the testis (Wrobel and Brandl, 1998). The testicular innervation pattern in the donkey is not uniform, is avoid of cholinergic fibers, and shows NPY as the most frequent peptidergic transmitter (Wrobel and Kamel-Moustafa, 2000). In bovine testis there was no evidence for a cholinergic innervation, and about half of the testicular nerves are NPY immuno-positive (Wrobel and Abu-Ghali, 1997).

The water buffalo (*Bubalus bubalis*) is a domesticated ruminant species of considerable economic interest, for both meat and milk production. This species exhibits seasonal sexual activity, and is known for its poor reproductive rate differently to bovine. Since there is only one paper, to our knowledge, regarding the structure and innervation of seminal vesicle in water buffalo (Abou Elmagd et al., 1992), further information on morphology and functions of its genital organs are highly desirable.

Thus, the present study was aimed at investigating the distribution and immunohistochemical properties of intrinsic innervation in the buffalo testis.

## Materials and Methods

### Sampling, fixation, sectioning

The present investigation was performed on fifteen testes from mature water buffalo (*Bubalus bubalis*). Samples of testis taken from different regions were immediately removed and fixed by vascular perfusion of Bouin's fluid or 4% paraformaldehyde, and post-fixed by immersion in the same fixatives. Then, same samples were dehydrated in an ethanol series, embedded in paraffin wax, and serially cut into longitudinal and horizontal sections 6–8  $\mu\text{m}$  thick; other samples were washed in 0.1 PBS, transferred into a graded series of saccharose (10%, 20%, 30%), immersed in Tissue-tek OCT compound, snap frozen in liquid nitrogen, and finally sectioned by cryostat, mounted on gelatin/chrome alum-coated slides and air dried.

## Immunohistochemistry

Simple immunohistochemical staining was performed using the peroxidase anti-peroxidase (PAP) method according to Sternberger (1986). Endogenous peroxidase activity was blocked by treating sections with 3% hydrogen peroxide for 20 min at room temperature (RT).

Then the section, rinsed for 15 min in 0.01 M phosphate buffered saline (PBS) at pH 7.4, were incubated with normal goat serum diluted 1:5 (Jackson ImmunoResearch Laboratories, Inc; West Grove, PA, USA; 005-000-121) for 30 min at RT to prevent background and successively incubated overnight at 4°C with polyclonal primary antisera (see Table 1). The section were then rinsed in PBS for 15 min and incubated with goat-anti rabbit IgG (1:50; Vector Lab, Burlingame, CA, USA; AI-1000) for 30 min at RT. Subsequently, they were rinsed in PBS for 15 min and incubated with PAP complex (1:100; UCB, Braine-l'Alleud, Belgium) for 30 min at RT. The peroxidase reaction was visualized with a solution of 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma; D 5905; 10 mg in 15 mL of 0.5 M Tris buffer pH 7.6, containing 1.5 mL hydrogen peroxide at 0.03 %). Each incubation was performed in a moist chamber. Finally, the sections counterstained with hematoxylin were dehydrated through an ethanol series, cleared in Xylene, mounted, observed and photographed using a Leitz Aristoplan light microscope. For fluorescence microscopy, the dewaxed sections were rinsed in PBS for 15 min,

**Table 1: Details of primary antibodies used in the study.**

ANTISERA	ANTIGEN	SOURCE	SPECIFICITY	DILUTION	HOST
Protein Gene Product 9.5 (PGP 9.5)	Human brain	Biogenesis	No cross reaction	1/16000	rabbit
Neurofilament Protein (NF)	NF 68 kD	Biogenesis	Cross reaction less than 2% with the 150 kD or 200 kD forms	1/500	rabbit
Calcitonine Gene Related Peptide (CGRP)	Rat CGRP	Peninsula IHC 6006	Cross reaction 10% with CGRP II no cross reaction with rat amylin	1/500	rabbit
Cholecystokinin (CCK)	CCK 26-33	Peninsula IHC 7181	Cross reaction with Gastrin	1/500	Rabbit
Neuron Specific Enolase (NSE)	Human brain	Dakopatts	Cross reaction with the isoenzyme of NSE which contain $\gamma$ -subunits	1/200	Rabbit
Neuropeptide Y (NPY)	Porcine NPY	Peninsula IHC 7172	Cross reaction with PYY	1/2000	Rabbit
Galanin (Gal)	Porcine Galanin	UCB	No cross reaction with NPY, CCK, Sub P, VIP, NT.	1/2000	Rabbit
Met-Enkephalin (Menk)	Human Met-Enk	UCB i672/002	No cross reaction	1/300	Rabbit
Leu-Enkephalin (Lenk)	Leu5-enkephalin	UCB i671/002	No cross reaction	1/500	Rabbit
Vasoactive Intestinal Polypeptide (VIP)	Porcine VIP	Incstar 20077	Slight cross reaction with GHRF, PHI and PP.	1/1500	Rabbit
Substance P (SubP)	Human SubP	Incstar 20064	No cross reaction	1/2000	Rabbit
Tyrosine-Hydroxylase (TH)	Rat pheochromocytoma	Chemicon AB 152	No cross reaction	1/500	Rabbit

incubated with normal donkey serum (1:5; Jackson Immunoresearch Laboratories; 017-000-121) for 30 min at RT and then incubated overnight at 4°C in a moist chamber with polyclonal primary antisera. The sections were then rinsed in PBS for 15 min and incubated with donkey-anti rabbit IgG conjugated to fluorescein isothiocyanate (FITC; 1:25; Jackson Immunoresearch Laboratories; 711-095-152) for 1 h at RT. Fluorochrome-stained sections were rinsed in three changes of PBS of 5 min each, mounted in phosphate-buffered glycerine (9:1) and examined with a Leitz Aristoplan microscope equipped for epifluorescence.

The specificity of the immunoreactivity (IR) was tested by successively substituting PBS for the primary antisera, or the anti-rabbit igG, or the PAP complex, in repeated trials. The cross-reactivity was tested by incubating the sections with antibodies that had been preincubated with excessive amounts of their homologous (up to 50 µg/mL antiserum in the final dilution) and heterologous (up to 100 µg/mL antiserum in the final dilution) antigens.

### **Histochemistry**

For acetylcholinesterase (AChE) histochemistry the modified, direct-coloring method of Kujat et al. (1993) was employed. Following a short incubation (1-2h) this method allows the specific visualization of cholinergic nerves, while following an incubation time of 25h (long incubation) the method reliably illustrates the general autonomous innervation pattern as completely as suitable pan-neuronal immunohistochemical markers.

NADPH diaphorase activity was localized by incubation with 0.25 mg/mL nitroblue-tetrazolium, 1 mg/ml βNADPH, and 0.5% triton X-100 in 0.1 M TRIS-HCl buffer at 37°C for 10-15 min in a dark box. The reaction was stopped by immersion in TRIS-HCl. Control sections included incubation in media in which the substrate was omitted and preincubation in the sulphhydryl inhibitor, 5,5'-dithio-bis-(2-nitrobenzoic acid).

### **Results**

The innervation pattern in the buffalo testis was determined by using histochemical and immunohistochemical methods and comparing a number of general neuronal markers: immunoreactivity for PGP 9.5, NSE, NF and the AchE technique (long incubation). Immunoreactions with antisera to NF and PGP 9.5 clearly depicted the overall nerve distribution. The immunostaining were equally strong in

larger nerve bundles (Figure 1A, B) as in the finest ramifications. However, PGP 9.5 also reacts with non-nervous structures such as spermatogenic cells in the tubular compartment of the testis, a fraction of the Leydig cells and the vascular endothelia. Immunostaining with antiserum to NSE often failed to show smaller and single nerve fibers. AChE reaction (long incubation) demonstrated the general autonomous innervation pattern similar to PGP 9.5 and NF immunohistochemistry.

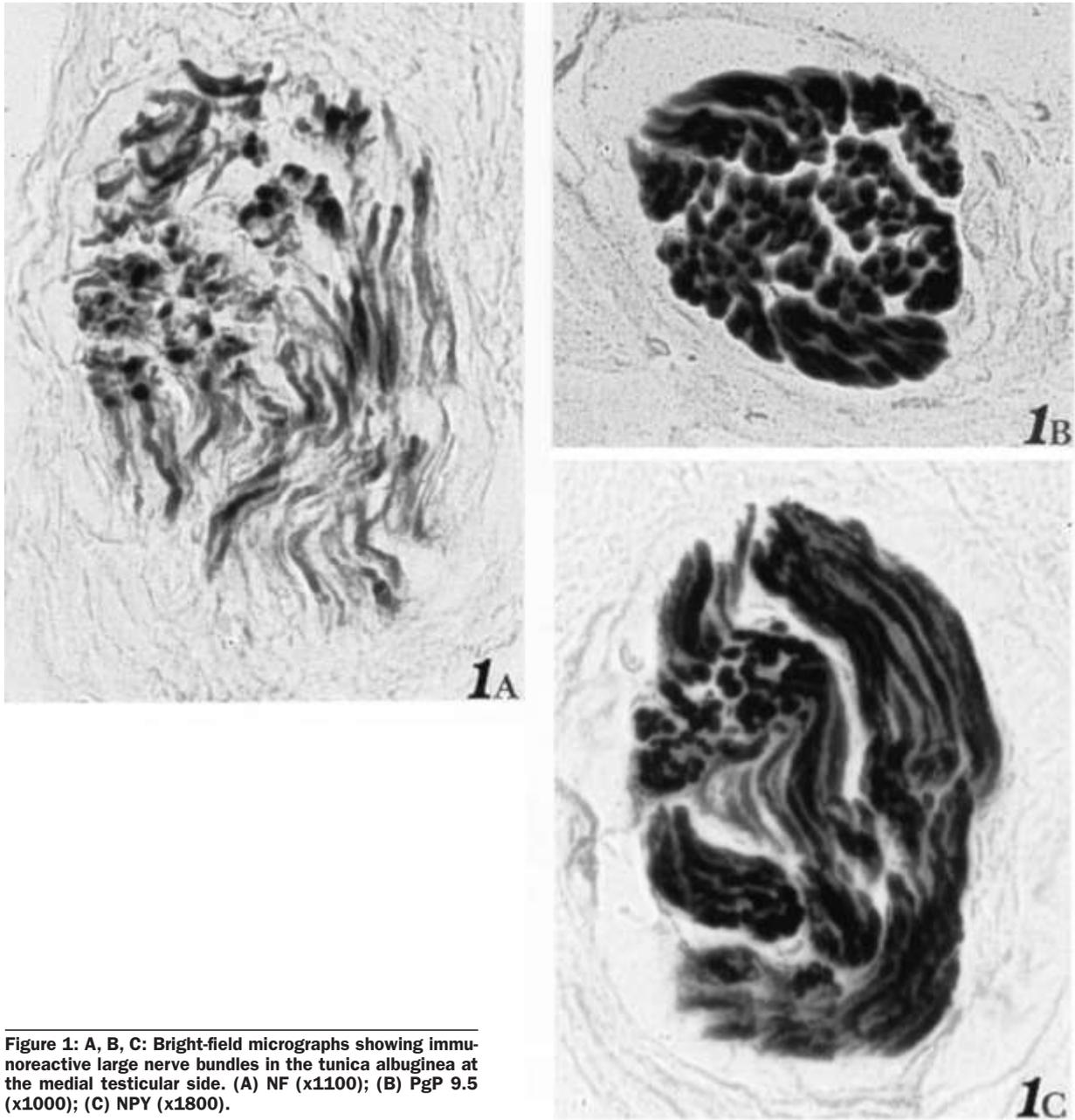
Nerves were concentrated in the tunica albuginea and septula testis, and did not show an uniform distribution (Figure 4). The tunica albuginea at the lateral and medial sides and at the free border of the testis is most densely innervated than at the epididymal border. At the cranial pole thick nerve bundles were observed between albugineal vessels and muscle bundles. Rare parenchymal nerves were found in perivascular position between seminiferous tubules and their occurrence is confined to lobules at the cranial and caudal testicular poles.

The occurrence of peptidergic nerve fibers has been studied with antibodies to NPY, Gal, Sub P, Met- and Leu-Enk, VIP, CGRP, CCK. NPY occurred in intense immunopositive nerve bundles (Figure 1C), in solitary varicose fibres (Figure 2A), and surrounding blood vessels. The highest density observed was similar to that observed with general markers. Nerves were concentrated in the tunica albuginea at the lateral and medial side and at the free border of the testis, and in the lobules at the cranial and caudal testicular poles.

Sub P immunoreactivity was occasionally detected in some thicker nerve bundles and solitary fibers, in the tunica albuginea (Figure 2B) and in the wall of blood vessels, showing a similar distribution but less intensity and density than NPY immunoreactivity.

The immunohistochemical reactions for the determination of CGRP, Met- and Leu-Enk, VIP, and Galanine were totally negative. TH immunoreactivity stained nerve fibers in the buffalo testis with a distribution pattern similar to that obtained with general neuronal markers (Figure 3A, B). The histochemical reaction for AchE (short incubation) was negative, so cholinergic fibers cannot be detected in the buffalo testis.

The histochemical NADPHd reaction stained rare nitrenergic nerve bundles and solitary fibers. The majority of NADPHd activity was confined to the vascular endothelium, and rarely to the interstitial Leydig cells, whereas the Sertoli and germ cells did not show any reaction.



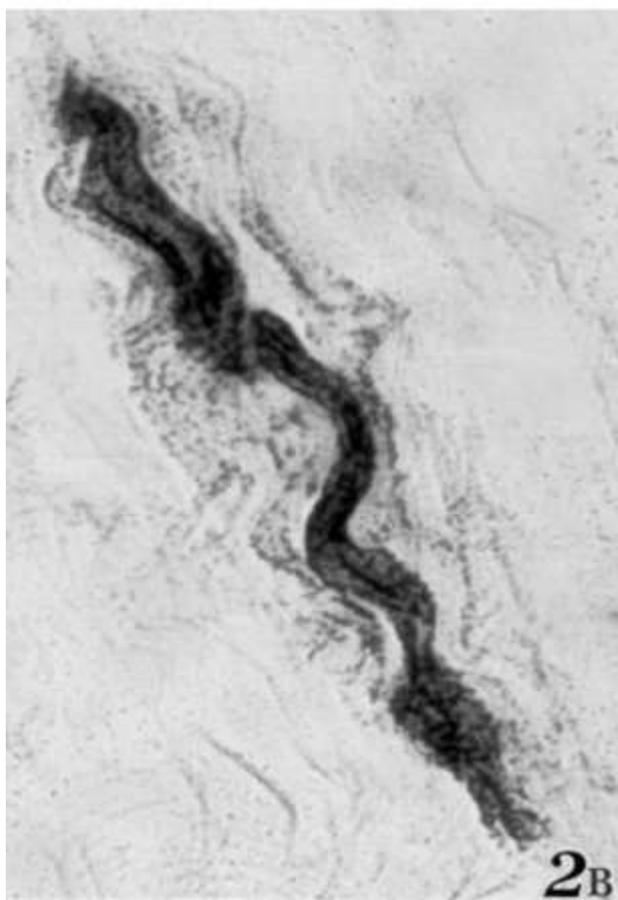
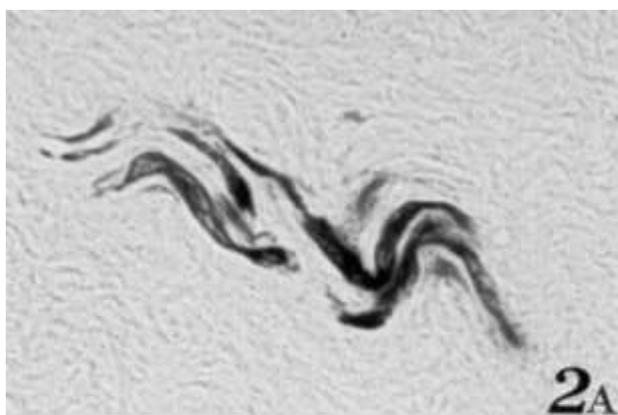
**Figure 1: A, B, C: Bright-field micrographs showing immunoreactive large nerve bundles in the tunica albuginea at the medial testicular side. (A) NF (x1100); (B) Pgp 9.5 (x1000); (C) NPY (x1800).**

## Discussion

In the present study the distribution pattern of the testicular nerves and their chemical coding were demonstrated using histochemical and immunohistochemical methods in the buffalo testis.

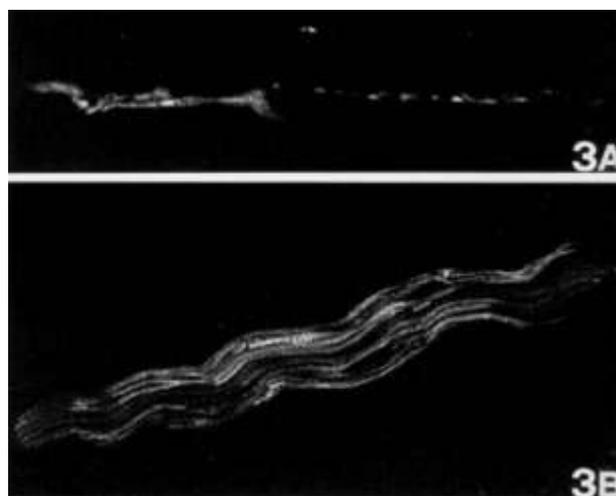
Similarly to other large ungulates, the distribution pattern of the testicular nerves showed conspicuous local variations in the buffalo testis. Nerves are concentrated in the tunica albuginea and septula testis, and rare parenchymal nerves were distributed in perivascular portion between seminiferous tubules at the cranial and caudal testicular poles. In the adult

bull the large vessels of the tunica albuginea display a discontinuous innervation: a vascular plexus is present in certain areas whereas other portions of the albugineal vascular tree are devoid of intrinsic nerves (Wrobel and Abu-Ghali 1997). The caudal half of the adult bovine testis is completely free of stromal, mediastinal and parenchymal nerves, and in the cranial quarter vascular nerves accompany the large centripetal arteries between the testicular lobes (Wrobel and Abu-Ghali 1997). In the adult donkey the tunica albuginea at the lateral and medial sides and at the free border of the testis is signif-

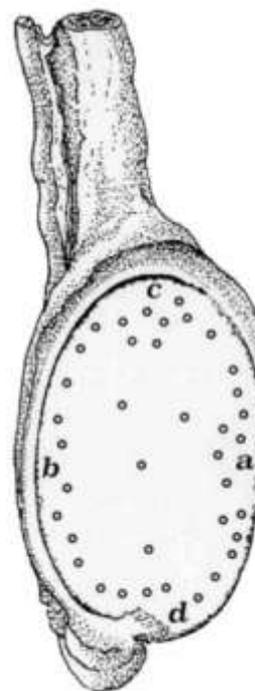


**Figure 2 A, B:** Bright-fields micrographs showing immunoreactive nerve bundles in the tunica albuginea at the lateral testicular side. (A) NPY (x1100); (B) Sub. P (x1600).

icantly less densely innervated than at the epididymal side and the testicular poles (Wrobel and Moustafa, 2000). In the adult pig testis is not completely innervated, and nerve fibers supply the vascular structures of the tunica albuginea and nearly all the septula testis and the mediastinum (Wrobel and Brandl,



**Figure 3 A, B:** Fluorescence micrographs showing TH immunopositive nerve fibers in the tunica albuginea at the lateral (A) and medial (B) testicular side. (A: x960; B: x820).



**Figure 4.** Diagram representing nerve density in the water buffalo testis. Caudal view of the right testis. *a*: free border; *b*: epididymal border; *c*: cranial pole; *d*: caudal pole. Cranial and caudal poles and the free and epididymal border (tunica albuginea and adjacent parenchyma) of the gonad are well innervated.

1998). Local variations in the density of testicular innervation were detected also in the testis of the toad, in which small fibres bundles as well as single nerve fibres were observed running close to blood vessels or independently in the intertubular tissue (Achi et al. 1995).

Our results on the peptidergic innervation of the buffalo testis shared only partially characteristics described for other mammals. In mammals there are some studies which altogether show marked species differences in the peptidergic innervation of the testis. In the rat testis the innervation appears to be scarce (Zhu et al 1995) or absent (Alm et al, 1980; Properzi et al 1992), while in the guinea pig a rather dense Sub P and VIP-IR innervation has been described (Alm 1980; Larsson 1977). Similar to our results, NPY is the dominating neuropeptide also in the male gonad of the bovine, pig and donkey (Wrobel and Abu-Ghali 1997; Wrobel and Brandl 1998; Wrobel and Moustafa 2000). On the contrary, NPY immunoreactivity is rather frequent in the nerves forming the vascular plexuses of testicular, albugineal and septal arteries, but not so frequent in the mediastinum and the testicular lobules of the cat (Wrobel and Gürtler 2001). NPY has been shown to stimulate vasoconstriction either directly or by potentiating responses to noradrenaline (Lundberg et al. 1982; Edvinson et al. 1983). With a modified AchE histochemical technique (Kujat et al. 1993) using a short incubation period (2h), no cholinergic fibres were detected in the buffalo testis. These results agree with those obtained in the testis of bull (Wrobel and Abu-Ghali 1997), boar (Wrobel and Brandl 1998), and donkey (Wrobel and Moustafa 2000). In contrast to the findings obtained in these four ungulates, Wrobel and Gürtler (2001) with the same histochemical method demonstrated a large amount of cholinergic nerve fibres in the testis of the cat. Furthermore, the absence of VIP-immunopositive fibres in the buffalo testis, in agree with the same results obtained in the human (Vaalasti et al. 1986) and bull (Wrobel and Abu-Ghali 1998), is a further indication for the absence of a parasympathetic innervation of the buffalo testis since cholinergic neurons are generally VIP-positive (Dail et al. 1990).

In conclusion, the present study showed a testicular nerve pattern distribution in the buffalo male gonad similar to the bull. Furthermore, the existence of a peptidergic innervation composed of two population of fibers containing NPY and Substance P support the concept of neural regulation of gonadal functions in this species, although the ultimate functional role of these nerve fibres remain to be determined.

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