Some Comparative Immunohistochemical Studies on the Ductus Deferens in the Donkey (*Equus asinus*) and Water Buffalo Bull (*Bubalus bubalis*).

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Abstract

The current work aimed to apply immunohistochemistry (IHC) to evaluate the differential distribution pattern of some biologically active proteins in the ductus deferens (DD) in two different animal species common in the Egyptian farms. Immunolocalization of S100, angiotensin converting enzyme (ACE), α-smooth muscle actin (α-SMA), connexin 43 (Cx43) and galactosyltransferase (GalTase) was practiced on paraffin-embedded sections of DD from clinically healthy adult five donkeys and five water buffalo bulls. The DD, in both species, was lined by a folded, high cuboidal (in donkey) or tall columnar (in buffalo) pseudostratified epithelium with stereocilia. IHC revealed that all antibodies used, displayed variable reactivities in the two species. Nerves and the periductal smooth muscles expressed a strong immunoreactivity (IR) for S100 and α-SMA respectively; both in the donkey and in the buffalo. Stereocilia of principal cells showed a marked ACE-immunostaining in the donkey but not in the buffalo. Cx43-binding sites were found between the basal portions of the ductal epithelium and the subjacent interstitium; as well as among the periductal smooth muscle cells (SMCs) only in the buffalo bulls. Distinct GalTase-binding sites were found in the ductal epithelium only in the donkey. In conclusion the present findings exhibited a species-specific pattern of distribution for most of proteins under study, suggesting a variable functional significance of the DD in the different animal species.

Keywords:

Introduction

The ductus deferens (DD) plays a key role in male fertility by providing an appropriate luminal environment for sperm before ejaculation (Hoffer, 1976; Silber, 1989; Carlin et al., 2002; Domeniconi et

al., 2007; Orsi et al., 2009). Moreover, it was reported that DD has a complex epithelium (Setchell and Brooks, 2006), showing absorptive, synthetic and secretory activities in rodents (Chinoy, 1985; Manin et al., 1995; Orsi et al., 2009) and in humans (Hoffer, 1976). Thus, the DD is not only a passage for sperm transport (Setchell and Brooks, 2006), but also is an important organ contributing positively to the conservation of sperm structure, maturation, survival and viability (Chinoy, 1985; Silber, 1989; Carlin et al., 2002). Several studies (Clermont and Hermo, 1988; Breton et al., 1996; 1998; Brown and Breton, 2000) applied the rodents as a mammalian model to study the structure and the function of the DD.

Though previous studies showed that the DD from different mammals revealed common basic structure; represented by a folded mucosa, a three-layered muscularis and a serosa or an adventitia (Hoffer, 1976; Murakami et al., 1982; Paniagua et al., 1982; Lohiya et al., 1988; Khan et al., 2003), and some species-specific characteristics (Murakami et al., 1982; Chinoy, 1985; Khan et al., 2003), a great need for comparative studies did exist for promoting anatomical, histological and functional backgrounds about the DD in animals.

The immunomarkers "proteins" under study were chosen according to certain functional significance. Angiotensin converting enzyme (ACE) was selected to identify the structures related to the transport of electrolytes and water (O'Mahony et al., 2000). Galactosyl-transferase (GalTase) was used to investigate the secretory activity. GalTase is a member of a functional family of intracellular, membrane-bounded enzymes that are responsible for the biosynthesis of carbohydrate fractions of glycoproteins (GPs) (Hennet, 2002). Some GPs have a significant role in the male fertility. Both of α–smooth muscle actin (α-SMA) (Alkafafy et al., 2009), connexin 43 (Cx43) (Dufresne et al., 2003) and S100 (Heizmann et. al., 2002) were used to investigate the reciprocal activities between the ductal epithelial and the periductal non-epithelial structures of the DD. The α-SMA is a significant tool in studying differentiation of SMCs in normal and pathological conditions (Skalli et al., 1989). Connexins are members of a large family of integral membrane proteins (Thomas et al., 2002), which oligomerize to form intercellular channels known as gap junctions. Ions, metabolites and second messengers diffuse directly from cell to another through these junctions (Segretain and Falk, 2004). The gap junctions facilitate the coordination of individual cells in an organ and provide a mechanism for regulating its biological function (Evans and Martin 2002). S100 is a multifunctional subfamily of Ca^{2+}-binding proteins that have a broad range of functions (Heizmann et. al., 2002) including motility, chemotaxis, neurite extension and secretion.

Therefore, the current study aimed to apply IHC to detect some of these interspecies differences. The immunolocalization of ACE, GalTase, α-SMA, Cx43 and S100 was used to investigate the reciprocal activities between the ductal epithelial and the periductal non-epithelial structures of the DD in the donkey and water buffalo bulls that usually found in the Egyptian farming life.

Materials and methods

Animals and tissues
The DD specimens were obtained from five clinically healthy adult male don-

keys (*Equus asinus*) and five Egyptian water buffalo bulls (*Bubalus bubalis*). Their ages ranged from 3 to 5 years. Specimens were taken directly after castration (of the donkeys) or slaughter (of the bulls).

Chemicals and methods
Specimens were fixed in Bouin's fluid and a mixture of methanol/glacial acetic acid (2:1). Bouin-fixed specimens were used for routine histological staining. For IHC specimens were fixed in Bouin's fluid (specimens used for detection of ACE, S-100, α-SMA and Cx-43) and in a methanol/glacial acetic acid mixture (specimens used for detection of GalTase). Tissue specimens were dehydrated in a graded series of ethanol, cleared in xylene, embedded in Paraplast wax[1] and sectioned at 5 µm thickness. Tissue sections were mounted on positively charged, coated slides[2].

General histological techniques
Many conventional stains were carried out to investigate the general histological structure. These included Hematoxylin and Eosin, Masson and Goldner's trichrome stains, Alcian blue 8GX (pH 1.0 and pH 2.5), Periodic acid-Schiff (PAS) reaction after McManus and Toluidine blue. All the staining techniques were performed according to Bancroft and Stevens (1990).

Morphometric measurements
The morphometric values of the different ductal components in both species were measured in transverse sections of the ductus deferens from all animals studied. Histomorphomertic measures were obtained in three different histological sections for each animal using the eyepiece micrometer and X 4 and X10 objective lenses. Descriptive statistics are given as means ± SD (standard deviation)[3].

Immunohistochemistry
Dewaxed and rehydrated sections were subjected to inactivation of endogenous peroxidases by incubation in 1% H_2O_2 for 15 minutes. Then after the sections were placed in 0.01 mol/L citrate buffer (pH 6) and heated in microwave (700 watt) for 10 minutes for antigen retrieval. The sections were blocked by PBS containing 5% bovine serum albumin (BSA) for an hour, and then each section was incubated with its corresponding primary antibody (types, sources and dilutions of antibodies, and the duration of incubation are shown in Table 1). The sections were washed by PBS for 5 minutes 3 times and incubated with biotinylated secondry antibodies (types, sources and dilutions are shown in Table 1), for 30 minutes at room temperature. The sections were washed by PBS for 10 minutes. Then the secondary antibody was detected with Vectastain ABC kit[4]; firstly each section is covered with 100 x dilution of A & B reagent in PBS (1 µl reagent A+ 1 µl reagent B + 98 µl PBS), then washed by PBS for 10 minutes 3 times and the colour was developed using DAB[5] reagent. Sections were counterstained with haematoxyline for 30 seconds, washed in water, dehydrated through graded ethanol, cleared in xylene and mounted with DPX permanent mounting medi[a6] and photographed by light microscopy

Positive and negative controls

[1] Sigma-Aldrich, St. Louis, MO, USA
[2] Menzel GmbH&Co KG, Braunschweig, Germany

[3] Microsoft_ Excel 2003 program
[4] Vector Laboratories Inc.
[5] Sigma-Aldrich, St. Louis, MO, USA
[6] Sigma-Aldrich, St. Louis, MO, USA

Immunohistochemical negative controls, where each primary or secondary antiserum or the ABC reagent was omitted, gave no positive staining. Positive controls were used according to the instructions provided by the manufacturers of the primary antibodies. For assessment of the immunolabelling a semi-quantitative subjective scoring was performed by three independent observers.

Results

Light microscopic observations

The wall of the DD, both in the buffalo (Fig. 1a, 1b) and in the donkey (Fig. 1c, 1d), was made up of three main layers: mucosa, muscularis and adventitia. The mucosa consisted of the epithelium and the underlying connective tissue lamina propria. The lining epithelium was high cuboidal in the donkey (Fig.1d), and tall columnar pseudostratified in the buffalo bull (Fig. 1b). The epithelium consisted of principal and basal cells, as well as a few migrating lymphocytes in both animal species. Together with the subjacent lamina propria, the epithelium was thrown up into numerous longitudinal mucosal folds. The number of folds ranged from 10 (in the buffalo) to 20 (in the donkey). The mucosal folds in the buffalo DD were relatively shorter and thicker (Fig. 1a, 1b), than those of the donkey (Fig. 1c, 1d). The tunica muscularis, in both animal species, consisted of three laminae: inner longitudinal, middle intermingled and outer longitudinal. While the inner layer was indistinct, both the middle and the outer layers were well-developed. The morphometric values of the different components of the DD in both species were summarized in Table 2. In brief, with the exception of the epithelium height; that was higher in the buffalo (64±10 µm vs 21±4 µm) DD; and muscularis'

thickness; that was nearly two-fold thicker in donkey DD (1155±240vs 712±155µm), other morphometric parameters were higher in the DD of the donkey than in the buffalo.

Immunohistochemical observations

The immunohistochemical findings of ACE, S-100, α-SMA, GalTase, and Cx-43 in donkey and water buffalo bulls DD were summarized in Table 3.

Angiotensin Converting Enzyme (ACE)

The basal cells of ductal epithelium of the buffalo exhibited a weak to moderate ACE-IR) (Fig. 2a). A marked immunostaining was evident in the stereocilia of the principal cells lining the DD of the donkey (Fig. 2b) but not of the buffalo (Fig. 2a). A strong reaction was distinct in the vascular endothelium in both species especially that of the subepithelial blood capillaries (Fig. 2a).

S100

Distinct S100 binding sites were localized in the nerve fibres in the lamina propria and the tunica muscularis; and in the subserosal nerves of the DD both in the buffalo bulls (Fig. 2c) and in the donkey (Fig. 2d). The positively reacting nerve network both in the subepithelial lamina propria and in the muscularis; showed further density in the male donkey (Fig. 2d) than in the buffalo bull. A weak S100-immunostining was recorded in the endothelium of the blood vessels in both species.

Alpha Smooth Muscle Actin (α-SMA)

The IR for α-SMA exhibited a strong signal in both periductal and vascular SMCs. This pattern of reactivity was similar in both species (Fig. 3a, 3b).

Galactosyltransferase (GalTase)

GalTase binding sites were evidently localized in the cytoplasm of the epithelium lining the DD of the donkey (Fig. 3c). Moreover, the vascular endothelium exhibited a weak reaction. On the other hand the buffalo showed no Gal-Tase-IR; either in the ductal epithelium or in the periductal structures.

Connexin 43 (Cx43)
Distinct dotty Cx43 binding sites were seen in the basal portions of the ductal epithelium and in the periductal muscle coat only in the buffalo (Fig. 3d).

Discussion

The information about the comparative histology of the mammalian DD exhibits a remarkable scarcity**.** Thus**,** the current study was carried out to provide a qualitative and quantitative comparative overview of the DD between the donkey and the buffalo. Similar to the case in other mammalian species (Hoffer, 1976; Murakami et al., 1982; Paniagua et al., 1982; Lohiya et al., 1988; Khan et al., 2003), the wall of the DD, both in donkey and buffalo, was made up of a mucosa, three concentric layers of SMCs and an adventitia. The epithelium, together with the subjacent lamina propria, was thrown up into numerous longitudinal folds. The number of folds ranged from 10 (in the buffalo) to 20 (in the donkey). The higher numerical density and heights of the mucosal folds in the donkey DD may confer a labyrintheal appearance to its lumen that may be of value in slowing down of the seminal stream and enhancement of the absorptive activities. Furthermore, the mucosal lining was found to be high cuboidal (21±4 µm, in the donkey) to tall columnar (64±10 µm, in the buffalo) pseudostratified epithelium with stereocilia. In the meantime, Khan et al.

(2003) showed that the DD of buffalo and goat characteristically possess four mucosal folds and that the lining epithelium was low columnar pseudostratified, this discrepancy might be due to age (Macmillan and Hafs, 1969; Abdel-Raouf and Hassan, 1981) or regional variations (Chinoy, 1985).

The donkey and buffalo bull DD had indistinct inner longitudinal and well-developed middle layers, like other mammals (Paniagua et al., 1982; Lohiya et al., 1988; Khan et al., 2003). The SMCs of the middle layer, in both species, showed an intermingled (circular, longitudinal and oblique) pattern of orientation (Wrobel, 1998). Yet, the circular profile predominates in buffalo DD. The outer longitudinal layer was well distinct and consisted of coarse bundles of SMCs particularly in the donkey. It was also noticeable that the muscularis of the donkey DD was nearly two folds thicker than that in the buffalo DD. Taken together the tunica muscularis displayed not only qualitative but also quantitative differences in the two species under study.

Immunohistochemical localization of S100 protein in the donkey and the buffalo DD, especially in the nerve fibres in the lamina propria, tunica muscularis and subserosa of the DD agrees with Slater et al., (2000) who recorded that S100 can be used as an axonal marker; and indicate the significance of S100 proteins in the vasal autonomic nerves which control the male reproductive function. The strong density of S100-IR in the nerve network both in the subepithelial lamina propria and in the muscularis, in accordance with a previous work (Mirabella et al., 2003), showed the presence of a very dense nerve network in both layers. Additionally, the dense nerve network distributed throughout the periductal

muscle coat reflected that the ductal motility is fully under the control of the autonomic nervous system (Dixon et al., 1998; Mirabella et al., 2003). Also, the higher density of the subepithelial nerve network in the donkey, and the broader propria (140±10 µm vs. 36±5 µm) might reflect the paramount implication of the autonomic nerves in the regulation of the DD function in the donkey. It is obvious that the sub-epithelial nerves of the DD are assumed to have a secreto-motor function (Dixon et al., 1998; Jen et al., 1999; Mirabella et al., 2003).

The marked GalTase-immuno-staining in the cytoplasm of the principal and basal cells lining the DD of the donkey but not of the buffalo may reveal some species-specific peculiarities. Although previous studies on the epididymis of both corresponding species (Alkafafy, 2009; Alkafafy et al., 2009) displayed positive GalTase-IR, the distribution pattern showed interspecies differences. Unlike the buffalo, the cauda epididymidis of the donkey expressed a strong IR suggesting that the distal segments of the male excurrent ducts, including the DD, may present some secretory activities (Manin et al., 1995; Orsi et al., 2009). Contrarily, in human; the GalTase activity was high in the intermediate caput and proximal corpus regions, but maintained low in cauda epididymidis and the vas deferens (Ross et al., 1993). GalTase plays a role in gamete interaction (Larson and Miller, 1997) and serves as a generalized gamete receptor during initial sperm-egg binding in mammals (Fayrer-Hosken et al., 1991). Accordingly, the absence of GalTase expression in buffalo bull DD combined with high testicular sperm output might provide species-specific mean to reduce the sperm-oocyte encounter and block of polyspermy.

Although a strong ACE-IR was evident in the stereocilia of the principal cells lining the DD of the donkey, those of the buffalo bull DD was devoid from ACE signals that is might be due to higher secretion of an ACE inhibiting substance (Hohlbru-gger et al, 1983) from bull epididymal epithelium. Nevertheless, the stereocilia of the epithelium lining the donkey cauda epididymidis (Alkafafy, 2009), the bovine caput epididymidis (Alkafafy and Sinowatz, 2009), the rabbit DD (Berg et al., 1986) and the human DD (Vivet et al., 1987; Pauls et al., 2003) displayed a strong ACE-IR. The basal cells of the DD epithelium of the buffalo displayed a moderate ACE-IR. Similar findings could be found in the epididymal basal cells in rabbits (Berg et al., 1986) but not in humans (Vivet et al., 1987). It is evident that this reaction was mostly in proximity with the strongly reacting subepithelial blood capillaries. It is worth noting that ACE is an ectoenzyme, which is synthesized and secreted by the epithelial cells of the epididymis and the DD (Berg et al., 1986). ACE stimulates angiogenesis *in vivo* (Fernandez et al, 1985) and acts as a growth factor (Naftilan et al., 1989) in cell culture systems; thus it may keep maintenance of the blood supply of the excurrent duct system.

Functionally, ACE is known to be crucial for male fertility in animal models (Pauls et al, 2003); it converts angiotensin-I, locally produced by male reproductive tract; into angiotensin-II (O'Mahony et al., 2000). Angiotensin potentiates electrical stimulated contraction and exerts a direct contractile effect on the muscle of DD (Sum and Cheung, 1995). Moreover, it can stimulate sperm motility, the acrosome reaction and binding to the zona pellucida and is also concerned with regulation of electrolyte and fluid transport (O'Maho-

ny et al., 2000). Our findings were also supported by recent studies indicated that the transepithelial movement of water and electrolytes takes place via protein water channels (aquaporins) immunolocalized in the apical surface of the excurrent ducts (Domeniconi et al., 2007; Skowronski et al., 2009).

The current results showed that the cytoplasm of the periductal SMCs presented a marked α-SMA-IR. This work is a contnuation of earlier findings reported the presence of α-SMA in the epididymal duct of the donkey (Alkafafy, 2009) and the buffalo bull (Alkafafy et al, 2009) and suggesting the maintenance of its function in DD likewise in the epididymis. Mewe et al. (2006) showed that the muscular auto-rhythmicity of the epididymal duct results from α-SMA-positive contra-ctile cells, in response to epithelium-derived excitatory prostaglandins. This auto-rhythmicity propulses spermatozoa through the epididymal duct, thereby ensuring sperm maturation. Similar mechanism has been proved on SMCs of the DD (Ruan et al., 2008).

In addition to the endocrine and the paracrine control, gap junctions and their constitutive proteins, connexins, are essential for the control of male fertility. Connexin 43 (Cx43) is the predominant testicular gap-junction protein (Sridha-ran et al, 2007). Additionally in the present study, a strong punctuate, dotty Cx43-IR was found in the basal portions of the ductal epithelium and throughout the periductal muscle coat only in the buffalo. These findings were supported by the detection of nexuses among SMCs in a previous work on the DD (Cobb and Bennett, 1969). Moreover, the epithelial Cx43-binding sites, reported in the buffalo DD, go in line with previous studies on the buffalo (Alkafafy et al., 2009), the stallion (Hejmej et al.,

2007) and the rat (Dufresne et al., 2003) epididymides. On the other hand, and in accord with Alkafafy (2009), neither the epithelial nor the muscular Cx43-binding sites could be seen in the donkey DD. The immunohistochemical findings in the present work assume that the lack of Cx43-IR, in the donkey, may be compensated by the S100-immunoreactive dense network of nerves both in the lamina propria and tunica muscularis. Occurrence of gap junctions (Cx-binding sites) might reveal the reciprocal relationships between the ductal epithelium and the periductal components, necessary for regu-lation of the different functions (Segretain and Falk, 2004; Palani et al., 2006; Hejmej et al., 2007) of the duct.

In conclusion, the immunohistochemical findings in the present work displayed a species-specific, distribution pattern for most of proteins under study, suggest-ing a variable functional significance of the DD in the two species. In accord with previous studies on humans and rodents our findings, particularly of the donkey, assume that the DD possess variable degrees of absorptive, synthet-ic and secretory activities. Therefore, the current results conclude that DD epithelia may play an active role in male fertility. Yet, this concept could not be generalized for all animal species, since some interspecies differences did exist. Thus, our study just highlighted the is-sue and further, extensive, comparative studies on different domestic mammals are highly recommended.

Acknowledgements

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Table 1: Identity, sources, and working dilutions of primary and secondary antibodies.

Primary antibodies					Secondary antibodies		
Against	Origin	Source	Dilution	Incubation time	Type	Source	Dilution
ACE	Chicken	Institute of Vet. Anat. II, LMU Munich	1:500	Overnight at 4°C	Biotinylated rabbit antichicken IgG	Rockland, USA	1:400
Gal-Tase	Chicken	Institute of Vet. Anat. II, LMU Munich	1:500	Overnight at 4°C	Biotinylated rabbit antichicken IgG	Rockland, USA	1:400
S100	Rabbit	Dako, Hamburg	1:400	½ hr at room temperature	Biotinylated pig anti-rabbit IgG	Dako, Hamburg	1:300
αSMA	Mouse	Dako, Hamburg	1:200	1 hr at room temperature	Biotinylated rabbit antimouse IgG	Dako, Hamburg	1:300
Cx 43	Mouse	BD Bioscience, Heidelberg	1:200	Overnight at 4°C	Biotinylated rabbit antimouse IgG	Dako, Hamburg	1:300

Table 2: Morphometric values of the different vasal parameters expressed as mean (µm) ± standard deviation.

Parameter	Donkey	Buffalo
Epithelium height	21±4	64±10
Mucosal fold height	278±49	127±32
Internal diameter[*]	800±34	530±65
Lamina propria	140±10	36±5
Muscularis	1155±240	712±155
Adventitia	370±65	303±42
External diameter[**]	4110±155	2610±143

*: The diameter extends between the basal laminae of a cross section of the DD.
**: The diameter extends between the outer adventitial borders of a cross section of the DD.

Table 3: Immunolocalization of different proteins in the ductus deferens of the donkey and the buffalo bull.

Protein	Donkey						Buffalo					
	Ductal Epithelium			Periductal Components			Ductal Epithelium			Periductal Components		
	BC	PC	IEL	PMC	BV	N	BC	PC	IEL	PMC	BV	N
S100	–	–	–	–	+	+++	–	–	–	–	+	+++
ACE	–	+++	–	–	++	–	+/++	–	–	–	+++	–
α-SMA	–	–	–	+++	+++	–	–	–	–	+++	+++	–
Cx43[*]	–	–	–	–	–	–	+++	+++	–	+++	–	–
GalTase	+	+++	–	–	+	–	–	–	–	–	–	–

[*]Immunostaining for Cx43 was confined to the base of the ductal epithelium. Basal cell (BC); principal cell (PC); intraepithelial lymphocyte (IEL); periductal muscle coat (PMC); blood vessel (BV) and nerve (N). Negative (–); weak (+); moderate (++); weak to moderate (+/++) and strong (+++) reaction.
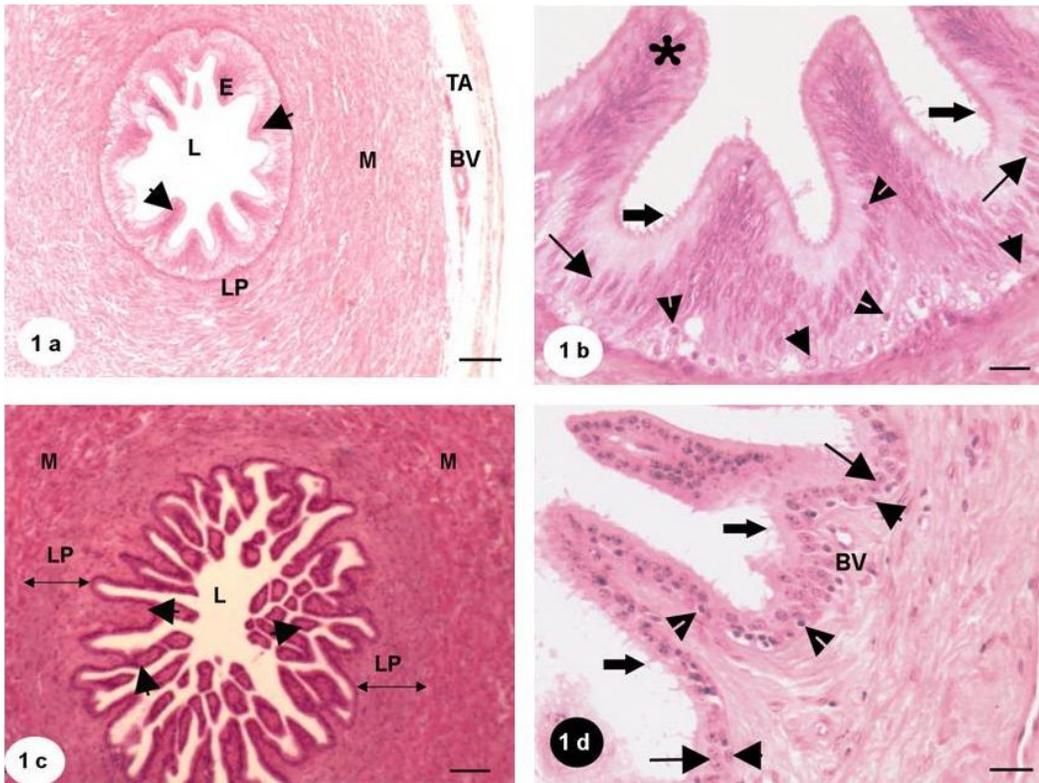
Fig (1)

(a) H&E-stained ductus deferens of the buffalo bull: ductal epithelium (E); ductal lumen (L); lamina propria (LP); muscularis (M); tunica adventitia (TA); blood vessel (BV); mucosal folds (arrow-heads).

(b) A higher magnification of H&E-stained ductus deferens of the buffalo bull: basal cells (arrow-heads); principal cells (thin-tailed arrows); stereocilia (thick-tailed arrows); intraepithelial lympho-cytes (notched arrowheads); mucosal folds (asterisks).

(c) H&E-stained ductus deferens of the donkey: extensively folded epithelium displaying high mu-cosal folds (arrowheads); lumen (L); lamina propria (LP); muscularis (M).

(d) A higher magnification of H&E-stained ductus deferens of the donkey: basal cells (arrow-heads); principal cells (thin-tailed arrows); stereocilia (thick-tailed arrows); intraepithelial lympho-cytes (notched arrowheads); subepithelial blood vessel (BV). Scale bars a, c–100µm; b, d – 25µm.
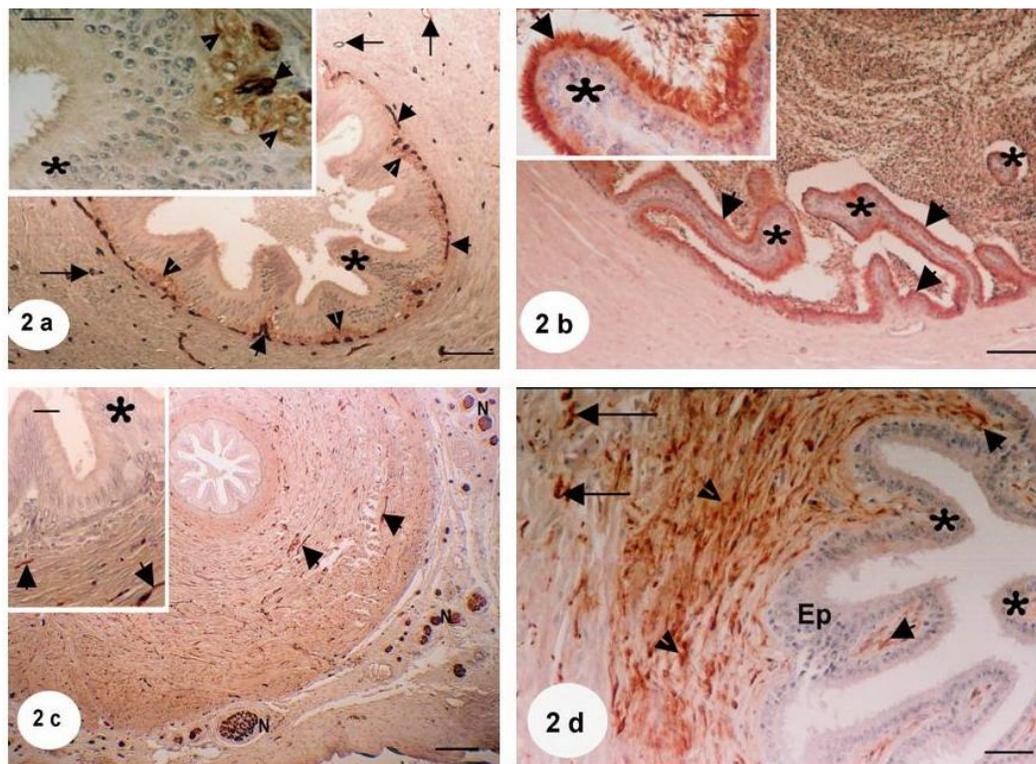
Fig (2)

(a) ACE-binding sites in the ductus deferens of the buffalo bull: basal cells (notched arrowheads) show moderate reaction; blood vessels (arrows) and subepithelial blood capillaries (arrowheads) show strong reactivity; mucosal folds (asterisks).

(b) ACE-binding sites in the ductus deferens of the donkey: strong reaction is evident in stereocilia (arrowheads);   mucosal folds (asterisks).

(c) Distribution of S100-binding sites in the ductus deferens of the buffalo bull: strong reaction in the nerve fibers (arrowheads) of propria and muscularis and in the nerves (N) in adventitia; mucosal folds (asterisks).

(d) Distribution of S100-binding sites in the ductus deferens of the donkey: negative reaction in the epithelium (Ep); strong reaction in the dense network of nerve fibers in lamina propria (notched arrowheads), in the connective tissue core (arrowheads) of mucosal folds (asterisks) and muscularis (arrows). Scale bars a, b,–100µm; c – 200µm; d –50µm; inserts' scale bars 25µm (a, b); 50µm (c).
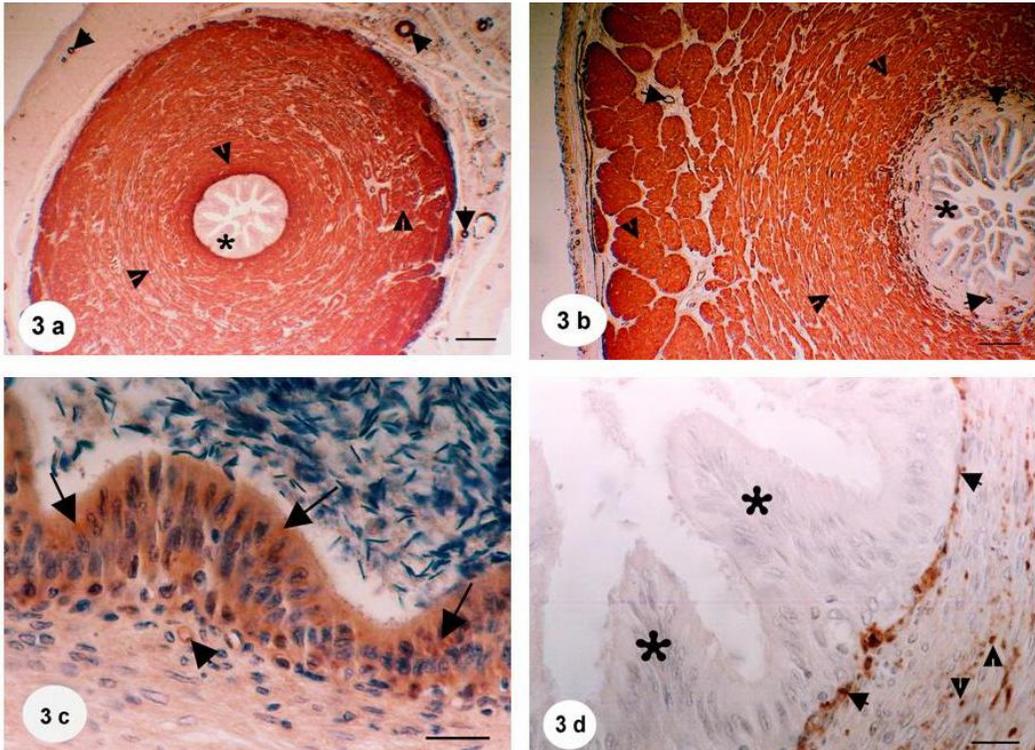
Fig (3)

(a) Distribution of α-SMA binding sites in the ductus deferens of the buffalo bull: strong reaction in periductal (notched arrowheads) and vascular (arrowheads) smooth muscle cells; mucosal folds (asterisks).

(b) Distribution of α-SMA binding sites in the ductus deferens of the donkey: strong reaction in periductal (notched arrowheads) and vascular (arrowheads) smooth muscle cells; mucosal folds (asterisks).

(c) Distribution of GalTase-binding sites in the ductus deferens of the donkey: the ductal epithelium displays moderate to strong reaction in the apical cytoplasm (arrows); weak reaction in a blood vessel (arrowhead).

(d) Distribution of Cx43-binding sites in the ductus deferens of the buffalo bull: distinct dotty reaction found amid smooth muscle cells (notched arrowheads) and between the basal portions (arrowheads) of the ductal epithelium and the lamina propria; mucosal folds (asterisks). Scale bars a, b –200µm; c, d – 25µm.