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LOCALISATION OF CYTOKERATIN AND SMOOTH MUSCLE ACTIN IN THE ACCESSORY GENITAL GLANDS OF CAMELS *(CAMELUS DROMEDARIUS)* DURING RUTTING AND NON-RUTTING SEASONS

A. S. ABD-ELMAKSOUD[1], S. M. EBADA[2] & M. B. SHOAIB[2]

[1]Department of Histology and Cytology; [2]Department of Anatomy, Faculty of Veterinary Medicine, Mansoura University, Egypt

Summary

Abd-Elmaksoud A. S., S. M. Ebada & M. B. Shoaib, 2012. Localisation of cytokeratin and smooth muscle actin in the accessory genital glands of camels *(Camelus dromedarius)* during rutting and non-rutting seasons. *Bulg. J. Vet. Med.*, **15**, No 4, 213–227.

The present study has disclosed for the first time the distribution of cytokeratin (CK) and α smooth muscle actin (αSMA) in the accessory genital glands of camel. In prostate, CK was localised in the cytoplasm of columnar cells of secretory acini and in the scanty cytoplasm of basal cells. In the ampulla of ductus deferens, the CK reaction was found in the pseudostratified columnar epithelium of mucosa and in the secretory columnar epithelium of submucosal glands. In the bulbourethral gland, CK reaction was exclusively observed in the pyramidal cells of type A and type C secretory units as well as in the lining epithelium of the duct system. αSMA was localised to the smooth muscle cells of the prostatic capsule, fibromuscular stroma and blood vessels. In the ampulla, αSMA reaction was seen in the smooth muscle of tunica muscularis, fibromuscular stroma and blood vessels. In the bulbourethral gland, α SMA was only localised to the smooth muscle cells of the capsule and blood vessels in both reproductive periods. Unexpectedly, neither the interlobular nor the intralobular connective tissue stroma of bulbourethral gland has reacted to αSMA. In conclusion, the distribution of CK and α SMA in the accessory genital glands of camels might point out to their roles in the male reproduction.

Key words: accessory genital glands, camel, immunohistochemistry

INTRODUCTION

Although the camel plays an important role as a domesticated mammal in the arid regions of Africa, Asia and Australia, many aspects of its reproduction are still unknown (Zayed *et al.*, 1995).

In dromedaries, the accessory sex glands are the prostate, the ampulla of ductus deferens, and the bulbourethral (Cowper's) glands, whereas camelids do not have vesicular glands (Mosallam, 1981; Hafez & Hafez, 2001). A detailed morphological description of these glands is given elsewhere (Ali *et al.*, 1976; Mosallam, 1981; El Wakeil, 2010).

Although seasonal changes in the spermatogenic activity are still a subject of dispute (Abdel-Raouf *et al.*, 1975; Tingari *et al.*, 1984), previous investigations generally agree that seasons of the year have an obvious effect on the morphology of the accessory genital glands in the camel (El Wishy *et al.*, 1972; Ali *et al.*, 1976, 1978; El Wakeil, 2010).

In Egypt, reproductive activity builds up during September and October and the animal is actually in rut during November–February. Activity falls in March, and regresses steadily from April onwards. June, July and August are the months of extreme inactivity (non-rut), but not total quiescence (Abdel-Raouf *et al.*, 1975; Marai *et al.*, 2009; El-Bahrawy & El Hassanein, 2011).

The cytoskeleton of eukaryotic cells is composed of three major protein families that form filamentous structures running throughout the cell, i.e microfilaments (actin isoforms), microtubules made of α- and β-tubulin, and the intermediate filaments (Ramaekers & Bosman, 2004).

Actin, one of the major cytoskeletal components in eukaryotic cells, is a 43-kDa globular monomeric protein that can polymerise into double-helical filaments. Actin occurs in two forms, the globular or G-actin which polymerises into the other form which is called filamentous or F-actin. In mammals, actin comprises highly conserved proteins that fall into three broad classes: α, β, and γ isoforms. It is mainly located in the cytoplasm, but is also present in the nucleus (Disanza *et al.*, 2005). Generally, the αSMA is regarded as a marker of terminal smooth muscle differentiation (Skalli *et al.*, 1986). Recent data suggest that αSMA plays a direct role in myofibroblast contractile activity through its N-terminal domain AcEEED (Chaponnier & Gabbiani, 2004). Previously, numerous approaches have studied the localisation of αSMA in the prostate of rats (Hayward *et al.*, 1996; Antonioli *et al.*, 2004, 2007) and humans (Castellucci *et al.*, 1996; Elbadawi *et al.*, 1997; Jennifer *et al.*, 2002; Tomas & Kruslin, 2004; Taboga *et al.*, 2008).

Cytokeratins (CKs) are the largest subgroup of intermediate filament proteins found in the intracytoplasmic cytoskeleton of epithelial tissue. The CK family is a highly complex multigene family of polypeptides, the molecular weight of which ranges from 40 to 68 kDa. There are two types of CKs: the acidic type I CKs and the basic or neutral type II CKs (Schweizer *et al.*, 2006). CKs act as protein scaffolds with structural and regulatory functions in a cell-type-specific manner, as underscored by keratinopathies (Omary *et al.*, 2004) and knockout mice (Magin *et al.*, 2004; Gu & Coulombe, 2007). Recently, new functions of cytokeratins in cell signaling and intracellular vesicle transport have also been discovered (Bragulla & Homberger, 2009). Several immunohistochemical studies have also detected CKs in the prostate of rats (Hsieh *et al.*, 1992), goats (Weijman *et al.*, 1992), dogs (Vos *et al.*, 1992; Lai *et al.*, 2008a, b) and humans (Achtstätfer *et al.*, 1985; Bártek *et al.*, 1986; Kitajima and Tökés, 1986; Wernert *et al.*, 1986, 1987; Srigley *et al.*, 1990; Sherwood *et al.*, 1991; Castellucci *et al.*, 1996).

To our knowledge, no data are available concerning the camel. Therefore, the present study was conducted to determine the seasonal changes in the camel accessory genital glands during rutting and non-rutting seasons using immunohistochemical technique.

MATERIALS AND METHODS

The present study was performed on the accessory genital glands of 10 sexually mature and apparently healthy camels. All samples (5 samples from animals during rutting months: November–January) and 5 samples from animals during non-rutting period: June–August) were collected within 30 min of slaughter in a local (Zagazig) abattoir.

Tissue preparation

Small samples of the accessory genital glands (0.5–1 cm) were fixed in Bouin's fluid for 24 h. Thereafter, fixed samples were extensively washed in 70% ethanol (3×24 h) to elute fixative before tissue processing to paraffin wax by routine methods. Using a Leitz rotatory microtome, 5 µm-thick sections were cut and mounted on both 3-aminopropyltriethoxysilane-coated and uncoated glass slides. Paraffin wax embedded sections were kept in an incubator at 40 °C until used for conventional hematoxylin and eosin (H&E) staining and immunohistochemical analysis.

Immunohistochemical staining

For the detection of CK and αSMA, a mouse monoclonal primary antibody against CK (Clone MNF116, M0821) and a mouse monoclonal primary antibody against αSMA (Clone 1A4, M0851) (DAKO, Hamburg, Germany) were used. Antigen localisation was achieved using the Dako LSAB®+ Kit, peroxidase (LSAB+ Kit, HRP) technique according to the manufacturer instruction. Briefly, 5 µm sections of paraffin-embedded tissues were dewaxed, rehydrated, and rinsed in PBS pH 7.4 (3×5 min). For antigen retrieval, the slides were heat treated in microwave oben at 750 W for two cycles of 7 min each in citrate buffer (PH 6.0). Thereafter the sections were allowed to cool at room temperature for 20 minutes. Endogenous peroxidase was blocked by soaking the sections in 3 % v/v hydrogen peroxide/ distilled water for 10 min at room temperature followed by washing them under running tap water for additional 10 min. Subsequently the slides were equilibrated in PBS pH 7.4 (2×5 min). Non-specific antibody binding was minimised by covering the slides with a serum-free protein blocking reagent (DAKO, Hamburg, Germany) for 10 min at room temperature. Sections were then incubated with primary antibody against CK and αSMA diluted 1:100 in antibody diluent (DAKO, Hamburg, Germany) for 1 h at room temperature. The slides were subsequently rinsed in PBS pH 7.4 (2×5 min) followed by incubation with diluted (1:300 in PBS) biotinylated secondary antibody (rabbit anti-mouse IgG) (DAKO, Hamburg, Germany) for 30 min at room temperature. Bound antibodies were visualised using a Dako LSAB®+ Kit, peroxidase (LSAB+ Kit, HRP) technique and diaminobenzidine (DAB) (DAKO, Hamburg, Germany). All incubations were performed in a humidified chamber. Sections were left unstained or counterstained in Mayer's haematoxylin, dehydrated, and mounted with DPX (Sigma, Munich, Germany). Negative controls were performed by omission of the primary antibody.

RESULTS

Cytokeratin

In this study the monoclonal mouse anti-human cytokeratin, clone MNF116, is a broad spectrum anti-keratin reagent reacting with intermediate and low-molecular-weight keratins (directed to CK5, 6, 8, 17, 19), so that cytokeratin isoforms could not be distinguished.

In rutting season, strong CK positive staining was observed in the cytoplasm of the columnar cells of secretory acini of prostate. In the basal cells, the intense positive staining for CK had a uniform distribution in the scanty cytoplasm (Table 1). However, CK reaction was absent in the capsule, fibromuscular stroma, and various blood vessels (Fig.1A, B). Simi-

larly, moderate CK staining was observed in the columnar and basal cells of secretory acini in the non-rutting periods. Although the CK was randomly distributed as a deposit of fine granules throughout the cytoplasm of columnar cells, more intense reaction was seen in the supranuclear region (Fig. 1C, D).

Although the ampulla ductus deferens consisted of four tunicae, the CK reaction was only confined to the tunica mucosa and to the ampullary gland of the submucosa in both rutting and non-rutting season (Fig. 2A–D). In rutting period, strong CK staining was localised in the cytoplasm of the pseudostratified columnar epithelium of the tunica mucosa. However, no reaction was seen in the underlying lamina propria. In the submucosa, strong CK staining was seen in the cytoplasm of columnar and basal cells of the secretory units while no staining was evident in the surrounding fibromuscular stroma (Fig. 2A, B). In non-rutting season, moderate CK reaction was also found in the pseudostratified columnar epithelium and in the secretory columnar epithelium of the ampullary gland (Table 1). However, the CK staining was more intense in the apical cytoplasm of the secretory columnar epithelium of the submucosal ampullary glands (Fig. 2C, D).

In the bulbourethral gland, mild CK reaction was solely observed in the pyramidal cells of type A and type C secretory units as well as in the lining epithelium of the duct system in both rutting and non-rutting periods. No CK staining was how-

Table 1. Expression of the cytokeratin (CK) and α smooth muscle actin (αSMA) in the accessory genital glands of camels during rutting and non-rutting seasons

	CK		αSMA	
	Rutting	Non-rutting	Rutting	Non-rutting
Prostate				
secretory acini	+++	++	0	0
basal cells	+++	++	0	0
capsule and fibromuscular stroma	0	0	+++	++
blood vessels	0	0	+++	++
Ampulla				
mucosal epithelium	+++	++	0	0
submucosal glands	+++	++	0	0
fibromuscular stroma	0	0	+++	+++
tunica muscularis	0	0	+++	+++
blood vessels	0	0	+++	+++
Bulbourethral gland				
type A and C secretory units	+	+	0	0
duct system	+	+	0	0
type B secretory units	0	0	0	0
capsule and blood vessels	0	0	+++	+++
inter- and intralobular connective tissue stroma	0	0	0	0

(0) no reactivity, (+) mild reactivity, (++) moderate reactivity, (+++) strong reactivity.

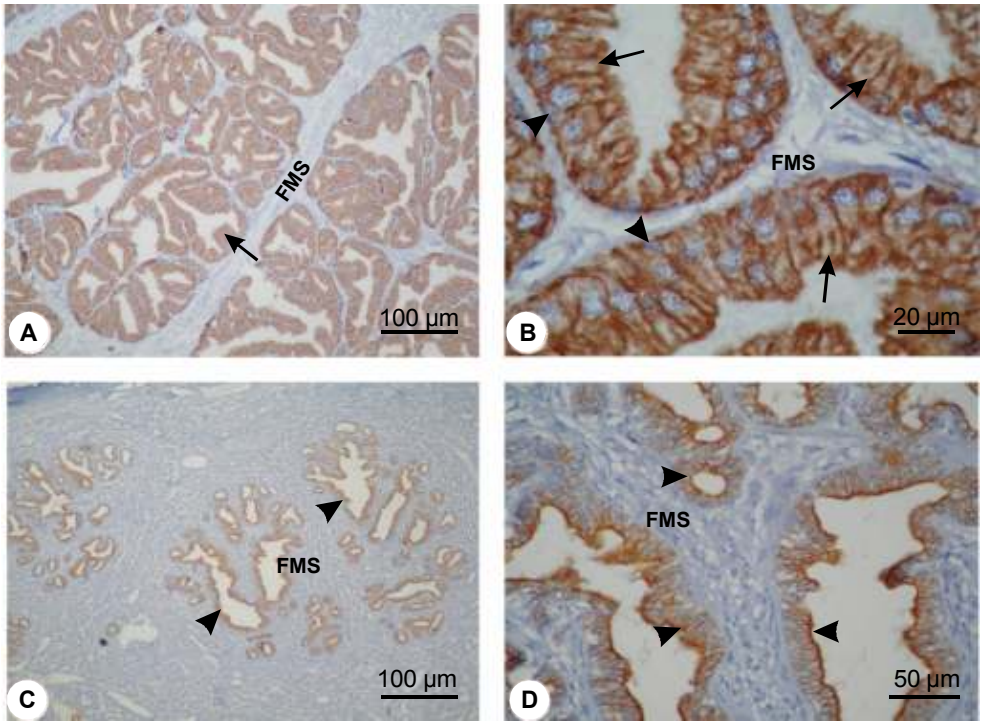

Fig. 1. Localisation of CK in the prostate of camel during rutting (**A** and **B**) and non-rutting (**C** and **D**) periods. **A** and **B**: strong CK reactivity was seen in the columnar (arrows) and basal (arrowheads) cells of the prostatic secretory acini. No expression could be detected in fibromuscular septa (FMS). **C** and **D**: Moderate CK reactivity was observed in the secretory epithelium of prostate (arrowheads) while no CK staining was evident in the fibromuscular septa (FMS).

ever detected in the cuboidal cells of the type B secretory acini and in the surrounding connective tissue stroma (Fig. 3A–D).

Generally, the glandular tissue and the CK positive staining of all accessory genital glands were comparatively less prominent in the non-rutting period.

Alpha-smooth muscle actin

In the rutting and non-rutting season, αSMA was only localised to the smooth muscle cells of the prostatic capsule and fibromuscular stroma immediately surrounding the secretory acini. Additionally, αSMA staining was seen in the smooth muscle cells of the prostatic blood vessels. αSMA reaction was apparently more intense in the fibromuscular stroma of rutting prostate (Table 1; Fig. 4A–D).

In the ampulla, strong αSMA reaction was seen in the smooth muscle of tunica muscularis and fibromuscular stroma surrounding the submucosal ampullary gland in both rutting and non-rutting season. Additionally, αSMA staining was seen in the smooth muscle cells of the ampullary blood vessels (Fig. 5A–D).

In the bulbourethral gland, strong αSMA was only localised to the smooth muscle cells of the capsule and blood vessels in both reproductive periods. Interestingly, neither the interlobular nor the intralobular connective tissue stroma has reacted to αSMA (Fig. 6A–D).

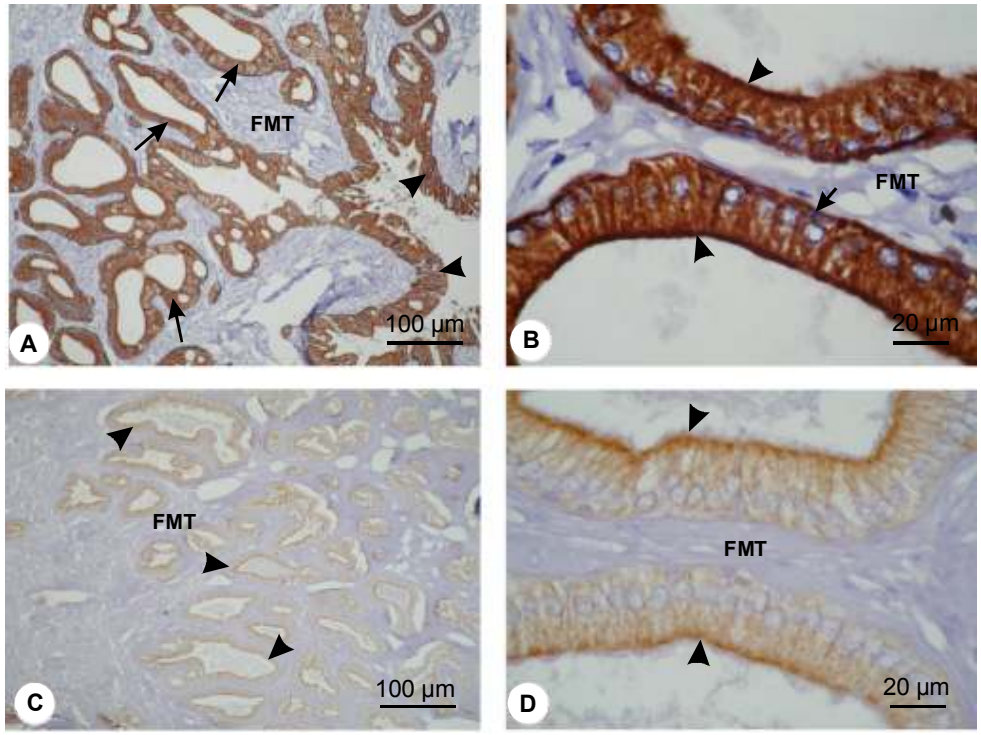

Fig. 2. Localisation of CK in the ampulla of camel during rutting (**A** and **B**) and non-rutting (**C** and **D**) periods. **A**: The CK reaction was only confined to the tunica mucosa (arrowhead) and to the ampullary gland of the submucosa (arrow). However, no reaction was seen in the fibromuscular tissue (FMT). **B**: In the submucosa, strong CK reactivity was seen in the cytoplasm of columnar (arrowhead) and basal (arrow) cells of the secretory units while no staining was evident in the fibromuscular tissue (FMT). **C**: Moderate CK reactivity was found in the secretory columnar epithelium of the ampullary gland (arrowhead). **D**: The CK staining was more intense in the apical cytoplasm of the secretory columnar epithelium of the submucosal ampullary glands (arrowhead).

Generally, no αSMA staining was evident within the lining epithelium of the secretory units of the accessory genital glands of camel either in rutting or non-rutting period.

DISCUSSION

The cytoskeleton carries out three broad functions: it spatially organises the contents of the cell; connects the cell physically and biochemically to the external environment; and generates coordinated forces that enable the cell to move and change shape (Fletcher & Mullins, 2010). Since the cytoskeleton is involved in virtually all cellular processes, abnormalities in this essential cellular component frequently result in disease (Ramaekers & Bosman, 2004).

Cytokeratins perform instrumental functions within epithelial cells to ensure not only their physical integrity but also their metabolic processes (Vaidya & Kanojia, 2007). The present study represents the first report that concerned the localisation of CK and αSMA in the accessory

genital glands of camel. In prostate, CK was generally localised in the cytoplasm of the columnar cells of secretory acini and in the scanty cytoplasm of basal cells in both rutting and non-rutting period. Conversely, no expression was seen in the capsule, fibromuscular stroma, and blood vessels. These findings are consistent with numerous approaches that investigated the localisation of CKs in the prostate of several mammalian species including rats (Hsieh *et al.,* 1992), goats (Weijman *et al.*, 1992), dogs (Lai *et al.*, 2008a, b) and humans (Achtstätfer *et al.*, 1985; Bártek *et al.*, 1986; Kitajima & Tökés, 1986; Wernert *et al.*, 1986, 1987; Sherwood *et al.*, 1991; Castellucci *et al.*, 1996).

In the ampulla of ductus deferens, the CK reaction was found in the pseudostratified columnar epithelium and in the secretory columnar epithelium in both rutting and non-rutting season. However, no reaction was evident in the surrounding connective tissue.

In the bulbourethral gland, CK reaction was exclusively observed in the pyramidal cells of type A and type C secretory units as well as in the lining epithelium of the duct system in both rutting and non-rutting periods. No CK staining was how-

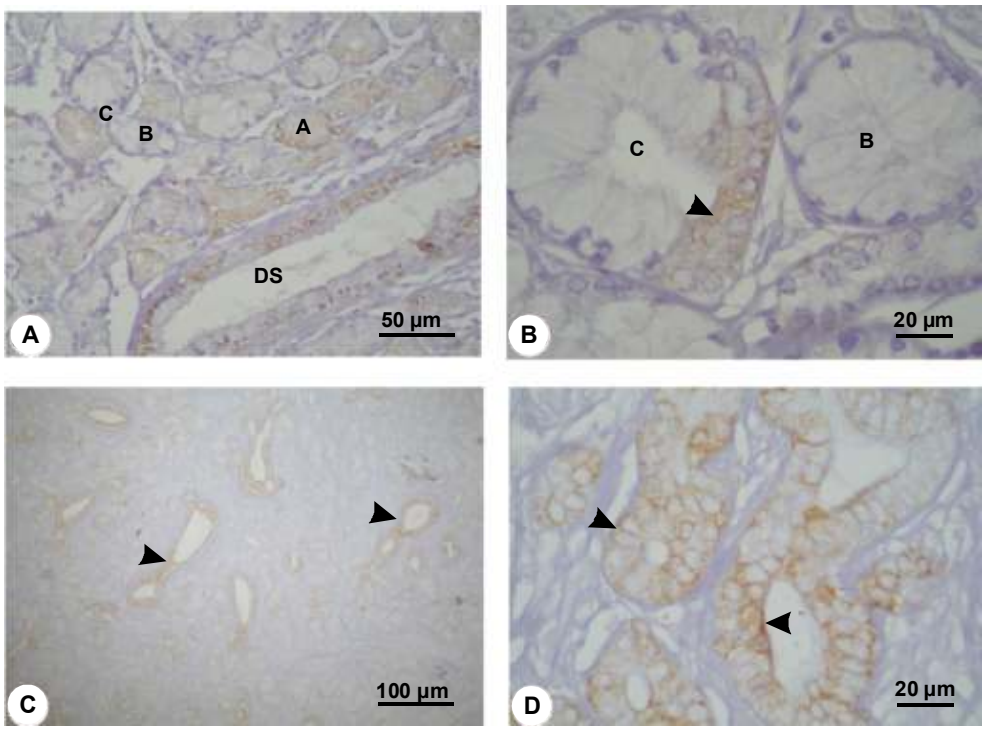

Fig. 3. Localisation of CK in the bulbourethral gland of camel during rutting (**A** and **B**) and non-rutting (**C** and **D**) periods. **A** and **B**: mild CK reaction was solely observed in the pyramidal cells of type A and type C secretory units (arrowhead) as well as in the lining epithelium of the duct system (DS). **C** and **D**: mild CK reaction was seen in the lining epithelium of the duct system (arrowheads). No CK staining was however detected in the cuboidal cells of the type B secretory acini and in the surrounding connective tissue stroma.

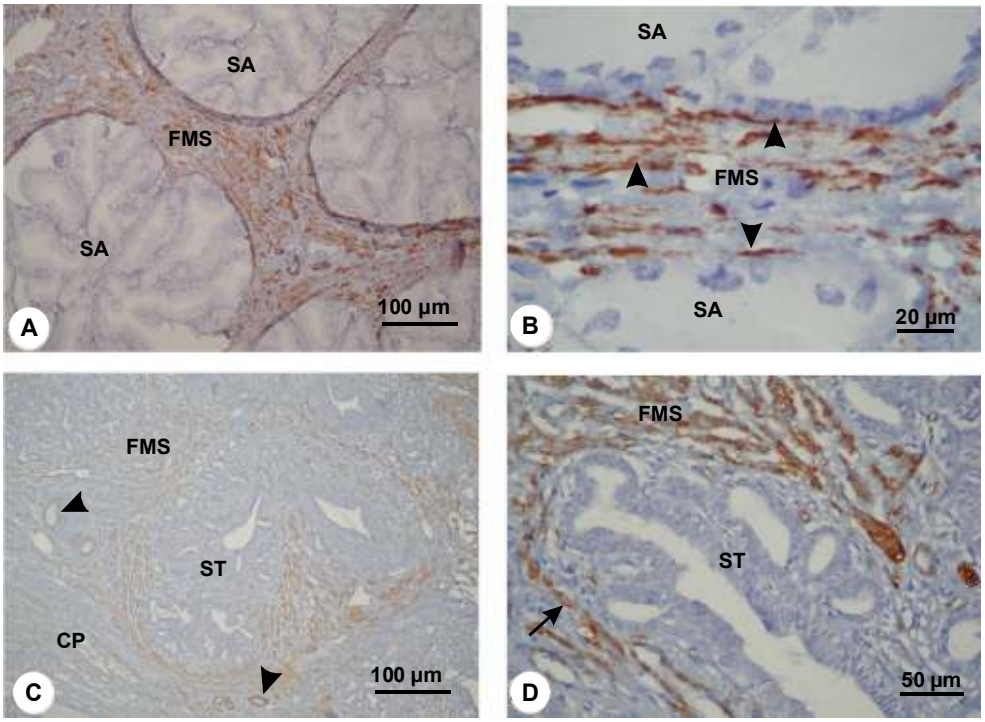

Fig. 4. Localisation of αSMA in the prostate of camel during rutting (**A** and **B**) and non-rutting (**C** and **D**) periods. **A** and **B**: αSMA reaction (arrowhead) was localised to the smooth muscle cells of the fibromuscular stroma (FMS) immediately surrounding the secretory acini (SA). **C** and **D**: αSMA staining (arrow) was seen in the fibromuscular stroma (FMS) surrounding the secretory tissue (ST), in the smooth muscle cells of prostatic blood vessels (arrowhead), and in the prostatic capsule (CP).

ever detected in the cuboidal cells of the type B secretory acini and in the surrounding connective tissue stroma. In humans, the available literature is contradictory. One approach stated that the bulbourethral glands are negative for high-molecular-weight cytokeratin K-903 (34beta E12) (Saboorian *et al.*, 1997). Conversely, another study reported that the high-molecular-weight cytokeratin is strongly reactive with the ductular epithelium and demonstrated an attenuated cell lining at the periphery of lobules (Cina *et al.*, 1997).

Taken together, the localisation of CK in the simple epithelium of accessory genital glands of camels in rutting and non-rutting seasons may point out to its crucial role in the male reproduction. In this concept, CK might regulate the secretory functions of such epithelia, act as signaling platforms, and/or protect epithelial cells against mechanical stress. This speculation is substantiated by the findings that the simple epithelia are commonly found lining glands and in organs involved in secretion and absorption, and the individual cells are often polarised, which suggests that the unique expression of simple epithelial cytokeratin in these cells is likely to have functional consequences related to polarised protein sorting, absorption, and

secretion (Toivola *et al.*, 2005; Oriolo *et al.*, 2007). Moreover, CKs are involved in cell signalling, cell transport, cell compartmentalisation and cell differentiation (Oshima, 2007; Vaidya & Kanojia, 2007). Additionally, the best-known function of CK is to provide a scaffold (through self-bundling and by forming thicker strands) for epithelial cells and tissues to sustain mechanical stress, maintain their structural integrity, ensure mechanical resilience, protect against variations in hydrostatic pressure and establish cell polarity (Coulombe & Omary, 2002; Gu & Coulombe, 2007). CK also influence cell metabolic processes by regulating protein synthesis and cell growth (Gu & Coulombe, 2007) and may be involved in the transport of membrane-bound vesicles in the cytoplasm of epithelial cells (Planko *et al.*, 2007).

In the non-rutting periods, the CK positive staining of all glands specially the prostate was comparatively less prominent as in the rutting period. This variation in CK expression could be attributed to the fluctuating level of androgen hormones during the rutting and non-rutting season. Generally, testosterone levels reach the basal level during the non-rutting season (August), while reach a maximum during the rut (February) (Marai *et al.*, 2009; El-Bahrawy & El Hassanein, 2011). Androgen appears to be a pleiotropic factor in

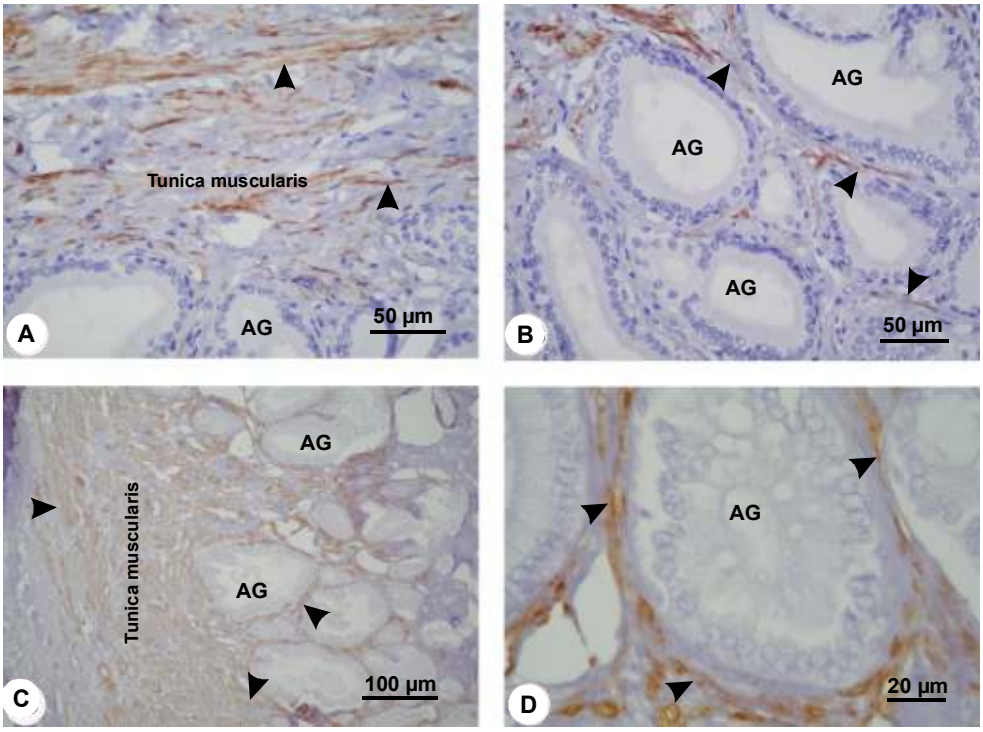

Fig. 5. Localisation of αSMA in the ampulla of camel during rutting (**A** and **B**) and non-rutting (**C** and **D**) periods. **A–D:** Strong αSMA reaction was seen in the smooth muscle of tunica muscularis and fibromuscular stroma (arrowhead) surrounding the submucosal ampullary gland (AG).

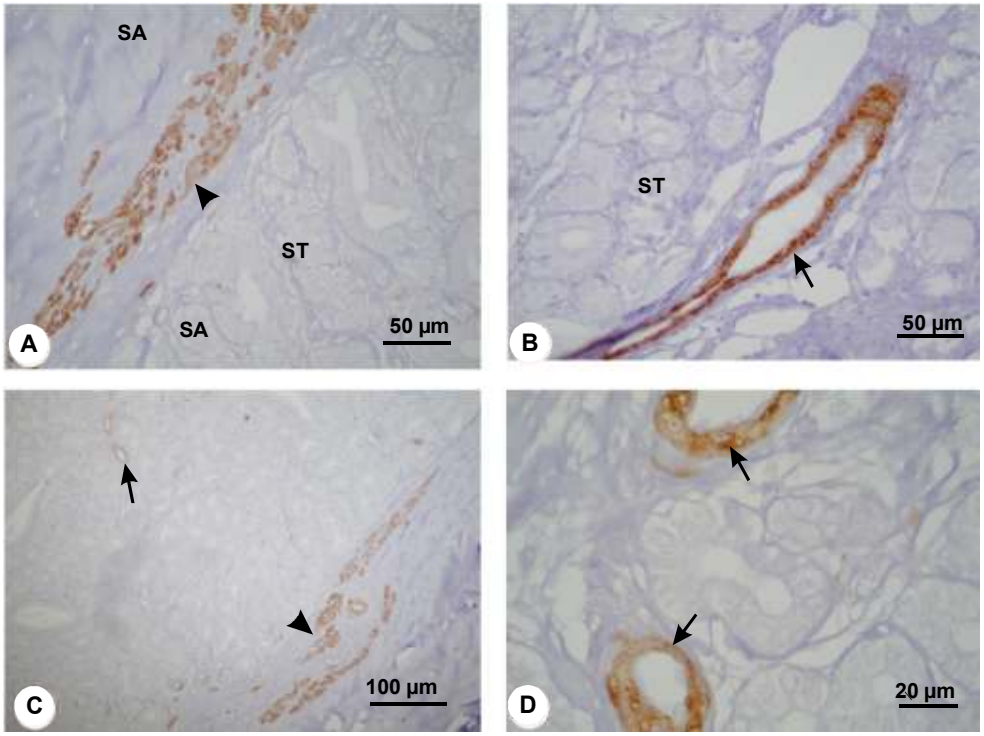

Fig. 6. Localisation of αSMA in the bulbourethral of camel during rutting (**A** and **B**) and non-rutting (**C** and **D**) periods. **A–D**: Strong αSMA staining was only localised to the smooth muscle cells of the capsule (arrowhead) and blood vessels (arrows).

regulating the expression of both luminal and basal specific CK mRNA expression in an organ-specific manner. In rat accessory sex organ, the levels of CK mRNAs expression are negatively regulated by androgen (Hsieh *et al.*, 1992). This discrepancy in the effect of androgen on the accessory genital glands may be species-specific.

Smooth muscle contractile activity is a major regulator of function of the vascular system, respiratory system, gastrointestinal system and the genitourinary systems. Therefore, malfunction of contractility in these systems leads to a host of clinical disorders (Kim *et al.*, 2008). αSMA is mainly found in cells having contractile functions and is therefore a powerful probe in the study of smooth muscle cell differentiation in normal and pathological conditions (Skalli *et al.*, 1986; van Nassauw *et al.*, 1993).

In the present study, αSMA was only localised to the smooth muscle cells of the prostatic capsule and fibromuscular stroma immediately surrounding the secretory acini. Additionally, αSMA staining was seen in the smooth muscle cells of the blood vessels. Nevertheless, no αSMA was evident within the lining epithelium of the secretory units of the camel prostate either in rutting or non-rutting period. Similar results are also described in the prostate of rats (Hayward *et al.*, 1996; Antonioli *et al.*, 2004, 2007) and humans (Castellucci *et al.*, 1996; Elbadawi *et al.*,

1997; Jennifer *et al.*, 2002; Tomas & Kruslin, 2004; Taboga *et al.*, 2008). Our results are also parallel to the previous morphological findings whereas as the interlobular stroma of the camel's prostate is less fibrous and more muscular (Ali *et al.*, 1978; Mosallam, 1981; El Wakeil, 2010).

In the ampulla, αSMA reaction was seen in the smooth muscle of tunica muscularis and fibromuscular stroma surrounding the submucosal ampullary gland in both rutting and non-rutting seasons. Additionally, αSMA staining was seen in the smooth muscle cells of the ampullary blood vessels. These findings confirm the previous morphological studies on the ampulla whereas the interstitial stroma in the glandular part of the ampulla ductus deferens in camel is formed of reticular fibres in common with smooth muscle fibres (Ali *et al.*, 1978; Mosallam, 1981; El Wakeil, 2010). Similar morphological reports are also described in several mammalian (Cooper & Hamilton, 1977; Riva *et al.*, 1982; Murakami *et al.*, 1986) and non-mammalian (Zalisko & Larsen, 1988) vertebrates. The presence of such great amount of smooth muscle fibres in the interlobular stroma of these glands lead us to think that they are related in some way to the evacuation of the ampullary and prostatic secretion.

In the bulbourethral gland, αSMA was only localised to the smooth muscle cells of the capsule and blood vessels in both reproductive periods. Unexpectedly, neither the interlobular nor the intralobular connective tissue stroma has reacted to αSMA. These results are concurrent with the previous morphological approaches on camel where the compound tubuloalveolar secretory end-pieces of the bulbourethral gland are only supported by abundant reticular fibres (Ali *et al.*, 1978; Mosallam, 1981; El Wakeil, 2010). On the contrary, αSMA reaction is seen at the periphery of the secretory acini of human bulbourethral gland (Cina *et al.*, 1997; Saboorian *et al.*, 1997). Moreover, myofibroblast cells are demonstrated in the secretory tissue of bulbourethral glands in rats (Nielsen, 1976), Japanese monkeys (Murakami *et al.*, 1981), and humans (Hellgren *et al.*, 1982; Riva *et al.*, 1988; Saboorian *et al.*, 1997). Collectively, the evacuation of camel bulbourethral gland may be depending on the capsular musculature and not on the interlobular stroma.

Our study also showed that the basal cells of the secretory acini of prostate and ampullary glands were positively stained with CK while negatively reacted with αSMA. These findings clearly indicate that the basal cells of these glands are not of myoepithelial origin whereas the secretory acini do not seem to need specialised myoepithelial cells to aid in the expulsion of acinar contents because of the abundant fibromuscular stroma. Similar results are also reported in humans (Srigley *et al.*, 1990). Generally, the immunocytochemical characterisation of myofibroblasts is based on a combination of different markers, such as expression of αSMA, vimentin, prolyl 4-hydroxylase and absence of cytokeratin, calponin and desmin immunostaining (Lazard *et al.*, 1993; van der Loop *et al.*, 1996; Wever & Mareel, 2003).

In conclusion, the distribution of CK in the accessory genital glands of camel during rutting and non-rutting seasons might indicate its critical role in the male reproduction. Moreover, the myofibroblasts in the accessory genital glands may provide the major force that allows these glands to evacuate their secretion.

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Correspondence:

Mahmoud Badran Shoaib
Department of Anatomy and Embryology,
Faculty of Veterinary Medicine,
Mansoura University,
Mansoura, Egypt
phone: 00201227578774
e-mail: mshoaib882004@yahoo.com