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SEASONAL STUDIES ON MORPHOLOGY AND IMMUNOHISTOCHEMICAL LOCALISATION OF S-100 AND ALPHA SMOOTH MUSCLE ACTIN PROTEINS IN POLL GLANDS OF DROMEDARY CAMEL

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ABSTRACT

The two poll glands rest subcutaneously behind the ear of the dromedary camel (*Camelus dromedarius*). Although they secrete a watery yellowish secretion with offensive odour during breeding seasons, their function is still largely unspecified. This study on camel poll glands morphology and immunoreactivity of S-100 and alpha smooth muscle actin (α-SMA) proteins during breeding and non-breeding seasons might shed some light on their function. During breeding season the gland was larger in size and darker in colour and its secretion increased compared to non-breeding season. No significant change was observed in the alveolar luminal diameter during seasonal reproductive cycle ($P>0.05$). However, alveolar diameter and epithelial height was significantly ($P<0.05$) increased during breeding season together with significantly increased inter-alveolar tissue thickness during non-breeding season. While S-100 reacted positively in the alveolar epithelium and inter-alveolar tissue, α-SMA reaction was negative in the alveolar epithelium and positive in the alveolar myoepithelial cells, smooth muscles and blood vessels. S-100 and α-SMA positive immunoreactivity increased during breeding season compared to non-breeding season. These results suggest that the poll gland secretory activity is correlated with male camel seasonal sexual activity. Moreover, S-100 and α-SMA are suggested to regulate cellular and muscular functions in the poll glands.

Key words: Anatomy, camel, histology, immunohistochemistry, poll gland, reproductive activity

The poll glands are symmetrical subcutaneous exocrine glands of the male dromedary camel which are located behind the ears in the neck region (Leese, 1927; Singh and Bharadwaj, 1978; Tingari and George, 1984; Ebada *et al,* 2012). The gland which has been considered as a tubule-alveolar modified apocrine sweat gland (Singh and Bharadwaj, 1978; Manivannan *et al,* 1996) is histologically similar to the mammary gland (Purohit and Singh, 1958). It is known to undergo cyclic activity, yielding a yellowish watery secretion with offensive odour, especially during the breeding season (Singh and Bharadwaj, 1978; Taha and Abdalla, 1980; Yagil and Etzion, 1980). The poll gland secretion has also been described as very copious with strong foetid smell which becomes black when exposed to the air and it dribbles down the neck of the rutting camel (Skidmore, 2004). The poll gland morphology and histochemistry have earlier been described (Purohit and Singh, 1958; Singh and Bharadwaj, 1978; Tingari and George, 1984; Tingari *et al,* 1984b; Manivannan *et al,* 1996; Atoji *et al,* 1998).

S-100 and alpha smooth muscle actin (α-SMA) are proteins suggested to affect the cellular absorption, cellular secretion and muscular contractile activities (Alkafafy *et al,* 2011b). S-100 is a calcium-binding protein which has earlier been considered specific to the nervous system as it was initially detected in brain glial cells and Schwan's cells of peripheral nerves (Bock, 1978). Later, S-100 has also been detected in other parts of mammalian body including the reproductive system (Amselgruber *et al,* 1992; Alkafafi *et al,* 2011b; Ibrahim, 2015; Ibrahim *et al,* 2017). Functionally, S-100 has many intracellular and extra cellular activities including modulation of enzymatic activity, motility and energy metabolism and cellular secretion (Heizmann *et al,* 2002). α-SMA, which has been used as a means for differentiation of normal and pathological smooth muscle cells, is mainly found in cells performing contractile functions (Skalli *et al,* 1989). In the camel epididymis α-SMA was mainly seen in the epididymal peri-tubular muscles and connective tissue as well as in the smooth

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muscles of epididymal blood vessels (Ibrahim *et al,* 2017; Abd-Elmaksoud, 2009). Immunoreactivity of S-100 and α-SMA has earlier been described in the testis and epididymis of camel and other mammalian species (Amselgruber *et al,* 1994; Abd-Elmaksoud, 2005; Alkafafy *et al,* 2011b; Alkafafy and Sinowatz, 2012; Alkafafy *et al,* 2016). Some seasonal immunohistochemical studies have been conducted on the efferent ductules (Ibrahim, 2015), testis (Alkafafy *et al,* 2016) and epididymis (Ibrahim *et al,* 2017) of the camel. According to Ibrahim *et al* (2017) S-100 and α-SMA show distinct variations in relation to reproductive activity in dromedary camel epididymis and they might change the structural and physiological states of this organ.

However, detailed immunohistochemical studies on the camel poll gland during the rutting and non-rutting seasons are scarce in the available literature. This study, therefore, was aimed to investigate the immunoreactivities of S-100 and α-SMA in the dromedary camel poll gland during breeding and non-breeding seasons. Complementary morphological investigation was also included.

Materials and Methods

Animals and tissue

Thirty adult and healthy dromedary camels (*Camelus dromedarius*) slaughtered at Buraidah Central Slaughterhouse, AL-Qassim, Saudi Arabia, were used in this study. Specimens from 15 animals during breeding season (between December and February) and from 15 animals during non-breeding season (between May to July) were used in this study. All experiments were approved by the Institutional Animal Ethics Committee (IAEC) of Qassim University, Saudi Arabia.

Anatomical study

For gross anatomical study three adult camel heads during both seasons were carefully dissected and the poll glands were exposed, grossly studied and their dimensions were measured.

Microscopic study

For histological, morphometric and immuno-histochemical studies glandular samples from 12 animals during each season were quickly collected after animal slaughter and fixed in 10% buffered formalin; the samples were then dehydrated in ascending series of ethyl alcohol, cleared in xylene and embedded in paraffin wax as described by Culling (1974). Paraffin sections (5µm thick) from each animal group were cut using a rotary microtome.

Histology

For histological observations the paraffin sections were conventionally stained with Haematoxylin and Eosin (H&E) following the procedure of Culling 35. Microscopic examination was carried out using Leica microscope (Leica DMD108-Germany).

Morphometry

Five H&E-stained sections of poll glands from each animal group were randomly chosen for measurements of alveolar diameter, epithelial height, luminal diameter and inter-alveolar connective tissue thickness. The measurements were carried out using Leica provided with Leica microscope, digital camera and measuring software (DMD108-Germany). The study only included regular transverse alveolar sections. The obtained morphometric data (expressed as means ± standard deviation) were analysed using Students T-test. $P< 0.05$ was considered as statistically significant.

Immunohistochemistry

Paraffin sections from each animal group were de-waxed using two changes of xylene and rehydrated in decreasing concentrations of ethyl alcohol. The sections were then washed in distilled water and phosphate buffer saline (PBS). Antigen unmasking/retrieval was performed using microwave heating (750 W) in 0.01 M sodium citrate buffer (pH 6.0) for 20 min. Immunohistochemical staining was carried out using Mouse and Rabbit Specific HRP/DAB (ABC) Detection IHC Kit (ab64264, Abcam, Cambridge, UK), as described by the manufacturer's instructions. Sections from both animal groups were incubated with rabbit polyclonal anti-S100 primary antibody (ab868, Abcam, Cambridge, UK), for overnight at 4°C and with rabbit polyclonal anti- α-SMA primary antibody (ab5694, Abcam, Cambridge, UK), for 3hr at room temperature. The dilutions for both anti-S100 (1:100) and anti- α-SMA (1:5000) primary antibodies were performed using a universal diluent (ab79995, Abcam, Cambridge, UK). Anti-S100 primary antibody cross-reacts with S100-equivalent protein in mouse, rat, cow, human and pig and anti- α-SMA primary antibody cross-reacts with α-SMA-equivalent proteins in rat, chicken, guinea pig, cow, dog, human and pig as stated by the manufactures. Negative control immunostaining was carried out by omitting the primary antibody. On the other hand, positive control immunoreactivity was performed according to the manufacturer's instruction for each primary antibody kit.

Results

Gross anatomy

The two poll glands rested dorsally on either side of the neck region, about 7 cm behind the occipital crest; a watery yellowish secretion with offensive odour exuded through the skin covering the gland, especially during the breeding season (Fig 1). The secretion colour changed to dark brown or black after being exposed to air. The gland was lobulated and its length ranged between 8-10 cm during rutting season and 5-8 cm during non-rutting season. Compared to non-breeding season, the glandular lobes were larger and many of them were darker in colour during breeding season, as they were filled with the glandular secretion.

Histology

The histological structure of the camel poll gland during breeding and non-breeding seasons is shown in Fig 2. The gland was surrounded by a connective tissue capsule that sent connective tissue septa which divided the gland into lobes and lobules consisted mainly of collagenous fibres, blood vessels and interlobular ducts (Fig 2a).

During breeding season (Fig 2a, b), the glandular lobules were occupied by large alveoli which were characterised by tall simple cuboidal epithelium and narrow lumina; the epithelium exhibited apical protruding blebs and it was surrounded by myoepithelial cells with dark nuclei followed by scanty inter-alveolar connective tissue. Intra-lobular ducts with tall simple cuboidal or columnar epithelium were also found in the lobules.

During non-breeding season the alveoli and intra-lobular ducts appeared smaller and their epithelium changed to simple squamous or low cuboidal, which resulted in increased luminal diameter (Fig 2c, d); the interlobular and inter-alveolar connective tissue was increased in comparison to that in breeding season.

Morphometry

The analysis of morphometric parameters of the camel poll gland during breeding and non-breeding seasons is shown in table 1.

During the breeding season, the alveolar diameter and epithelial height were significantly increased in comparison with non-breeding season ($P<0.05$). On the other hand, significantly increased inter-alveolar tissue thickness was observed during non-breeding season as compared to breeding season ($P<0.05$). However, the luminal diameter did not show any significant change during seasonal reproductive cycle ($P>0.05$).

Table 1. Seasonal morphometric measurements (µm) of the camel poll gland structures.

Parameters	Breeding season	Non-breeding season
Alveolar diameter	$64.45\pm4.04_a$	$52.34\pm6.83_b$
Epithelial height	$14.63\pm0.93_a$	$9.50\pm1.55_b$
Luminar diameter	$44.75\pm1.87_a$	$38.46\pm2.81_a$
Inter-alveolar tissue thickness	$13.80\pm1.58_a$	$32.37\pm2.78_b$

Values (mean ± SD, N = 12).
Values within the same row with different subscripts are significantly different ($P<0.05$)

Immunohistochemistry

Seasonal variations in the immunoreactivity of S-100 and α-SMA during breeding and non-breeding seasons are shown in Table 2 and Fig 3 and 4.

S-100

In the breeding season the glandular alveoli and glandular ducts exhibited numerous epithelial cells with intense S-100 immunostaining, especially in their supra-nuclear region and apical protruding blebs (Fig 3a, b). In the glandular blood vessels the S-100 immunoreaction was intense in the endothelium and moderate in the tunica media. The inter-alveolar connective tissue and myoepithelial cells appeared with weak S-100 reaction.

During non-reproductive season, decreased intensity of S-100 reaction was observed in the alveolar epithelium, ductal epithelial and blood vessel endothelium (Fig 3c). In the glandular connective tissue, blood vessel tunica media and myoepithelial cells the reaction intensity was negative to weak.

Negative control immunostaining showed negative reaction in the different parts of the poll gland during breeding and non-breeding seasons (Fig 3d).

α-SMA

During both seasons there was positive α-SMA immunoreactivity in the myoepithelial cells, peri-alveolar smooth muscles and tunica media of inter-alveolar and interlobular blood vessels. The reaction was stronger during breading season, especially in the myoepithelial cells and smooth muscles of blood vessel (Fig 4a), than that in non-breeding season (Fig 4b). However, the epithelial cells, inter-alveolar connective tissue and inter-lobular connective

Table 2. Seasonal immunohistochemical reactions of S-100 and α-SMA in the camel poll gland structures.

Protein	Season	AE	DE	MECs	IASM	CT	VE	VSM
S-100	Breeding	+++	+++	++	+	+	+++	++
	Non-breeding	++	++	+/-	+/-	+/-	++	+/-
α-SMA	Breeding	-	-	++++	++	++	+++	++++
	Non-breeding	-	-	++	+	-	+	+

Alveolar Epithelium (AE); Ductal Epithelium (DE); Myoepithelial Cells (MECs); Inter-alveolar Smooth Muscles (IASM); Connective Tissue (CT); Vascular Endothelium (VE); Vascular Smooth Muscles (VSM).Very strong (++++); strong (+++), moderate (++); weak to moderate (+/++); weak (+), weak to negative (+/-) and Negative (−) reaction.

tissue exhibited negative α-SMA immunoreactions throughout the entire reproductive cycle of the camel.

Negative control immunostaining produced negative reaction for α-SMA antibody during breeding and non-breeding seasons (Fig 4c).

Discussion

The present study showed that the paired poll gland was located dorsal to the poll (neck) region of the male dromedary camel where a yellowish watery secretion with offensive odour was observed during the breeding season. This is in line with earlier findings of a number of authors (Leese, 1927; Singh and Bharadwaj, 1978; Taha and Abdalla, 1980; Yagil and Etzion, 1980; Tingari *et al,* 1984b; Ebada *et al,* 2012). According to Yagil and Etzion (1980) the presence of androgens has been proved in the secretions of the poll glands of the rutting dromedary camel. The poll gland secretion has also been suggested to contain some types of pheromones that might be used to border a mating area for the male in the herd (Skidmore, 2004). The gross anatomical change of poll glands in this study showed darker glandular colour and increased glandular size in the breeding season. It has been mentioned that seasonal structural variations observed in the poll glands indicate their increased activity during December-March (Singh and Bharadwaj, 1978). These seasonal structural changes were considered as testosterone-dependent (Aguilera-Merlo *et al,* 2005).

The present results revealed seasonal histological and morphometric variations in the poll gland. During the breeding season the glandular alveoli showed narrow lumina and simple cuboidal or columnsar epithelium surrounded by a thin inter-alveolar connective tissue. During non-breeding season the alveoli and intra-lobular ducts were smaller and their epithelium changed to simple squamous or low cuboidal, which resulted in relatively increased luminal diameter and thicker inter-alveolar connective tissue in comparison to those in breeding season. Similar findings have also been reported by Tingari *et al* (1984b)

The histological findings in this study were confirmed by morphometric measurements which showed significantly increased alveolar diameter and epithelial height during breeding season than those in non-breeding season. Also in the breeding season, the inter-alveolar tissue thickness was significantly decreased. On the other hand, the luminal diameter did not show significant change in relation to seasonal reproductive activity. This is in accordance with records by Tingari *et al* (1984b). It has been suggested that increased poll gland activity could be indicated by higher alveolar epithelial lining and greatly reduced inter-alveolar connective tissue during December -March in winter as compared to the period between April and August in summer (Singh and Bharadwaj, 1978). Moreover, it has been noted that during peak reproductive activity in November

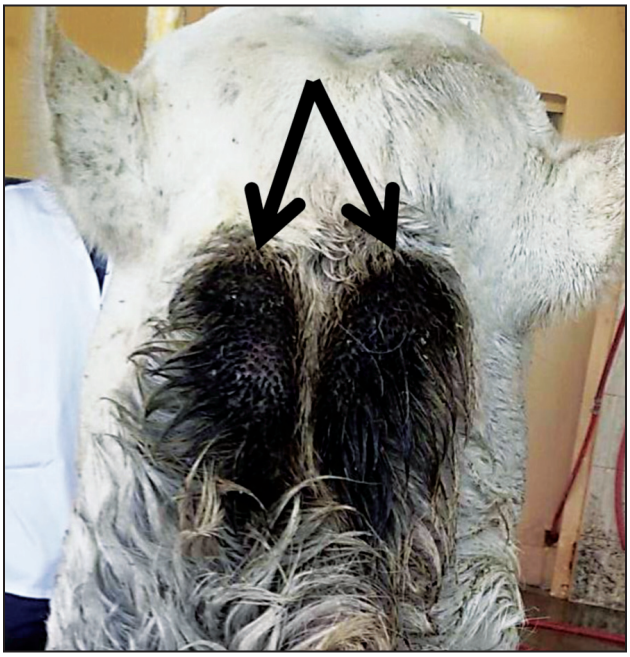

Fig 1. Dark brown secretion of the paired poll gland exuding through the skin of the rutting dromedary camel (Arrows).

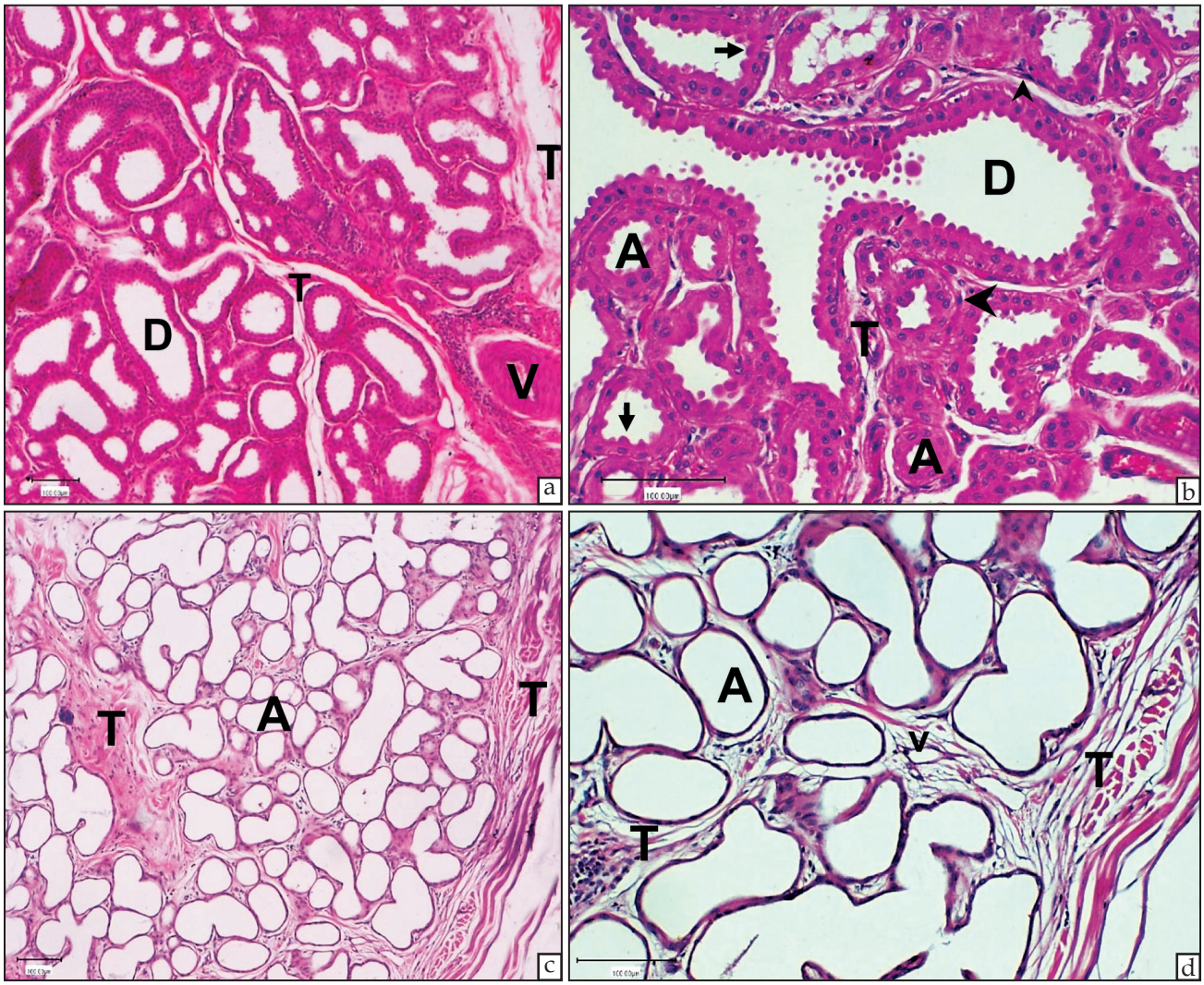

Fig 2. H & E-stained sections of camel poll gland. Fig a, b: show the glandular alveoli (A) in breeding season with apical blebs (Arrows) and myoepithelial cells (Arrowheads); note the high alveolar epithelium, narrow lumina and thin inter-alveolar and inter-lobular connective tissue (T); note the intra- and inter-lobular ducts (D) and blood vessels (V). Fig 2c,d show the glandular alveoli (A) in non-breeding season with low alveolar epithelium, wide lumina, and thick inter-tubular connective tissue (T). Scale bars: 100 µm.

and December, the poll gland lobules are separated by thin strands of connective tissue and the alveoli and intra-lobular ducts are taller and lumina are narrower than those during non-reproductive period (Tingari *et al,* 1984b). However, it has been stated that no considerably decrease was found in the connective tissue of active poll glands and the main structural difference between the active and inactive poll gland resides in the epithelial heights of their secretory units (Taha and Abdalla, 1980). Further, the poll gland secretory phase is characterised by narrow lumina and tall epithelial cells of alveoli and intra-lobular ducts (Tingari *et al,* 1984b).

The present study showed positive immunoreactivity of S-100 in the epithelial lining and connective tissue of the poll gland. A number of studies indicated S-100 localisation in the mammalian testis (Haimoto *et al,* 1987; Amselgruber *et al,* 1992; Amselgruber *et al,* 1994; Cruzana *et al,* 2003; Alkafafy *et al,* 2016), epididymis (Ibrahim *et al,* 2017; Cruzana *et al,* 2003) and efferent ductules (Ibrahim, 2015). The intensity of S-100 reaction in the current study increased during breeding season, especially in supra-nuclear region and apical protruding blebs of the alveolar epithelium. More obvious S-100 binding sites in the supra-nuclear region and apical blebs have also been reported during the rutting season in camel poll gland (Ebada *et al,* 2012). S-100 reaction in this study was also increased in the myoepithelial cells, glandular stroma and blood vessels during breeding

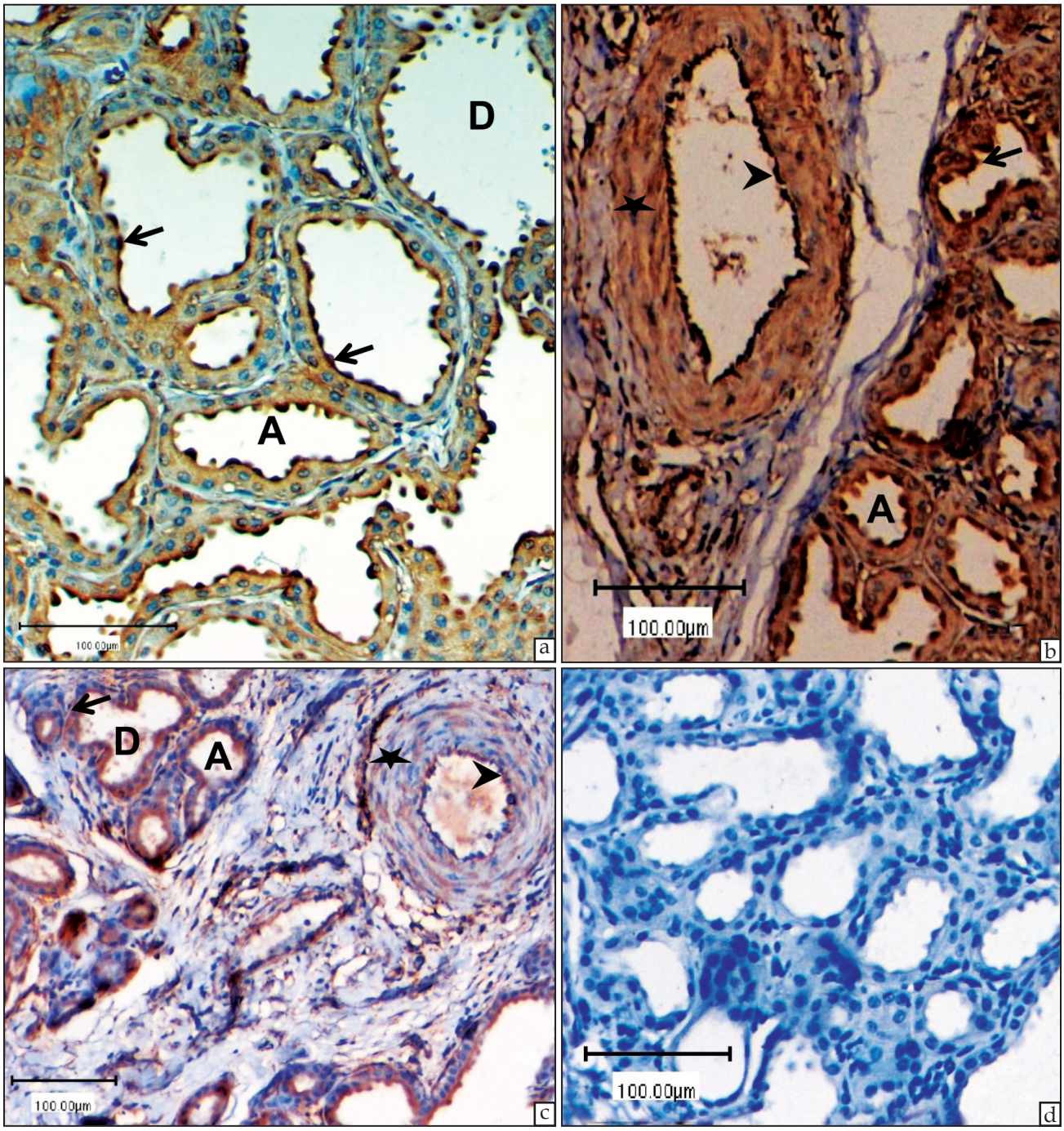

Fig 3. S-100 immuno-stained sections of camel poll gland. In breeding season (Fig 3a, b) there is strong S-100 immuno-reaction in the apical epithelium of alveoli (A) and ducts (D) (Arrows) as well as blood vessel endothelium (Arrowheads); note the blood vessels tunica media (Stars) with moderate S-100 immuno-reaction. In non-breeding season (Fig 3c) S-100 immuno-reaction shows less intense in the alveolar (A) and ductal (D) epithelium; weak to negative reaction is shown in blood vessels tunics media (Stars). Fig 3d represents the negative S-100 reaction in the control sections from breeding and non-breeding seasons. (Scale bars: 100 µm).

season than in non-breeding season. According to Ebada *et al* (2012) variable S-100 immune-reaction was observed in the glandular myoepithelial cells and peri-alveolar connective tissue, whereas weak to moderate reaction appeared in the inter-alveolar connective tissue in camel poll glands. Similarly, Kahn *et al* (1985) recorded positive S-100 immunoreaction in the myoepithelial cells of normal salivary glands using immunoperoxidase and immunofluorescence methods. Moreover, a positive reactivity of S-100

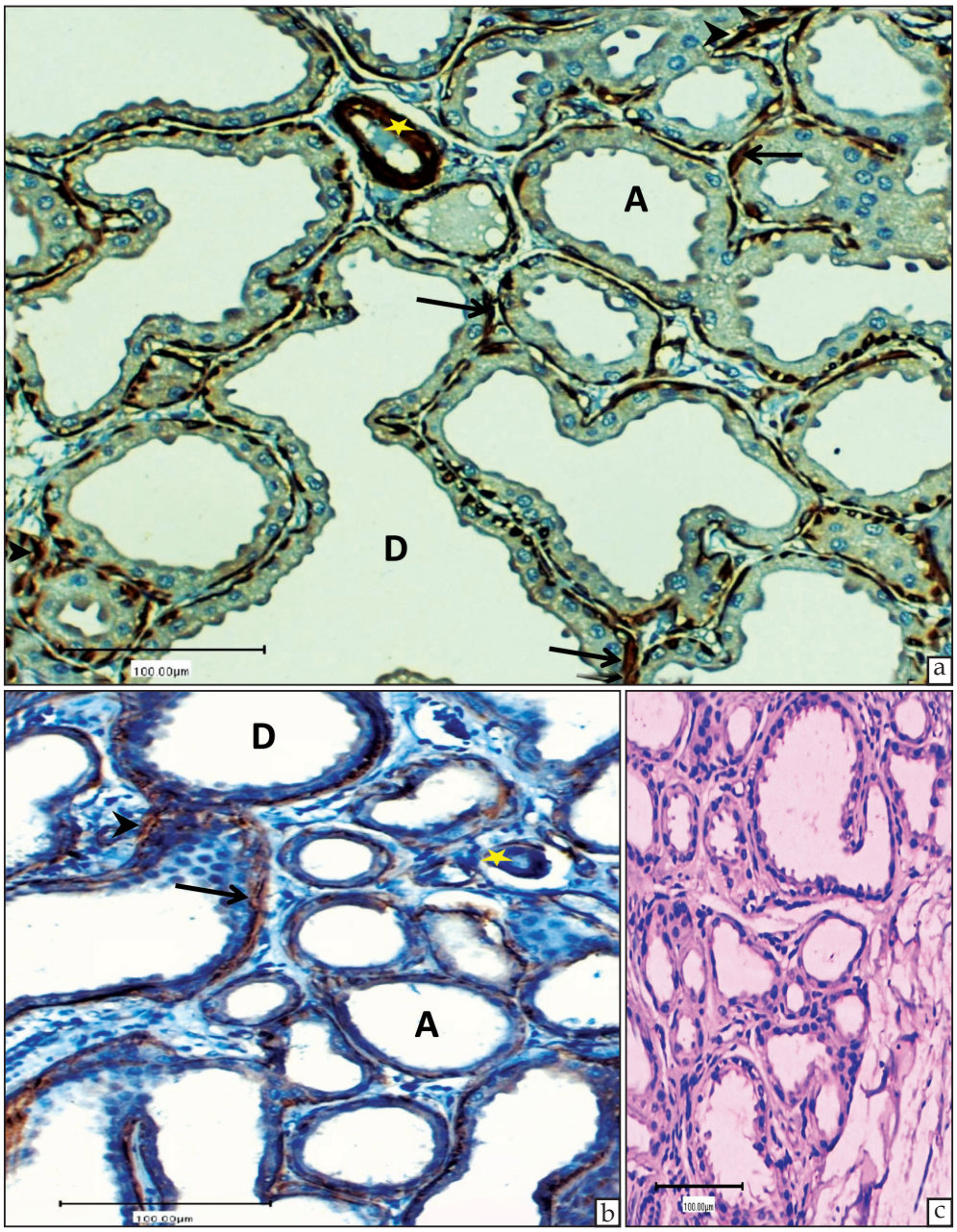

Fig 4. α-SMA immuno-stained sections of camel poll gland. In breeding season (Fig 4a) the glandular myoepithelial cells (Arrows), inter-alveolar smooth muscles (Arrowheads) and tunica media of blood vessels (Stars) show strong α-SMA immuno-reaction than those in non-breeding seasons (Fig 4b). The connective tissue (T) and epithelium of alveoli (A) and ducts (D) show negative α-SMA in both seasons. Negative control sections do not react with α-SMA in both seasons Fig 4c). (Scale bars: 100 µm).

in myoepithelial cells has also been expressed in canine sweat glands. According to Heizmann *et al* (2002) S-100 is a multifunctional protein with many intracellular and extracellular functions including motility, enzymatic activity, energy metabolism, chemotaxis, neurite extension and secretion. It has also been mentioned that S-100 protein might play a role in the absorptive and secretory activities in the testis (Amselgruber *et al,* 1994; Cruzana *et al,* 2003). Moreover, S-100 protein is considered to improve the secretory and absorptive functions in the epithelial cells and to improve smooth muscle contractility in the epididymis of rutting camels (Ibrahim *et al,* 2017). Similarly, increased S-100 immunoreaction in the poll gland epithelial cells in the breeding season observed in this study might indicate increased secretory and absorptive functions. Furthermore, increased intensity of S-100 protein immunoreactions

in the glandular myoepithelial cells and tunica media of blood vessels during breeding season recorded in this study could also indicate their increased muscular contractility. Additionally, it has been stated that positive immunoreactivity to S-100 protein in the epididymal vascular endothelium of Egyptian water buffalo indicates its contribution to the processes of transcytosis (Alkafafy *et al,* 2011b). Therefore, it could also be suggested that the transcytosis process is increased in the poll glands during breeding season as there was increased S-100 immunoreactivity in their vascular tissue.

In the present study, there was positive α-SMA immunoreaction in the myoepithelial cells and tunica media of inter-alveolar and interlobular blood vessels of poll glands. The reaction was stronger in the breeding season than in non-breeding season, especially in the myoepithelial cells. The study, however, showed negative α-SMA immunoreactivity in the epithelial cells and weak to negative reaction in the inter-alveolar connective tissue throughout the entire annual reproductive cycle. Positive α-SMA reaction in the myoepithelial cells and inter-alveolar blood vessels of camel poll glands has also been stated earlier (Ebada *et al,* 2012). Most myoepithelial cells of camel poll glands have been reported to exhibit positive α-SMA reaction (Atoji *et al*, 1998). Localisation of α-SMA has also been reported in myoepithelial cells of mammary gland of bovine (Haaksma *et al,* 2011) and human (Zancanaro *et al,* 1999) as well as in rat sweat gland (Gugliotta *et al,*1988) and apocrine tubules of Japanese serow (Atoji *et al,*1995).

According to Sato *et al* (1989), myoepithelial cells are proved to be involved in cellular contraction which results in rapid sweat excretion on stimulation of sweat glands; they added that increased luminal hydrostatic pressure in the walls of sweat glands secretory units can be relieved by the mechanical support of myoepithelial cells. These authors report that myoepithelial cells are capable of producing both nitric oxide (probably induces relaxation) and acetylcholine which induces contraction. Therefore, increased intensity of immunoreactivity of myoepithelial cells to both S-100 and α-SMA during breeding season could be an indication of increased cellular activity and contractility. They might also play a role in conduction of cellular secretion from the secretory units of poll glands to the lumen and then to the outer surface via the hair follicles. Increased contractility during breeding season has also been reported in the smooth muscle of camel epididymis (Ibrahim and Singh, 2014) and efferent ductules (Ibrahim, 2015).

Based on the morphological and immunohistochemical variations, it could be concluded that the camel poll glands are active throughout the year, though their activity increases during cold months of winter and decreases in hot months of summer; this indicates a correlation with the glandular secretion and the male camel sexual activity. Further, the S-100 and α-SMA proteins are suggested to be involved in the control of secretory activities of the poll gland.

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