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IMMUNOHISTOCHEMICAL STUDIES ON THE DROMEDARY CAMEL (*Camelus dromedarius*) MAMMARY GLAND DURING LACTATION AND NON-LACTATION PERIODS

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ABSTRACT

The current study was undertaken to evaluate the validity of immunohistochemistry in the proportional labeling of the diverse components of the lactating and non-lactating mammary gland in the camel (*Camelus dromedarius*). Paraffin-embedded sections of lactating and non-lactating mammary glands were stained by conventional and histochemical techniques. Primary antibodies against S100, alpha smooth muscle actin (α-SMA) and cytokeratin (Ck) were applied on paraffin sections. The spatial distribution of different proteins in the diverse compartments of lactating and non-lactating mammary tissue displayed a variable immunoreactivity (IR). The luminal epithelial cells showed binding sites only for S100 and Ck 8. The myoepithelial cells exhibited either a consistent IR (α-SMA) or variable IR (Ck 5 and S100). In conclusion, the intensity and distribution pattern of all proteins in the lactating gland are greater than in the non-lactating one. The functional relevance of the findings is interpreted. S100 and Ck proteins participate in secretory activities and in maintaining cellular integrity of luminal cells during lactation and non-lactation phases, respectively. Immunolocalisation of α-SMA highlights the contractile capacities of myoepithelial cells reflecting their contractile function, Ck preserves their structural and physiological integrity at different phases, whereas the S100-IR displayed by them, especially during non-lactation phase, may lend support to the notion that myoepithelial cells provide a regenerative potential of the mammary epithelium.

Key words: Camel, immunohistochemistry, involution, lactation, mammary gland

The dromedary (one-humped) camel is a domesticated multipurpose animal, used for its supply of milk, meat, hides and for transport (Farah, 2004).

Abundant literature on the histology and histochemistry of the mammary glands in humans (Welsch *et al,* 2007) and various animal species (Monterio-Riviere, 1998; Welsch *et al,* 1998; Hellmén and Isaksson, 1997; Li *et al,* 2006; Patel *et al,* 2007; Adriance *et al,* 2005; Faraldo *et al,* 2006; Veltmaat *et al,* 2003; Sun *et al,* 2010; Alkafafy *et al,* 2012) are available but those related to camels are scarce. Available literature shows studies on histological structure (Nosier, 1974) and morphometric characteristics under different physiological conditions (Kausar *et al,* 2001) of mammary gland of dromedary camels. However, histochemical characteristics of the camel mammary gland are lacking. Present study was aimed to examine the comparative immunohistochemical labeling of various cellular components of the lactating and non-lactating camel mammary gland and to determine the structural-functional relationships.

Materials and Methods

Animals and tissues

Mammary gland tissues were collected from five lactating and five non-lactating apparently healthy camel (*Camelus dromedarius*) immediately after slaughter at a slaughterhouse in Zagazig, Egypt.

Histology

Small specimens from the mammary glands were fixed in Bouin's solution for 24 hours, then washed and preserved in 70% ethanol. The samples were dehydrated in ascending grades of ethanol, cleared in xylene and embedded in Paraplast wax (Sigma-Aldrich, St. Louis, MO, USA) and sectioned

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at 5µm thickness. Tissue sections were mounted on positively charged and coated slides (Thermo Scientific, Menzel-Gläser GmbH, Braunschweig, Germany). Sections were stained with iron Haematoxylin and Eosin and Crossman's Trichrome stains. Processing and staining methods were detailed by Bancroft *et al* (1996).

Immunohistochemistry

Dewaxed and rehydrated sections of mammary glands were subjected to inactivation of endogenous peroxidases by incubation in 1% hydrogen peroxide (H_2O_2) for 15 minutes. Then the sections were placed in 0.01 mol/L citrate buffer (pH 6) and heated in a microwave oven at 750 W for two cycles of 7 minutes each for antigen retrieval. After the sections were allowed to cool to room temperature for 20 minutes, they were rinsed in phosphate buffered saline (PBS) containing bovine serum albumin (pH 7.6) for 5 minutes to block non-specific binding sites. Subsequently, the sections were incubated with the specific primary antibody (types, sources and dilutions are shown in Table 1) for 1 h in a humidified chamber at room temperature. The sections were then washed with PBS for 5 minutes 3 times and incubated with biotinylated secondary antibodies (types, sources and dilutions are shown in Table 1) for 30 minutes at room temperature, followed by incubation with peroxidase-labeled streptavidin for 15 minutes. Immunolocalisation of bound antibodies was visualised by incubation of the sections with 3,3'diaminobenzidine (DAB) reagent (Sigma-Aldrich, St. Louis, MO, USA). The sections were counterstained with Mayer's haematoxylin, dehydrated and mounted with DPX (Sigma-Aldrich, Munich, Germany). The methods of processing and immunohistochemical staining were adopted after Kumar and Rudbeck (2009).

Positive and negative controls

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.

Labeling assessment and photomicrography

For assessment of the immunolabelling a semi-quantitative subjective scoring was performed by three independent observers. Digital photomicrographs for general histology and immunohistochemistry were taken using an imaging system consisting of a Leica DM LB light microscope (Leica Microsystems, Wetzlar, Germany) and digital camera (Leica EC3, Leica Microsystems).

Results

Histological findings

Sections of the lactating gland showed variably shaped lobules. The lobules consisted of tubulo-alveolar secretory units draining into small intralobular ducts, which leave the lobule and open into a large interlobular duct. The alveoli were tightly packed within a lobule with little inter-alveolar connective tissues (Fig 1).

Sections of non-lactating gland, either stained with haematoxylin and eosin or with trichrome stain, showed islets of glandular parenchyma within abundant connective tissue. Higher magnification of these parenchymal islets displayed some ducts and reduced alveoli with or without lumina, enclosed by some myoepithelial cells. Alveoli consisted of randomly aggregated luminal epithelial cells at the center and peripheral myoepithelial cells (Fig 2).

Immunohistochemical findings

The main immunohistochemical findings are summarised in Table 2.

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

Antibodies Against	Primary antibodies				Secondary antibodies		
	Type	Origin	Source	Dilution	Type	Source	Dilution
α-SMA	Monoclonal anti-human SMA, clone 1A4 (M0851)	Mouse	Dako Cytomation, Glostrup, Denmark	1:50	Polyclonal Rabbit Anti-Mouse Biotinylated Igs (E 0464)	Dako Cytomation, Glostrup, Denmark	1:300
Ck	Monoclonal anti-human cytokeratin, clone MNF116 (M 0821)	Mouse	Dako Cytomation, Glostrup, Denmark	1:100	Polyclonal Rabbit Anti-Mouse Biotinylated Igs (E 0464)	Dako Cytomation, Glostrup, Denmark	1:300
S100	Polyclonal anti-S100 (Z 0311)	Rabbit	Dako Cytomation, Glostrup, Denmark	1:400	Polyclonal Swine Anti-Rabbit Biotinylated Igs (E 0353)	Dako Cytomation, Glostrup, Denmark	1:300

S100

Sections of lactating camel mammary gland displayed a strong S100-IR in the luminal alveolar epithelium, but not in the surrounding myoepithelial cells. On the other hand, sections of non-lactating gland showed weakly reactive luminal epithelium in both alveoli and ducts. However, variable IR varied from negative to weak staining was displayed by myoepithelial cells enclosing alveoli and ducts, respectively. The blood vessels within the interstitium were negative (Fig 3).

α-SMA

Sections of lactating gland showed strong α-SMA-IR in the myoepithelial cells surrounding the alveoli and excretory ducts and negative luminal alveolar and ductal epithelium. Sections of non-lactating camel mammary glands displayed distinct α-SMA-binding sites in the myoepithelial cells surrounding the secretory units, but not in those enclosing the excretory ducts or in the luminal epithelium. The vascular SMCs exhibited strong IR (Fig 4).

Ck

In the current study we used a broad spectrum anti-keratin reagents (Monoclonal anti-human cytokeratin, clone MNF116), capable of reacting with intermediate and low molecular weight keratins. Thus, it offers the reactivity with Ck5 (in myoepithelial cells) and Ck8 (in luminal cells). Sections of lactating gland showed diffuse Ck-IR in some luminal epithelial cells lining the alveoli and the ducts or on their intercellular and apical cell membranes. Myoepithelial cells displayed variable IR ranging from weak to moderate staining. Immunostained sections of non-lactating glands displayed moderate Ck-IR in myoepithelial cells surrounding the secretory units, which are mostly lined by cells presenting weak to moderate IR. Some luminal cells showed diffuse weak to moderate immunostaining, while only the apical surfaces of some others exhibited strong reactivity (Fig 5).

Discussion

The histological structure of the lactating camel mammary gland agrees with previous studies on different species (Monterio-Riviere, 1998; Welsch *et al,* 1998; Kausar *et al,* 2001; Patel *et al,* 2007; Alkafafy *et al,* 2012). The non-lactating camel mammary gland showed scattered islets of glandular parenchyma embedded within abundant connective tissue. The parenchymal islets displayed reduced alveoli with or without lumina. Similar findings were reported in non-lactating mammary glands from buffaloes (Patel *et al,* 2007) and camels (Kausar *et al,* 2001), where the number of alveoli was greatly reduced as a result of the proliferation of connective tissue stroma in the inter-alveolar spaces (Patel *et al,* 2007).

In accordance with previous immunohisto-chemical studies on mammary, salivary, eccrine sweat glands and camel poll gland (Haimoto *et al,* 1987; Alkafafy *et al,* 2012; Ebada *et al,* 2012), S100 was localised at high levels in exocrine cells of lactating camel mammary glands. The family of S100 proteins belongs to the EF-hand Ca^{2+}-binding proteins, which are involved in a wide range of cell functions including exocrine secretion (Schafer and Heizmann, 1996; Heizmann *et al,* 2002; Cruzana *et al,* 2003; Ebada *et al,* 2012). In general, the presence of S100 protein in mammary glands may indicate its potential role in the regulation of their secretory function. On the other hand, the exocrine cells from non-lactating glands displayed relatively reduced S100-IR. This reduction in IR included both intensity and frequency of binding sites. However, the persistence of S100-IR in the luminal cells might be of functional significance as indicated from the findings reported by Welch

Table 2. Immunolocalisation of different proteins in the camel mammary gland.

Proteins	Lactating						Non-lactating					
	Parenchyma			Interstitium			Parenchyma			Interstitium		
	AE	DE	Myo	CT	Blood VE	Vessel SMCs	AE	DE	Myo	CT	Blood VE	Vessel SMCs
S100	+++	+	–	–	–	–	–/+	–/+	–/+*	–	–	–
α-SMA	–	–	+++	–	–	+++	–	–	–/++**	–	–	+++
CK	+++	++	+/++	–	–	–	+/++	+/++	++	–	–	–

Alveolar epithelium (AE); ductular epithelium (DE); myoepithelium (Myo); connective tissue (CT); vascular endothelium (VE); smooth muscle cells (SMCs). Negative (–); weak (+); moderate (++); weak to moderate (+/++) and strong (+++) reaction.

* Variable S100-IR in myoepithelial cells bordering the alveoli (+) and ducts (–) in non-lactating gland.

** Variable α-SMA-IR in myoepithelial cells bordering the alveoli (++) and ducts (–) in non-lactating gland.

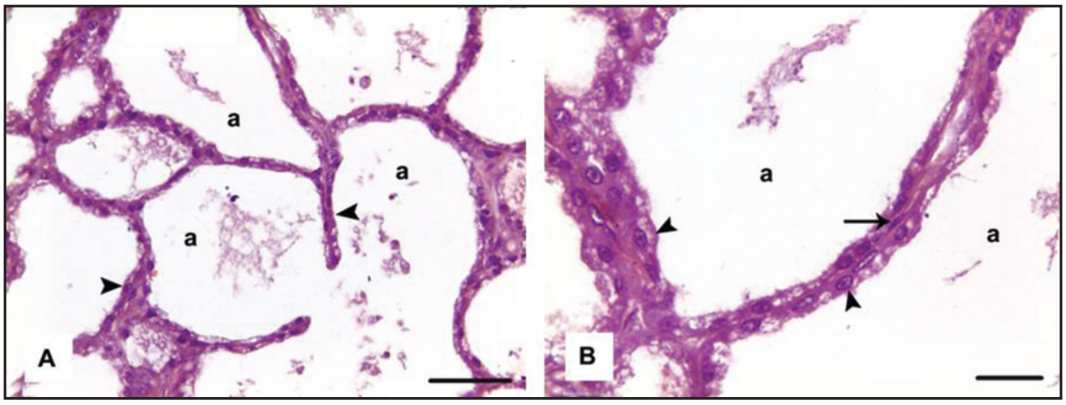

Fig 1. **A,** H & E stained section of lactating camel mammary gland showing the wide glandular alveoli (a) distended with milk, and lined by alveolar epithelium (arrowheads). **B,** Higher magnification of some alveoli (a) lined by alveolar epithelium consisted of luminal cells (arrowheads) and basal myoepithelial cells (arrow) Scale bars = 50 µm (A) and 20 µm (B).

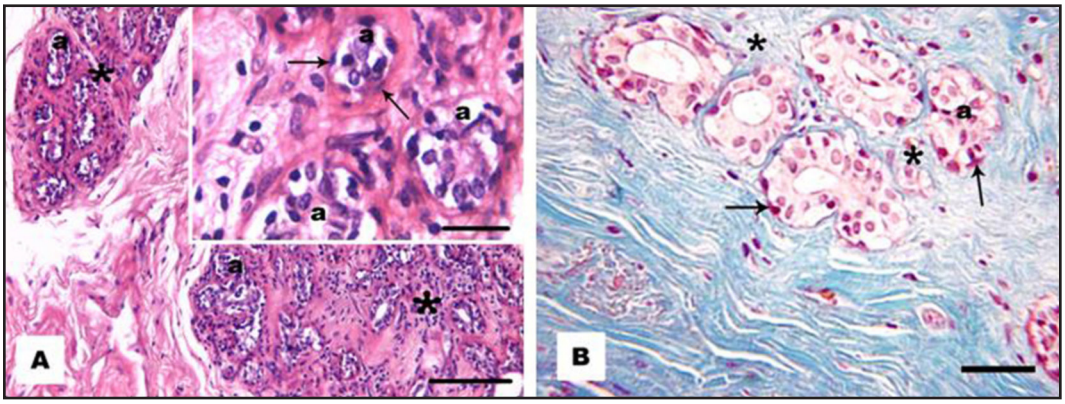

Fig 2. **A,** H & E stained section of non-lactating camel mammary gland showing islets of glandular parenchyma (asterisks) within abundant connective tissue. The inset shows a higher magnification of a part of a parenchymal islet displaying reduced alveoli (a) with or without a lumen. Alveoli consist of randomly aggregated luminal epithelial cells and myoepithelial cells (arrows) **B,** trichrome-stained section of non-lactating gland showing isolated cluster of glandular parenchyma where the ducts and alveoli (a) were enclosed by some myoepithelial cells (arrows). The parenchymal structures were separated from each other by abundance of connective tissue (asterisks). Scale bars = 100 µm (A) and 20 µm (in the inset and in B).

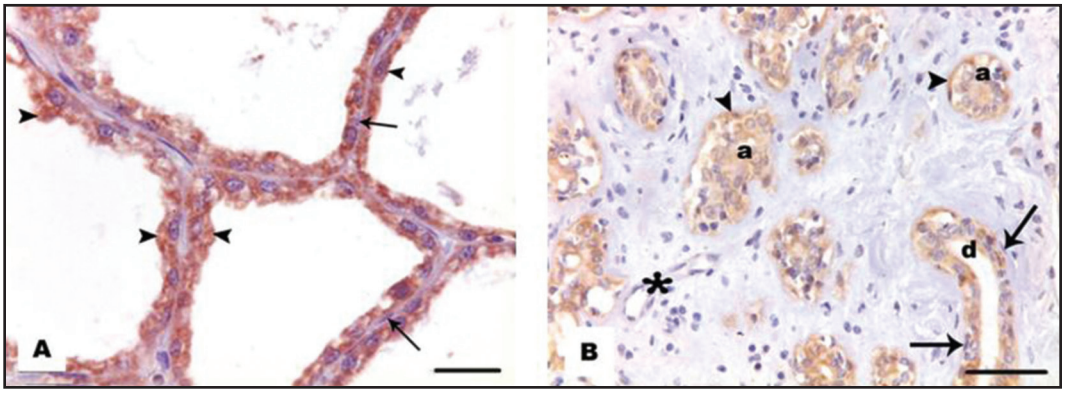

Fig 3. **A,** Photomicrograph of S100-stained section of lactating camel mammary gland displaying a strong S100-IR in the luminal alveolar epithelium (arrowheads) but not in the basal myoepithelial cells (arrows). **B,** S100-stained section of non-lactating gland showing weakly reactive luminal epithelium both in alveoli (a) and ducts (d). Variable IR varied from negative (arrows) to weak (arrowheads) was displayed by myoepithelial cells enclosing ducts and alveoli, respectively. The blood vessel (asterisk) within the interstitium was negative. Scale bar = 20 µm (A) and 50 µm (B).

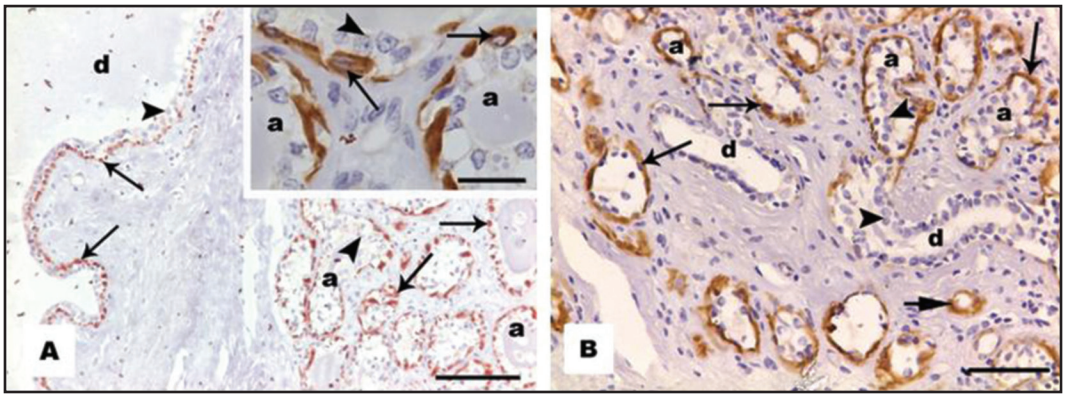

Fig 4. **A,** Photomicrograph of α-SMA-immunostained section of lactating camel mammary gland showing strong IR in the myoepithelial cells (arrows) surrounding the alveoli (a) and excretory ducts (d) and negative luminal alveolar and ductal epithelium (arrowheads). **B,** α-SMA-immunostained section of non-lactating camel mammary gland displaying distinct α-SMA-binding sites in the myoepithelial cells (arrows) surrounding the secretory units (a) but not in those enclosing the excretory ducts (d) or in the luminal epithelium (arrowheads). The vascular SMCs (longhead arrow) exhibited strong IR. Scale bar = 100 μm (A), 20 μm (inset, A) and 50 μm (B).

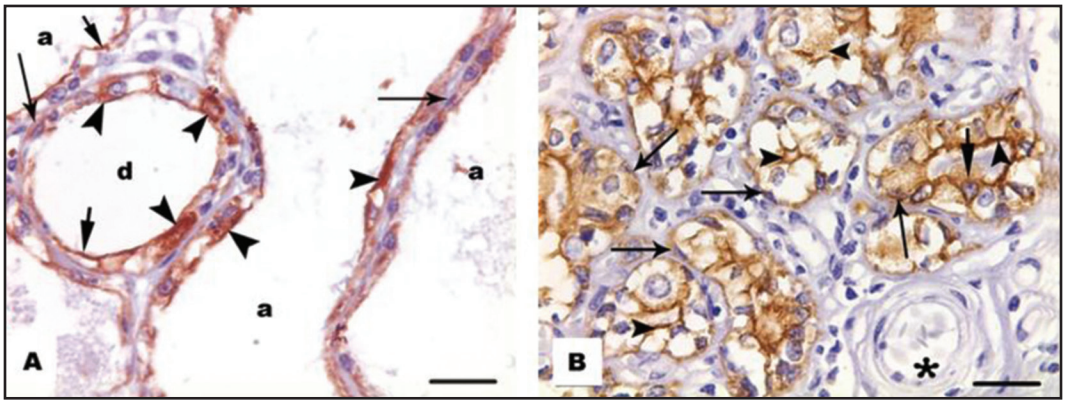

Fig 5. **A,** Photomicrograph of Ck-immunostained section of lactating camel mammary gland showing diffuse IR in some luminal epithelial cells (arrowheads) lining alveoli (a) and ducts (d) or in their intercellular and apical cell membranes (longhead arrows). Myoepithelial cells (arrows) displayed variable IR ranged from weak to moderate. **B,** Ck-immunostained section of non-lactating camel mammary gland displaying moderate Ck-IR in myoepithelial cells (arrows) surrounding the secretory units, which are mostly lined by cells exhibiting weak to moderate IR. Some luminal cells (longhead arrow) showed diffuse weak to moderate immunostaining, while only the apical surfaces (arrowheads) of the other some exhibited strong reactivity. Blood vessels (asterisk) within the inter-alveolar connective tissue failed to exhibit Ck-IR. Scale bar = 20 μm (A and B).

et al (2007) in non-lactating human mammary glands, which are thought to secrete a number of antimicrobial peptides. Additionally, the bovine psoriasin, a member of the family of S100 proteins, exhibits antibacterial activity suggesting a significant role in the local host defense mechanisms in the mammary gland (Regenhard *et al,* 2010).

Though S100 protein was considered as a specific marker for myoepithelial cells, it was immunolocalised equivocally in myoepithelial and epithelial cells of the canine mammary gland (Möller and Hellmén, 1994). Thus it was concluded that S100 is not a specific marker for myoepithelial cells. Similar to previous results reported in human sweat glands (Noda *et al,* 1988) and in lactating bovine mammary glands (Alkafafy *et al,* 2012), the myoepithelial cells of the lactating camel mammary gland failed to display S100-IR, lending support to this conclusion. In contrast with the current observations, the myoepithelial cells in dog sweat glands (Ferrer *et al,* 1990) and camel poll glands (Ebada *et al,* 2012) showed positive immunolocalisation with the anti-protein S100. On the other hand, myoepithelial cells in the non-lactating gland, in the current work, displayed variable IR varied from negative (in ducts) to weak (in alveoli). The negative S100-IR in the myoepithelium enclosing the ducts may be explained on the basis of transient inactivity and reduction in contractility (i.e. no excretion), as supported by the absence of immunoreaction for α-SMA in the

periductal myoepithelial cells (Fig 4 B). On the other hand, the existence of S00-IR in perialveolar myoepithelial cells, despite its weakness, might reflect phenotypic changes relevant to the certain physiological activities. It is worth mentioning that myoepithelial cells may actively participate in mammary morphogenesis through influencing the proliferation, survival and differentiation of luminal cells, and modulating stromal-epithelial interactions (Faraldo *et al,* 2005), and thus provide a regenerative potential of the mammary epithelium (Moumen *et al,* 2011).

Several cytokeratins are lineage markers within the mammary epithelium. Among these, both Ck5 and Ck14 are found to be localised in myoepithelial cells, whereas Ck8/Ck18 expression were localised in luminal cells of mouse mammary glands (Mikaelian *et al,* 2006). Similar findings were established in a previous work on human mammary glands (Sommers *et al,* 1989). In the current study we used a broad spectrum anti-keratin reagents (Monoclonal anti-human cytokeratin, clone MNF116), capable of reacting with intermediate and low-molecular-weight keratins. These included Cks 5, 6, 8, 17 and 19. Thus, it offers the reactivity with Ck5 (in myoepithelial cells) and Ck8 (in luminal cells) as reported in the present findings, in lactating and non-lactating glands. However, the labelling showed remarkable variation both in intensity and in frequency of binding sites. The myoepithelial cells bordering the lactating alveoli and ducts displayed more distinct Ck-IR than did those surrounding the non-lactating gland. This reduced reactivity seems to be related to temporary phenotypic changes coinciding with the gland inactivity (Silberstein *et al,* 1992; Deugnier *et al,* 1995). Similarly, the luminal cells lining the alveoli and ducts of lactating gland exhibited more intense IR than did those lining the non-lactating gland. Similar findings were reported by Alkafafy *et al* (2012) in lactating bovine mammary glands. The bovine mammary myoepithelium expressed a distinct Ck14-IR, while the luminal epithelia failed to display such immunostaining.

It is well known that Cks are of particular importance for epithelial flexibility to mechanical stress by forming a stable network, which is attached to specific cell-cell contacts of the desmosome type (Schmidt *et al,* 1994; Fuchs and Cleveland, 1998). At the same time they are dynamic structures to avoid interference with processes critical for tissue repair and homeostasis such as mitosis, stratification, migration and secretion (Windoffer and Leube, 1999). Additionally, disturbance of this network results in reduced tissue coherence and increased cell fragility (Windoffer and Leube, 1999). This might explain the present findings reported in luminal and myoepithelial cells in non-lactating gland that always maintained a moderate Ck-IR. This seems to preserve the isolated parenchymal clusters within the stromal dominance during periods of inactivity, ensuring initiation of a new cycle of activity in the next season.

It is worth noting that antisera against α-SMA and against other actin-associated contractile proteins have previously been used to identify myoepithelial cells in normal and pathological conditions (Bussolati, 1980; Skalli *et al,* 1986; Haaksma *et al,* 2011; Alkafafy *et al,* 2012; Ebada *et al,* 2012). In the current study, α-SMA was immunolocalised strongly in the perialveolar and the periductal myoepithelial cells in sections from lactating glands, and only in the perialveolar myoepithelial cells in sections from non-lactating glands. Distinct α-SMA-IR was also expressed by the vascular SMCs. These findings are in accordance with the reported localisation of α-SMA in mice (Haaksma *et al,* 2011) and cows (Alkafafy *et al,* 2012) mammary glands and poll glands in male camels (Ebada *et al,* 2012). On the other hand, the failure of the periductal myoepithelial cells in non-lactating glands to α-SMA-IR might be attributed to certain phenotypic changes (Deugnier *et al,* 1995) occurring during transient physiological states of gland inactivity. The phenotypic changes of myoepithelial cells may be controlled by extrinsic factors such as the extracellular matrix, hormones, and growth factors which accumulate in the periductal extracellular matrix of the mammary gland (Silberstein *et al,* 1992; Deugnier *et al,* 1995).

In conclusion, the spatial distribution of different proteins in the diverse compartments of lactating and non-lactating mammary tissue displayed a variable immunoreactivity. Overall, the intensity and distribution pattern of all proteins investigated were greater in the lactating gland than in the non-lactating one. The luminal epithelial cells revealed binding sites only for S100 and Ck, reflecting their participation in secretory activities and in maintaining cellular integrity during lactation and non-lactation phases, respectively. The myoepithelial cells exhibited either a consistent IR (α-SMA) or a variable IR (Ck and S100). Immunolocalisation of α-SMA emphasises the contractile capacities of myoepithelial cells, Ck preserves their structural and physiological integrity at different physiological phases, whereas the S100-IR displayed by them,

especially during non-lactation phase, may lend support to the notion that myoepithelial cells provide a regenerative potential of the mammary epithelium.

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