HISTOLOGICAL FEATURES AND MUC1 DISTRIBUTION IN THE PALPEBRAL CONJUNCTIVA OF THE DROMEDARY CAMEL (*CAMELUS DROMEDARIUS*)

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ABSTRACT

The conjunctiva is important adnexa of the eye that maintains the stability of the tear film. The objective of current study is to describe the histological features of palpebral conjunctiva of the dromedary, emphasizing the distribution of Muc1 using indirect tissue immunofluorescence technique. Conjunctival samples of ten adult, apparently healthy dromedary camels of both sexes were collected, fixed, embedded in paraffin and stained with H&E for general histological structure, Masson´s trichrome for fibrous tissue, Periodic acid–Schiff (PAS) for goblet cells and immunofluorescence for the distribution of Muc1. The palpebral conjunctiva of the dromedary camel is lined with stratified squamous to stratified columnar epithelium. Large numbers of goblet cells were identified associated with conjunctival epithelial cells. The lamina propria consisted of loose superficial cell layer and a deeper tarsal plate. Within the lamina propria, there were lymphoid follicles, numerous tarsal glands and palpebral seromucoid glands. The palpebral conjunctiva and associated palpebral glands were stained positive for PAS and Muc1. In conclusions, the eye of the dromedary camel is protected with a palpebral conjunctival tissue that is richly populated with PAS positive cells and numerous Muc1-producing cells.

Key Words: *Palpebral, Conjunctiva, Camel, Histology, Immunofluoresence, Muc1.*

INTRODUCTION

The conjunctiva is the mucous membrane of the ocular surface and composed of non-keratinized squamous epithelium with goblet cells interspersed between the epithelial cells (Banks, 1993; Aughey and Frye, 2001; Samuelson, 2007; Gillan, 2008). Additionally, the abundant blood supply of the conjunctiva delivers protective substances, such as antibodies, complement, and white blood cells to the eye to combat infections and remove dead or damaged tissue (Nichols, 1996). The exposure to the open environment also makes it susceptible to several pathological conditions (Fahmy, 2003; Sandikci *et al.,* 2005). The tear film mucous layer on the ocular surface consists of several high molecular weight glycoconjugates including mucins, which are secreted mainly by the conjunctival goblet cells and is essential for normal vision. This layer plays an important role in protecting the ocular surface from exogenous agents and provides lubrication (Ríos *et al.,* 2000; Sugiura *et al.,* 2010, Wizert *et al.,* 2014).

It is well-established that the stratified squamous epithelia of the human cornea and conjunctiva normally express Mucl, the membrane-spanning mucin, as one of the ocular surface mucins. Muc1 has a protective effect for the conjunctival tissue (Srinivasan *et al.,* 2013). Genetically engineered Muc1 null mice curiously display a patent propensity for development of blepharitis and conjunctivitis (Inatomi *et al.,* 1995; Kardon *et al.,* 1999).To our knowledge Muc1 expression was not investigated in the conjunctival tissue of the camel. Therefore, the objective of the current study was to describe the histological characteristic of the dromedary conjunctiva and to evaluate conjunctival expression of Muc1 by immunofluorescence methods.

MATERIALS and METHODS

The materials used for this study, were collected from 10 adult apparently healthy dromedary camels of both sexes (4 males and 6 females) at Alomran Slaughter House, Al-Ahsa, Saudi Arabia. These animals were derived from herds maintained in open yards outside Al-Ahsa Sheep and Camel Market, about 10 kilometers outside the city´s metropolitan area. Prior to slaughtering, they were housed in the market yards for several days after coming from their natural habitat in the desert. Immediately after slaughtering, the globes and ocular adnexa were collected and immersed in 10% neutral buffered formaldehyde solution. Fixed tissues were further processed byroutine paraffin-embedment techniques, cut into 5 µm sections, stained with hematoxylin and eosin (H&E), Masson's trichrome orperiodic acid Schiff

stain (PAS) and examined with light microscopy according to Bancroft and Cook (1994).

For immunofluorescence stainingparaffin-embedded tissues were sectioned (5 µm thick) and mounted on positively charged slides (Superfrost®, Thermo Scientific Portsmouth, New Hampshire, USA). Tissue sections were then cleared with xylene (2x10minutes), hydrated with ethanol and followed by PBS. The tissues were then blocked with goat serum for at least 1 hour at room temperature followed by incubation with a primary antibody (Rabbit polyclonal anti-Human Muc1 antibody, ab79226, Abcam, Cambridge, MA 02139-1517 USA) diluted in a blockingsolution (1/100) overnight at 4°C. The slides were then washed, 3X5 minutes each, and incubated in a darkenvironment with goat polyclonal secondary antibody (Rabbit IgG - H&L, FITC, ab6717, Abcam, 1/1000) for 1 hour at room temperature. The tissue slides were then washed with PBS (3x5 minutes), mounted and stored at 4°C in a dark environment until examination. Histological photos were obtained under bright field for conventional histological stained sections (BX 41 microscope coupled with a DP-12 camera, Olympus Corp., Tokyo, Japan) and under dark field for antibody linked immunofluorescence stained sections (DM6000-Bmicroscope with a fluorescence axis coupled with a DEC-420, Leica Microsystems, Germany).For negative controls, primary antibodies were substituted with PBS.

RESULTS

Conjunctival epithelium

The palpebral conjunctiva of the dromedary camel was lined with epithelia that varied from stratified squamous to stratified columnar. Goblet cells were present in all forms of epitheliaexcept over the lymphoidfollicles, where the epithelium is transformed to low stratified squamous. PAS-stained sections showed numerous dispersed goblet cells among the epithelium. Immunostaining with anti-Muc1 antibodies showed strong immunofluorescence reaction (Fig. 1).

Laminapropriamucosae

The lamina propria mucosa of the palpebral conjunctiva was composed of two layers, a superficial loose layer and a deep fibrous one. This connective tissue layer accommodates lymphatic follicles, tarsal and palpebral glands. The camel eyelids have an ill-developed tarsus at the center of the eyelid that is infiltrated by some bundles of connective tissue. This layer was very weak or negative for Muc1 immunostaining (Fig. 2).

Lymphoid follicles

On the palpebral surface of the eyelids, there were severallymphoid follicles, specifically near the medial canthus. These follicles morphologically appeared as basophilic patches of a single follicle or of a group of two or more follicles (Fig. 3, a). The epithelium over these follicles was low stratified and lacks goblet cells (Fig.3, b). Immunostaining of these follicles showed a very strong reaction to Muc1 (Fig.3, c).

Tarsal and palpebral glands

Embedded within the tarsus, numbers of large multilobular sebaceous-like tarsal glands were observed. The cells at the periphery of sebaceous adenomeres had more acidophilic cytoplasm than that observed at thecenter. In addition, the latter cells were vacuolated and showed some nuclear pyknosis. These glands showed weak immunofluorescence activity for Muc1 than thoseof mature adenomeres, which were negative (Fig. 4).

Seromucoid palpebral glands were also present in the eyelid of the dromedary. These glands appeared as isolated lobules or as aggregations of 3-6 lobules of variable sizes. These lobules were embedded within connective tissue and their ducts opened into the palpebral conjunctiva. The cells of these glands were pyramidal in shape and had spherical nuclei, found next to the base and their cytoplasm showed granular PAS-positive contents. On the other hand, cell of the intercalary and interlobular ducts were PAS-positive only at the apical portions of their cytoplasm. These glands also showed positive immunoreactivity to Muc-1, but less strong as that seen in the conjunctival epithelium (Fig. 5).

Third Eyelid

The bulbar surface of the third eyelidcontained some PAS-positive cells and demonstrated more intense reaction for Muc1 immunostaining, while the palpebral surface lacks PAS-positive cells and showed less Muc1-immunoreactivity (Fig. 6).

Figure Legends

Fig. 1:

Histological features and immunofluorescence for Muc1 of the palpebral epithelium. (a) Stratified squamous epithelium, H&E; bar = 50 µm (b) Stratified columnar epithelium, H&E; bar = 20 µm (c) Additional stratified squamous epithelium, Masson´s trichrome; bar = 50 µm (d) Stratified columnar epithelium showing the intensity of the goblet cells, PAS; bar = 50 µm (e) Goblet cells; PAS; bar = 50 µm (f) Intense immunoreactivity against Muc1 in the epithelial lining of the palpebral membrane; bar = 100 µm.

Fig. 2:

Histological features and immunofluorescence for Muc1 of the palpebral connective tissue. (a) Superficial layer (1) and tarsal body (2). Part of the tarsal gland is shown (arrow), Masson´s trichrome; bar = 100 µm (b) Immunofluorescence for Muc1 at the conjunctiva showing intense immunoreactivity at the epithelium (arrow) while the connective tissues underneath were negative for Muc1; bar = 200 µm.

Fig. 3:
Conjunctival lymphoid follicles (a) Two follicles in the lamina propria of the conjunctiva, H&E; bar = 200 µm (b) The epithelial lining of the lymphatic follicles is low stratified epithelium (arrow), lacks goblet cells while the goblet cells (arrowheads) are shown on the epithelium on either side of the follicles, PAS; bar = 100 µm (c) Immunofluorescence anti-Muc1 was weak in the epithelium while the cells in the lymphoid follicles were positive for the stain; bar = 200 µm.

Fig. 4:
Histological and immunofluorescence images of the tarsal glands. (a) Superficial, PAS; bar = 200 µm (b) Superficial, Muc1; bar = 200 µm (c) Deep, PAS; bar = 200 µm (d) Deep, Muc1. Insertion in (c) showing early stage, right, and late stage, left, of the holocrine secretion; bar = 200 µm.

Fig. 5:
Hitological and immunofluorescence staining for the palpebral glands. (a) Palpebral seromucoid glands formed from several lobules, the main duct opens at the palpebral surface (insertion), PAS (4x); X40 (b) One lobe of the palpebral gland showing some intralobular ducts (arrows) and a higher magnification of one of the adenomeres (insertion); PAS; bar = 200 µm (c) Muc1 immunofluorescence in the palpebral gland, general view showing part of the conjunctival epithelium (arrow); bar = 200 µm (d) A higher magnification of the palpebral gland showing the low intensity of the immunoreaction for Muc1 in the adenomeres (arrow) while the ducts were intensely stained (arrowhead); bar = 200 µm.

Fig. 6:
Histologic and immunofluoresence of the third eyelid. (a) Third eyelid stained with PAS, goblet cells (arrowhead) can be observed at the bulbar surface (arrow);bar = 200 µm (b) Third eyelid stained with Muc1 immunofluoresence; notice that bulbar surface (arrow) was positive for stain while the palpebral surface showed very low immunoreactivity. In this section, part of the cartilage of the third eyelid is shown (CT); bar = 200 µm.

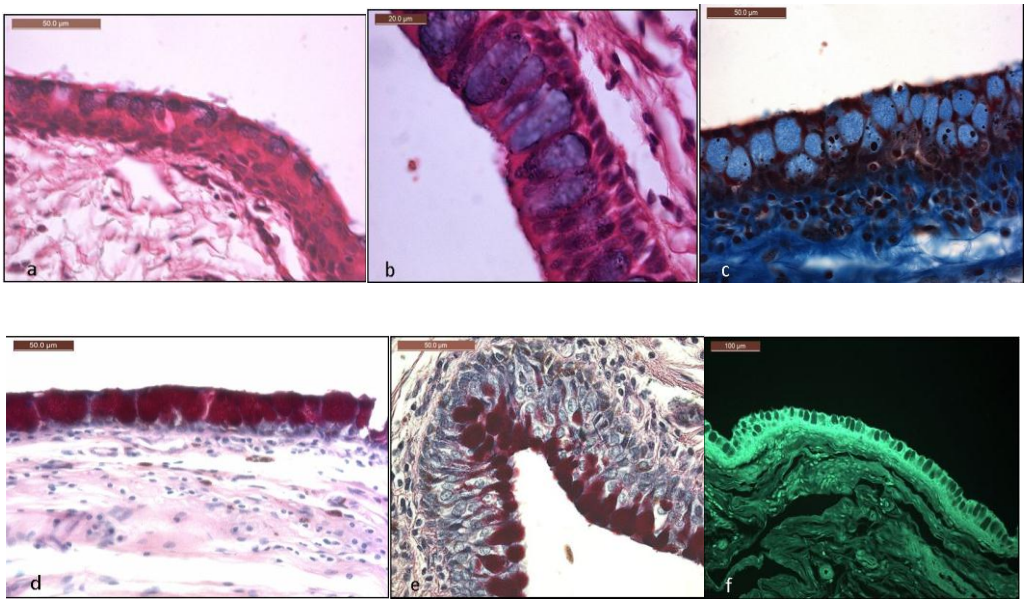


Fig. 1.

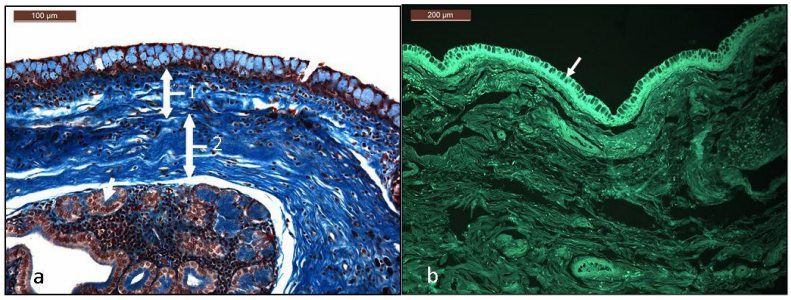


Fig. 2.

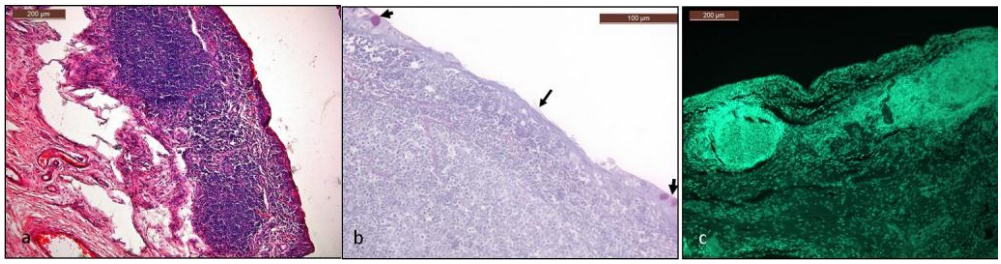
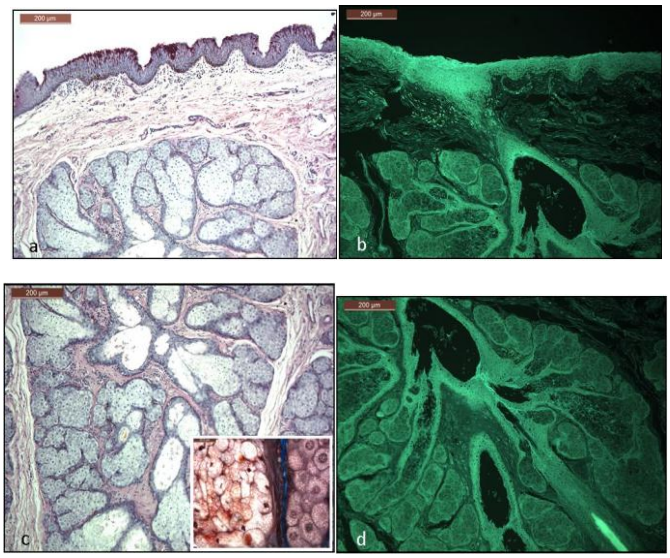


Fig. 3.



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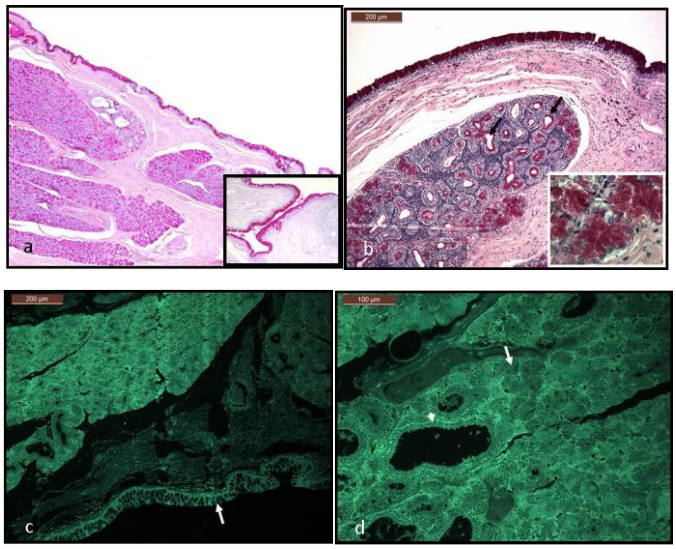

Fig. 5.

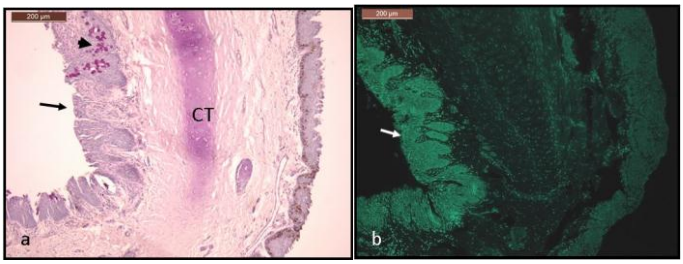

Fig 6.

DISCUSSION

The dromedary camel lives in a habitat of extreme temperature and moisture conditions, which constantly challenges its conjunctival tissue. This study focused on the histological features of the palpebral conjunctiva of the dromedary camel. Furthermore, the previous immnunofluorescence studies showed the distribution of a well-known, mucosa-associated anti-adhesion molecule, Muc1.

In the dromedary camel palpebral conjunctiva, the lamina epithelialis mucosae vary from stratified squamous to stratified columnar. Variation in the conjunctival epithelium was reported in other domestic animal species (Banks, 1993; Samuelson, 2007).In the horses, astratified columnar to cuboidal epithelium was observed on nearly the entire surface of the conjunctiva, and a stratified squamous-type epithelium was present at the palpebral and bulbar edges (Bourges-Abella *et al.,* 2007). Different types of palpebral conjunctival epithelial linings also have been reported in sheep. In the latter species, the lining epithelium may reached 12 layers of stratified squamous near the limbus of the eyelid, while close to the fornix of the eyelid, it is quite variable with cells appearing columnar in some areas and cuboidal in others (Getty, 1975).

PAS staining showed that the palpebral conjunctiva in the dromedary was intensely covered with goblet cells. In other domestic animals, the goblet cells are most numerous at the fornix (Samuelson, 2007). In rodents, the distribution of goblet cells within the palpebral conjunctiva is variable. For instance, in guinea pigs, goblet cells were reported to occur as solitary cells, and the highest density was detected on the palpebral conjunctiva. In gerbil, however, goblet cells were observed either as solitary or in clusters, but their distribution was detected throughout the entire conjunctiva (Voigt *et al.,* 2012). In general, the reduction of goblet cells on lymphonodulareareas was common to every mammal investigated sofar (Samuelson, 2007; Voigt *et al.,* 2012).

In the present study, the lamina epithelialis of palpebral conjunctiva showed very strong Muc1 expression. Similarly, but less intensely, Muc1 was also expressed by the palpebral glands. In addition, this study showed that these glands were also PAS-positive. The PAS and immunofluorescence results of the present study suggest that besides the goblet cells, the palpebral glands may also contribute to the amount of Muc1 present on the conjunctival epithelium of the dromedary camel. The low intensity of Muc1 immunostaining might be due to cross reactivity between Muc1 and other secretory mucins, which is a possibility since at least four out of the 20 mucin gene products, Muc1, Muc2, Muc4 and Muc5AC, were detected in the tear film of the dog (Royle *et al.,* 2008).

Mucins are a group of glycoproteins with a high molecular weight that contain tandem repeats of amino acids rich in serine and threonine, which serve as sites for O-glycosylation. Up to 80% of the mass of mucin molecule can be made up of O-glycans. Two types of mucins are known: secreted and membrane-associated; both are present on the apical side of wet-surfaced epithelia (Blalock *et al.,* 2007). In fact, mucins form the macromolecular scaffolding of the mucus gel of the tear film. Composition of this gel reflects the competing needs of ocular surface, which are transparency, stability, hydration, and protection (Royle *et al.,* 2008). Of the membrane-associated mucins,Mucs 1, 3A, 3B, 4, 11, 12, 15, 16, 17, and 20, three Mucs (1, 4, and 16) have been identified as major membrane-associated mucin on the ocular surface (Govindarajan and Gipson, 2010). Among the membrane mucins, Muc1 is the most ubiquitously expressed. It is expressed by bothcorneal and conjunctival epithelia, as well as by the lacrimal gland and lacrimal duct epithelia (Govindarajan and Gipson, 2010). Interestingly, Round *et al.* (2002), studied the biophysical characteristics of ocular mucins and concluded that the mucosal gel maintains a barrier against the penetration of foreign bodies and, at the same time, must allow diffusion of molecules at the cell surface. This reported phenomenon might explain the basis for the conjunctiva being selected as a good route for some vaccinations as well as being a physical hindrance for other particles.

In the dromedary camel the lamina propria of the conjunctiva is composed of two layers, a superficial layer of loose connective tissue and a deeper fibrous layer. The lamina propria was negative for Muc1 immunofluorescence. This result was in accordance with previous literature, which describes Muc1 as a transmembrane protein that is expressed at the apical surface of many normal secretory epithelial cells but is not expressed in connective tissues (Julian *et al.,* 2009). The less-developed tarsal plateobserved in the dromedary camelof the present study is in accordance with that observationreported in other domestic animals, in which the tarsal plate is represented by a few bundles of collagen fibers (Banks 1993; Aughey and Frye, 2001).

Numerous tarsal glands were observed in the dromedary camel eyelid margins. These glands produce the lipid portion which forms the outermost layer of tear film, which acts as a hydrophobic barrier that preventing the evaporation of water from the tear film (Butovich *et al.,* 2012). These animals´ natural habitats typically receive low amounts of precipitation and possess high temperatures. Thus, it is conceivable that this observation is an evolutionary

ocular adaptation to its natural environment. Signs of nuclear pyknosis was noticed in the central adenomeres, which might be an indication of holocrine mechanism of secretionon the ocular surface. Various stages of cellular differentiation also were detected in the tarsal glands of cattle, human beings, primates and rabbits (Jester *et al.,* 1981; McMahon *et al.,* 2014).

In this study, a highly organizedconjunctival lymphoid follicles was detected which considered as part of the conjunctiva-associated lymphoid tissues (CALT). The overlying follicle-associated epithelium was distinct from the other epithelia that cover the palpebral conjunctiva, while the low stratified squamous epithelium was observed, goblet cells were absent from this area. In a comprehensive study, lymphoid follicles were detected in twelve animal species (Chodosh *et al.,* 1998). However, the existence of CALT in rats and mice has been contradictory. While some studies did not detect CALT in stimulated or unstimulated laboratory mice and rats, others have described lymphoid follicles in the third eyelid of mice (Steven and Gebert, 2009; Sakimoto *et al.,* 2002). CALTseems to play an important role in the protection of ocular surface by initiating and regulating immune responses (Steven and Gebert, 2009). The conjunctiva is constantly challenged by potential pathogens and harmful substances. Therefore, effective defense mechanisms are necessary to maintain the structural and functional integrity of this delicate tissue. In the present study, immunostaining of conjunctiva-associated lymphoid follicles was very strong to Muc1. Although, Muc1 was originally thought to be an exclusive feature of normal and tumor secretory epithelial cells, the expression of Muc1 was later reported on variety of normal and neoplastic hematopoietic cells (Fattorossi *et al.,* 2002). Various reports have shown that Muc1 can be detected in bone marrow, peripheral blood and lymph nodes. Muc1 expression was detected in plasma cells of the axillary lymph node while its expression in hematopoietic tissues was detectable by RT-PCR and Western blot (Zhong *et al.,* 2001; Dent *et al.,* 1999). There are also accumulating evidence that resting human T-cells express basal levels of Muc1, which was suggested to act as a negative regulator of T cell activation (Zhong *et al.,* 2001; Chang *et al.,* 2000).

Our results showed that goblet cells were distributed on the bulbar surface of the third eyelid. This was similar to our previous findings where some goblet cells were found interspersed among the epithelial cells that covered the bulbar surface, especially near the base of the third eyelid (Al-Ramadan and Ali 2012). Interestingly, goblet cells of the third eyelid of dogs and horses were found to perform a humoral immune response through the accumulation and transcytosis of IgA, which is produced by IgA-producing plasma cells. The latter cells were found to be densely populated in the subepithelial spaces (Schlegel *et al.,* 2003).

Our investigation described several morphological features of the ocular surface of the dromedary camel, with emphasis on the palpebral conjunctival tissue, including the third eyelid. It shows that the ocular surface of this species is richly populated by goblet cells and by several Muc1-producing cells, particularly the conjunctival epithelium and associated lymphoid tissue follicles. These features might be part of a robust protective mechanism of the dromedary´s ocular surface, which was probably a necessary evolutionary adaptation to exist in their natural environment.

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الخصائص النسيجية وتوزيع الميوسين-١ في الملتحمة الجفنية للجمل وحيد السنام

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الملتحمة هي لاحقة عينية مهمة تساعد على استقرار الطبقة الدمعية. تهدف الدراسة الحالية الى وصف السمات النسيجية في الملتحمة الجفنية للأبل العربية، مع التركيز على توزيع الميوسين-١ باستخدام التألق المناعي غير المباشر (الإميونوفلوريسنت). جمعت العينات المستخدمة في هذه الدراسة من عشرة إبل بالغة من كلا الجنسين، ثم تم تثبيت العينات وتضمينها وصبغها بصبغات الهيماتوكسولين- أيوسين لمعرفة البنية النسيجية العامة، وصبغة ماسون الثلاثية لتمييز الأنسجة الليفية وتفاعل شيف- حمض البريوديك للكشف عن الغليكوجين والسكريات المتعددة والتألق المناعي للكشف عن الميوسين-١. ظهرت الملتحمة الجفنية للأبل وحيدة السنام ببطانة ظهارية حرشفية مصففة أوعمودية مصففة. كما تم التعرف على اعداد كبيرة من الخلايا المخاطية موزعة بين الخلايا الظهارية. اما طبقة الصحيفة الرئيسية الملاصقة فتتكون من نسيج ضام رخو وفي العمق توجد الصفيحة الجفنية. وجد ايضا في طبقة الصحيفة الرئيسية جريبات ليمفاوية وغدد جفنية وغدد جفنية مصلية مخاطية. وقد إصطبغت الملتحمة الجفنية والغدد الجفنية بصبغتي الشيف- حمض البريوديك والميوسين-١. ونستنتج من هذه الدراسة ان عين الابل محمية بملتحمة جفنية غنية بالخلايا الكأسية، بالإضافة إلى العديد من الخلايا المنتجة للميوسين-١.