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MORPHOLOGICAL AND IMMUNOLOGICAL CHARACTERISTICS OF CONJUNCTIVA-ASSOCIATED LYMPHOID TISSUE IN BACTRIAN CAMELS (*Camelus bactrianus*)

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ABSTRACT

The morphological and immunological characteristics of conjunctiva-associated lymphoid tissue in bactrian camels (*Camelus bactrianus*) was studied in samples taken from complete conjunctival sacs (n=20) and observed by light microscopy, scanning electron microscopy, transmission electron microscopy, and immunohistochemistry. The CALT in the camel were formed by solitary or aggregate lymphoid follicle, few scattered lymphoid cells, which were mainly distributed in conjunctival fornix. The histological structure of secondary lymphoid follicle was consisted of follicle-associated epithelium (FAE), dome area (DA), follicular area (FA) and parafollicular area (PFA). FAE consisted of flattened associated epithelial cells, microfold cells (M cells) and intraepithelial lymphocytes. The germinal centres of the lymphoid follicles were comprised of lymphoblasts, follicular dendritic cells (FDC), few macrophages and plasma cells. The cytoplasm of these cells contained the usual organelles together with frequent vesicles. Prominent lymph vessels were always presented together with numerous high endothelial venules (HEV). B and T lymphocytes, macrophages were distributed in lymphoid follicle of CALT. The positive FDC were only found in germinal centres. These results suggested that CALT in camel had the cytologic basis of normal mucosa reaction. As an active component of the eye-associated lymphoid tissue in camel, the CALT were inductive sites for the common mucosal immune system, as well as an important components in ocular defense.

Key words: Bactrian camel, conjunctiva, histology, immunology, lymphoid tissue, morphology

The eye-associated lymphoid tissue (EALT) is consisted of conjunctiva-associated lymphoid tissue (CALT) and lacrimal drainage-associated lymphoid tissue, which is recognised as a component of the mucosal immune system (Knop and Knop, 2007). The mucosa associated lymphoid tissue (MALT) exists as two forms. One is 'organised' lymphoid tissue and the other is 'diffuse' lymphoid tissue (Kraehenbuhl and Neutra, 1992). Species-specific differences were observed in the components of CALT. Conjunctival lymphoid follicles have also been normally found in poultry and mammal, but individual and age of the animal or ocular health may be caused by the distribution and quantity of discrete lymphoid follicles (Chodosh and Kennedy, 2002; Chodosh *et al,* 1998b; Fix and Arp 1991a; Fix and Arp, 1991b). There are rare diffuse lymphatic cells in conjunctiva of rat and mouse under normal conditions (Chodosh *et al,* 1998b; McMaster *et al,* 1967; Setzer *et al,* 1987).

Comparative research in specific features of CALT of different animals might aid in better understanding the EALT pathology and immunology(Liebler-Tenorio and Pabst, 2006). Bactrian camels with special abilities and attributes live in the cold semi-deserts or even deserts regions of China and Mongolia (Jirimutu *et al,* 2012). In bactrian camels, morphological and immunological characteristics of gut-associated lymphatic tissue (GALT) (Qi *et al,* 2011; Wang, 2003; Xu *et al,* 2010; Zhang *et al,* 2012), nasopharynx-associated lymphoid tissue (NALT) (Yang and Wang, 2013a; Yang *et al,* 2011) and larynx-associated lymphoid tissue (LALT) (Yang and Wang, 2013b; Yang *et al,* 2010) have been described. No study until now has described the presence, topographical distribution, and organisation of bactrian camels CALT. The authors investigated in bactrian camels eyes with gross anatomy, histological and immunohistochemistry methods to gain insight into the basic comparative anatomy and histology, which will be contributed to future physiological and pathological studies.

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Materials and Methods

Complete conjunctival sacs (n=20), from 10 adult (5-10 years) healthy bactrian camels of either sexes were obtained from the slaughterhouse of the Right Alasan Banner Food Company in Inner Mongolia (autonomous region of China), after the animals had been killed by exsanguination for human consumption. All experimental procedures were approved by the welfare authority of the Right Alasan Banner of Inner Mongolia (autonomous region). The specimen were investigated by different techniques:

Light microscopy

Conjunctival sacs (n=6) were dissected free and flushed with normal saline. Samples for light microscopy (LM) were fixed in 10% formaldehyde for 72 h, dehydrated, cleared and embedded in paraffin. Embedded tissues were cut into 8-µm thick sections and stained with haematoxylin and eosin (H&E), Gomori reticulin stains for reticular and collagen fibres, toluidine blue for mast cells. Four conjunctival sacs were fixed in 2% acetic acid for 24 h to visualise the lymphoid follicles (Cocquyt *et al,* 2005; Cornes, 1965). The presence and distribution of lymphoid follicle were detected.

Scanning and transmission electron microscopy

Fresh tissues from conjunctival sacs (n=6) were thoroughly washed and processed for scanning and transmission electron microscopy (SEM and TEM) (Yang *et al,* 2009). Small pieces of the CALT from 3 conjunctival sacs for TEM were pre-fixed in 3% glutaraldehyde buffer (pH=7.2) for 3 h. The tissues were washed in 0.1 M phosphate buffer (3×30 min), cutted into 1 mm^3 pieces and post-fixed with osmium tetroxide for 1 h. After the samples were washed in 0.1 M phosphate buffer (3×30 min), then dehydrated in ascending grades of ethanol and embedded in epon 812. Semi-thin sections of each tissue were collected and stained with toluidine blue. Ultra-thin sections (50–70 nm) were then collected on copper grids. The ultra-thin sections were stained with a saturated solution of uranyl acetate for 30 min, followed by lead citrate for 7 min in a CO_2-free environment. Sections were then washed in CO_2-free water, dried and examined under a transmission electron microscope (JEOL, JEM-1230). Tissue samples for SEM were fixed as described above. After dehydration in an increasing alcohol series, samples were freeze-dried at vacuum and coated with platinum. The mounted specimens were observed under a scanning electron microscope (JEOL, JSM-680LA).

Immunohistochemistry

The fragments from conjunctival sacs (n=8) were fixed in 10% formaldehyde. After dehydration using an increasing alcohol solution series, the tissue was embedded in paraffin and made into a thin section about 8 µm in thickness with a 100 µm interval were made from all tissues using a microtome. The sections were deparaffinised and pre-treated with microwaving at medium power in 0.01 M sodium citrate buffer (pH 6.0). Then, the endogenous peroxidase was inhibited with 3% H_2O_2 in PBS for 30 min. Non-specific binding was blocked with 10% normal goat serum in PBS. Immunohistochemical markers were tested by CD20, CD3, CD68, and follicular dendritic cells (FDC) (monoclonal mouse anti-human antibodies from DAKO®). All primary antibodies were diluted 1:100 in 0.1 M phosphate buffer containing 0.5% Triton X-100 and incubated overnight at 4°C. Secondary antibodies were biotinylated goat anti-mouse IgG incubated for 3 h at room temperature. Visualisation was achieved by incubating with diaminobenzidine (DAB) and haematoxylin counterstaining. Negative control sections were performed in a similar manner, except the primary antibody was substituted with PBS. Images were captured using a digital camera (Leica DFC320). The positively reacting cells in different areas were counted with 400 × magnification in 3 different fields. Percentage of positive staining cells was assessed as follows; +++, >50% of cells stained; ++, 10–50% of cells stained; +, <10% were stained; -, negative.

Results

In the bactrian camel's unstained native conjunctival sacs, lymphoid follicles appeared as elevations with indefinite circumscription during macroscopic inspection and restricted to fornical zones in the upper and lower eyelids (Fig 1-A). Clear opaque white lymphoid follicles of different size, which formed prominent aggregations, were distributed in the similar zones of the conjunctival surface after fixation in 2% acetic acid for 24 h. The length and width of the aggregations were about 30 and 10 mm, respectively. There were 180 follicles with 3 mm in diameter in the aggregations (Fig 1-B). The size of the follicles was on the increase from conjunctival fornix towards the lacrimal punctum. Densely aggregated follicles resembled Peyer's patches (PP) in the intestine. Solitary follicles were occasionally observed outside the aggregations.

A large number of lymph follicles were observed in the lamina propria mucosae of the

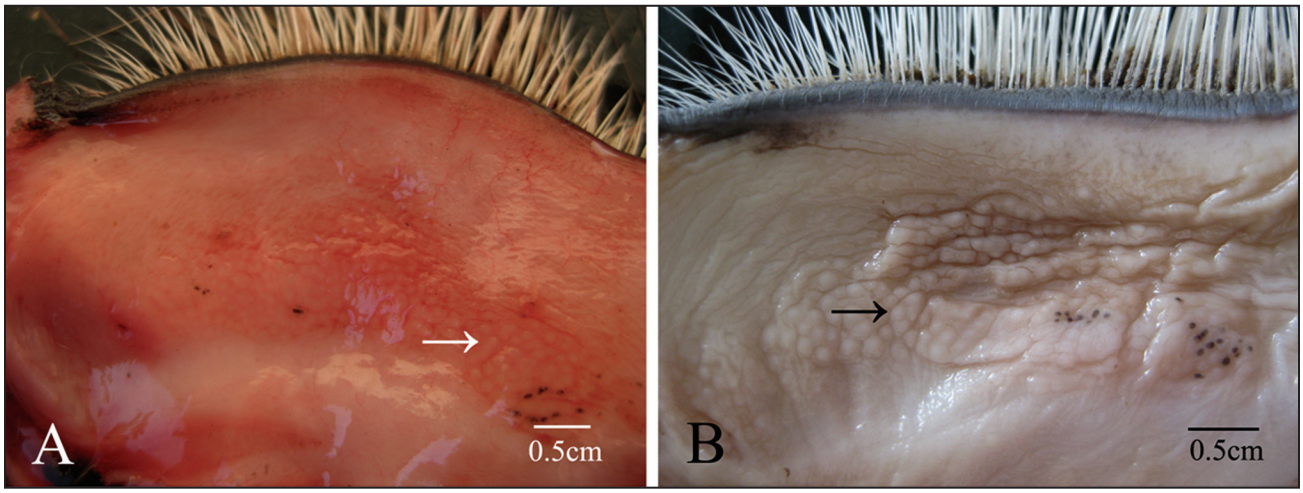

Fig 1. A. Macroscopic view of the camel's unstained native conjunctival tissues shows lymphoid follicles (arrow) with indefinite circumscription which are restricted to fornical zones. B. Opaque white lymphoid follicles (arrow) are distributed as over the similar zones of the conjunctival surface after fixation with acetic acid.

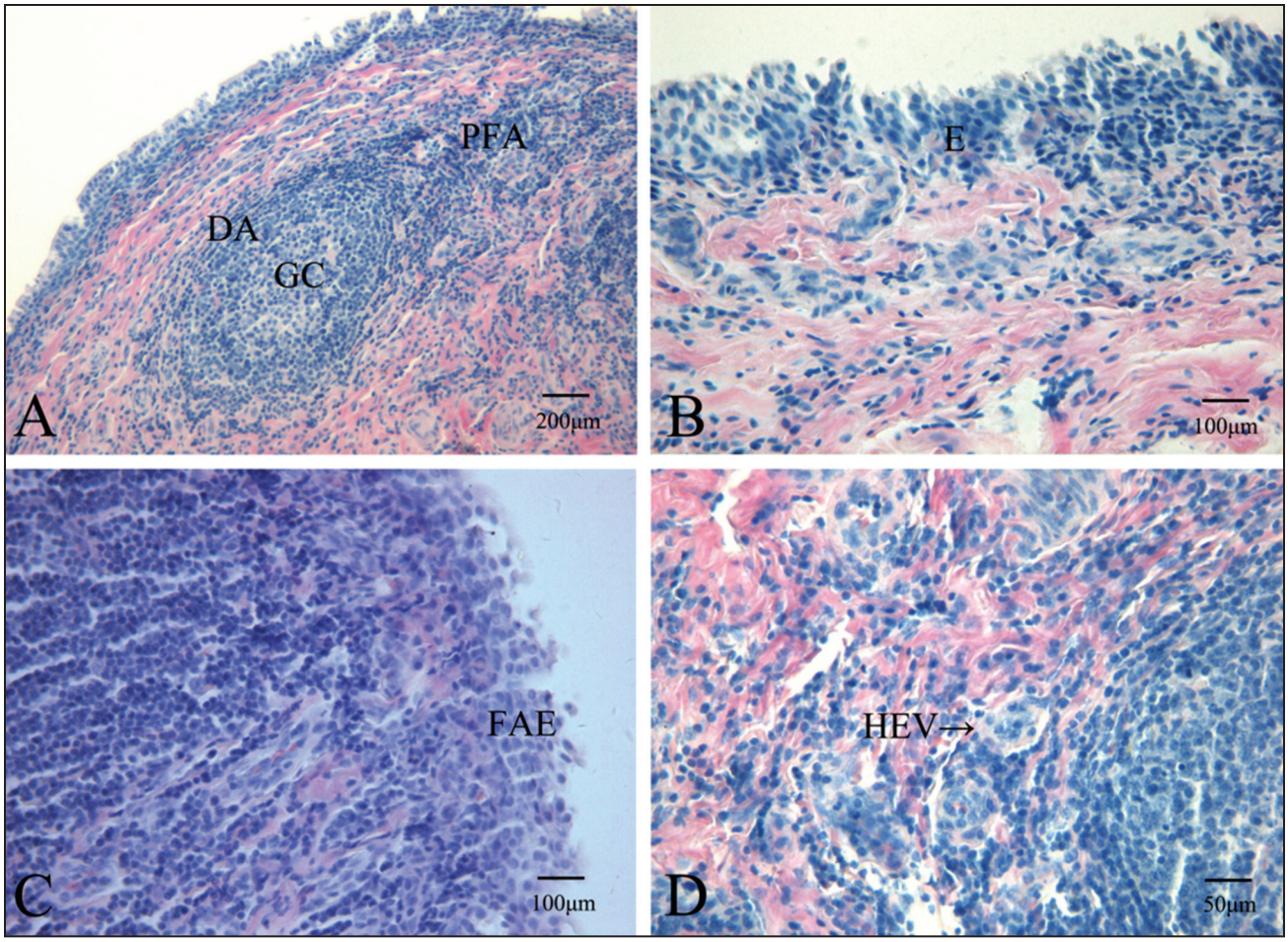

Fig 2. A-D. H&E stains showing microstructure of lymphoid follicle in conjunctiva. Follicle-associated epithelium (FAE); dome area (DA); parafollicular area (PFA); high endothelial venules (HEV); germinal centres (GC).

camel's conjunctiva. Most of the follicles belong to secondary lymphoid follicles. Numerous lymphocytes were diffusely located in the camel's normal conjunctiva. Secondary lymphoid follicle was comprised of 4 areas: follicular area (FA), parafollicular area (PFA), dome area (DA) and follicle-associated epithelium (FAE) (Fig 2-A). No goblet cells were observed in the FAE

which contained lymphocytes arranged in groups. Because of maximal lymphocyte infiltration into the epithelium, the epithelial basement membrane appeared discontinuous on the apex over each follicle (Fig 2-B). Subepithelial lamina propria mucosae were discontinuous, where the epithelium directly connected with lymphoid tissue (Fig 2-C). There were lymphocytes in the lumen of HEV. HEV mainly distributed in PFA. Few HEV were observed in the interfollicular areas (Fig 2-D).

The meshwork which was formed by reticular fibres and a few collagenous fibres was used to support each follicle. The shapes and sizes of the follicle were different (Fig 3-A). Only rare reticular fibres distributed in the germinal centres (Fig 3-C). The meshwork has more density in lymphocyte infiltrated epithelium than other area (Fig 3-B). Mast cells were observed in tunica mucosa of the conjunctiva.

Scanning electron microscopy

SEM images micrograph revealed aggregated prominent round to oval hemispheres on the surface of the camel's conjunctiva with occasional shallow depression (Fig 4-A). The oval hemispheres encircled by polygonal shape cells, and these surfaces were densely packed with numerous short microvilli (Fig 4-B). Flattened epithelial cells were covered by irregular microfolds and have prominent, raised junctions with adjacent cells (Figs 4-C and D). There were many small openings between flattened epithelial cells (Fig 4-E). Few bacillar bacteria are occasionally bound to the surface of CALT M cells in the scatter or clusters (Fig 4-F).

Transmission electron microscopy

The epithelial cells overlying the lymphoid follicles were often flattened, had short irregular microvilli, and were tightly connected to each other by junctional complexes containing desmosomes.

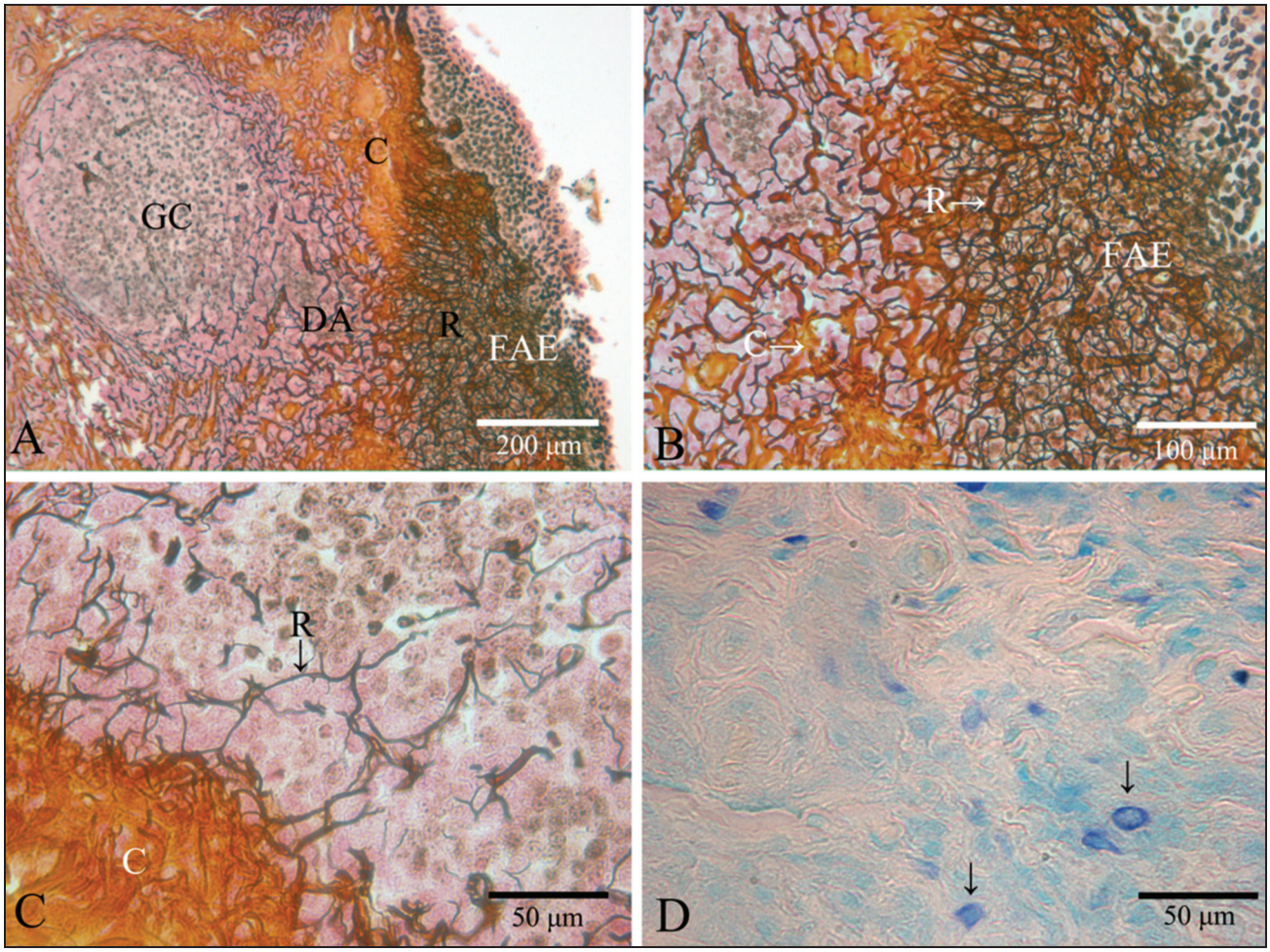

Fig 3. A-C. Gomori reticulin stains showing reticular (black) and collagen fibres (yellow-brown) fibres. Follicle-associated epithelium (FAE), dome area (DA) and parafollicular area (PFA) are supported by reticular (R) and collagen (C) fiber network in the mucosa of conjunctiva. A few reticular fibres are diffuse in the germinal centres (GC). D. Mast cell (→) stained with toluidine blue (TB) were observed in tunica mucosa of the conjunctiva.

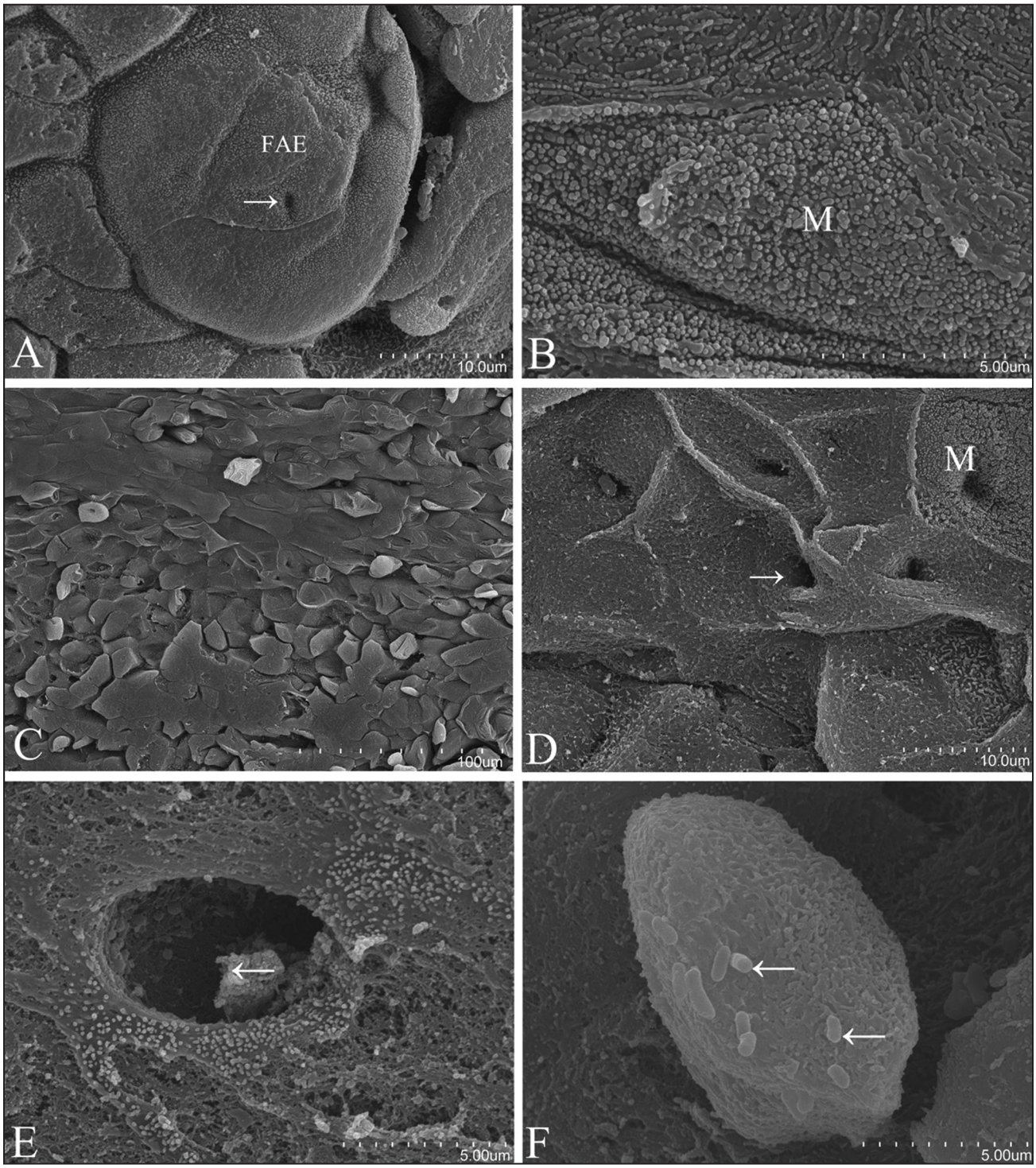

Fig 4. A. SEM images reveal aggregated prominent round to oval hemispheres on the surface of the camel's conjunctiva with occasional shallow depression (→). B. Regularly arranged microvilli on the surface of M cells. C-D. SEM images micrograph reveals the surface around the FAE. Flattened epithelial cells are covered with irregular microfolds and have prominent, raised junctions with adjacent cells. E. There are small openings between flattened epithelial cells. F. Few bacillar bacteria are occasional bound to the surface of CALT M cells in scatter or clusters.

TEM images of FAE showing small vesicles (→) occured beneath the outer cell membrane but inside the cell. Some short microvilli (m) were present at the surface (Fig 5-A). The apical surface of M cells was covered with blunt microvilli and microfolds. The dissociative ribosomes, oval or elongated mitochondria, even a spot of rough endoplasmic reticulum formed cisternae were free in the cytoplasm

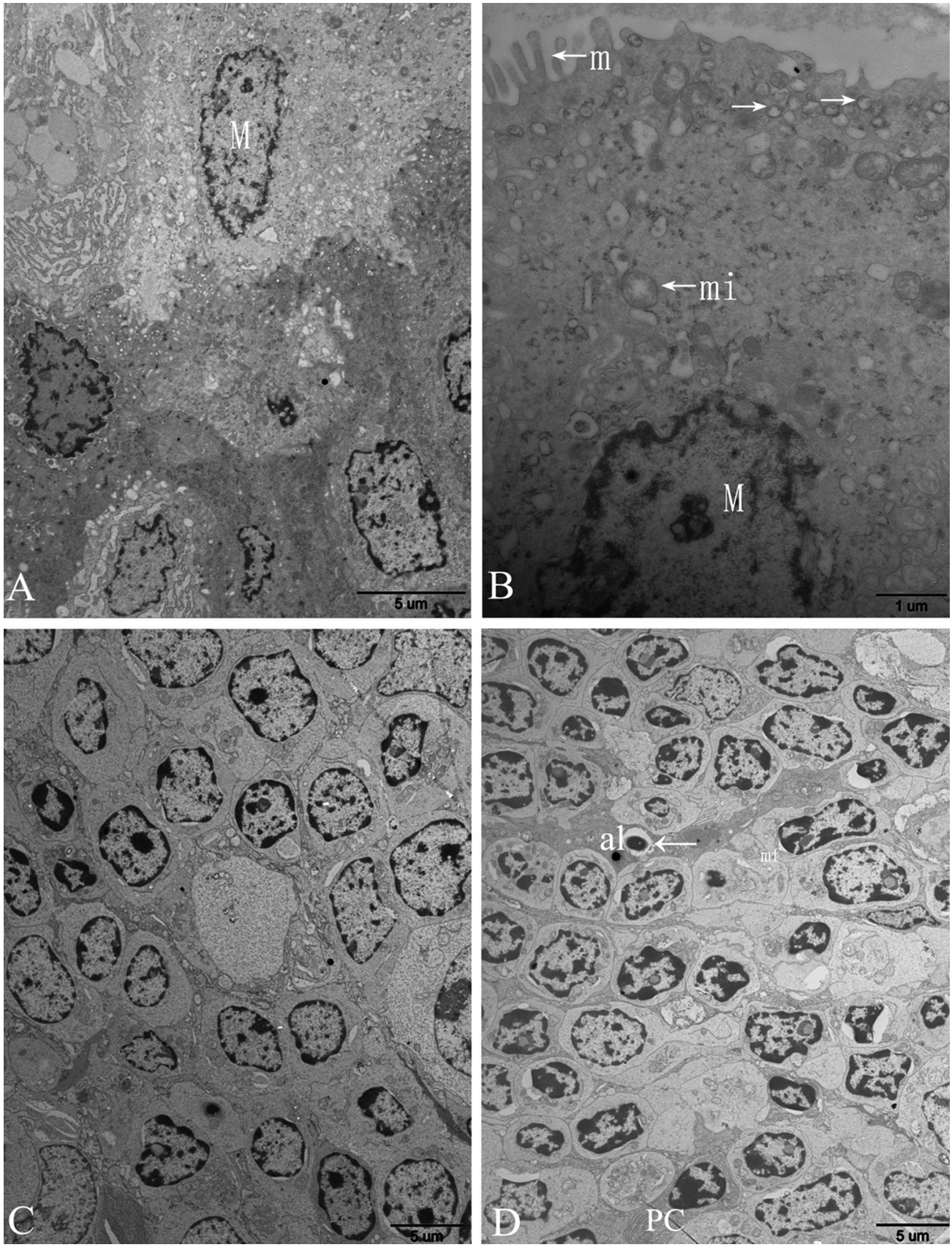

Fig 5. A and B. TEM images of FAE showing small vesicles (→) occur inside the M cells. Some short microvilli (m) are present at the surface. C. GC packed lymphoid cells. D. The nuclei of apoptotic lymphocytes are small and dark.

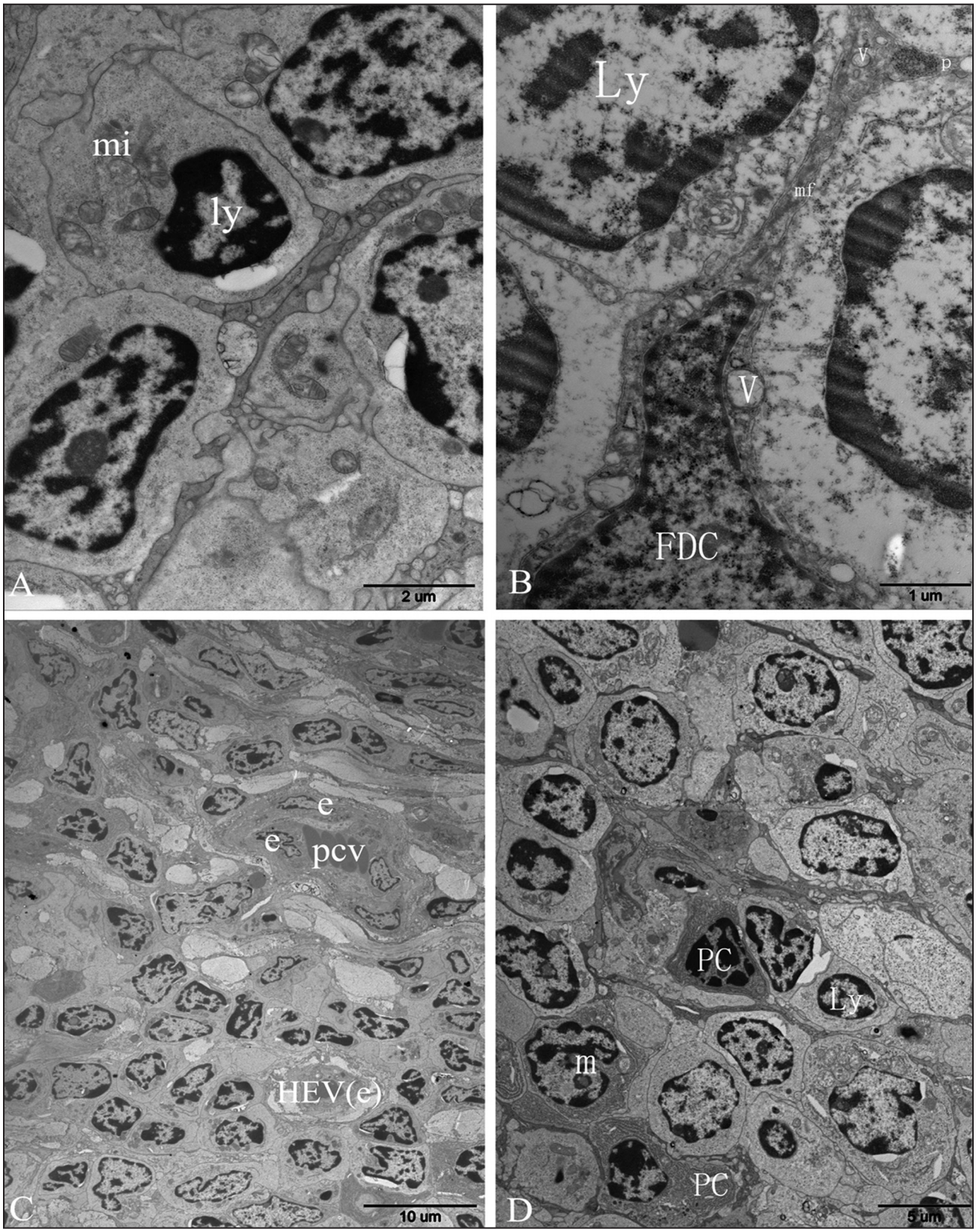

Fig 6. A. TEM micrograph of lymphoid cells (Ly) in the germinal centre. B.TEM images of the process of the FDC, closely adjacent to the lymphoblast, is seen to contain microfilaments (mf), mitochondria (mi), multiple vesicles (v), the process (p) of the FDC.C. TEM images of an ordinary post-capillary venule (pcv), the endothelial cells (e) and high endothelial venules (HEV) in parafollicular area (PFA). D. TEM images of PFA showing Ly, plasma cell (PC) and macrophage (m).

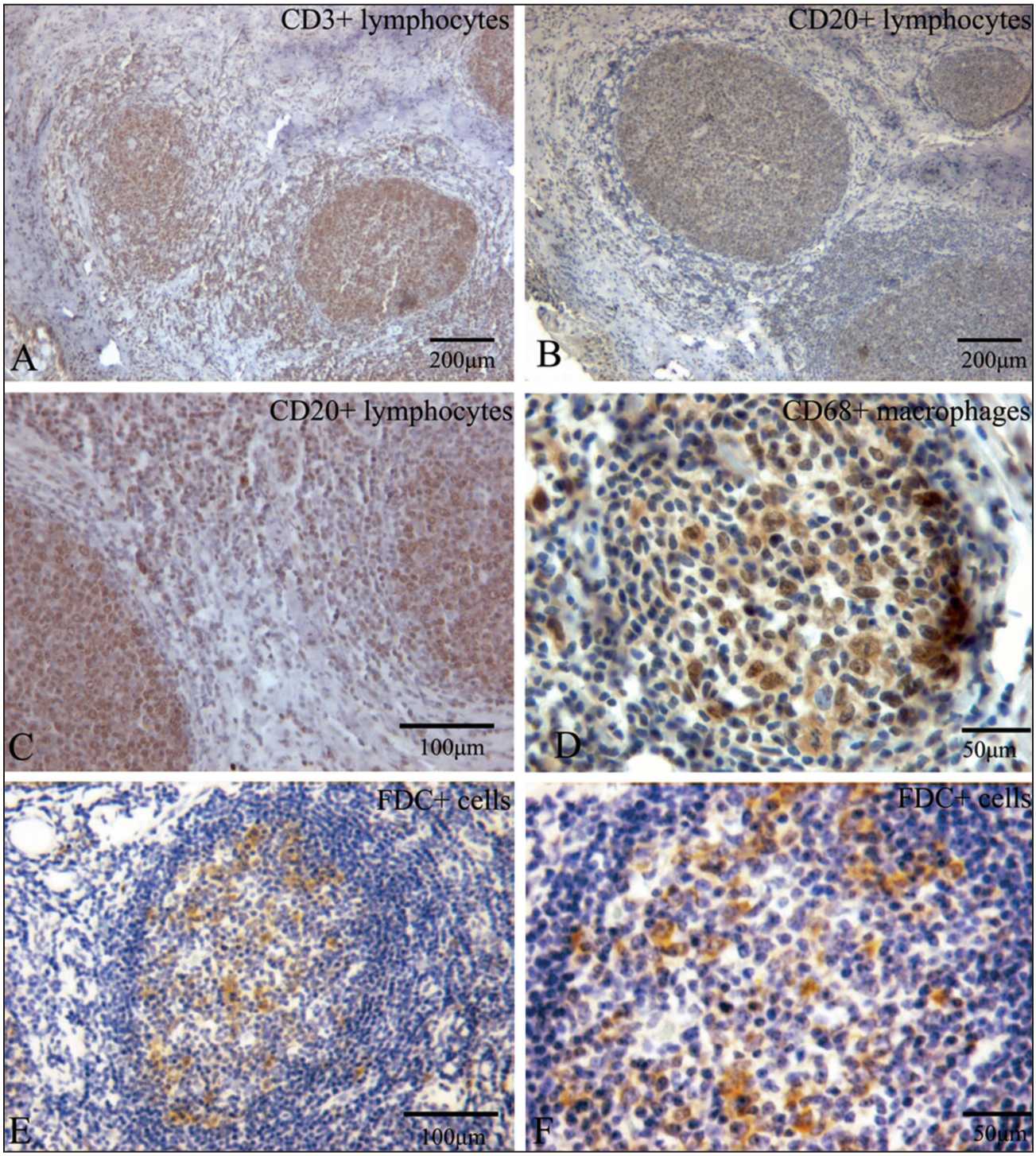

Fig 7. A-D. Distribution of CD20+, CD3+ and CD68+ positive immunocytes in germinal centres and parafollicular area of the CALT. E and F. FDC+ positive immunocytes are only found in germinal centres.

of M cells. There were distributed abundant mitochondria and numerous small sized vesicles from supranuclear region to the luminal surface (Fig 5-B). No cells with the distinctive appearance of goblet cells were observed on the FAE. The follicle mainly composed of lymphoblasts, tangible body macrophages, plasma cells, FDC and apoptotic cells (Fig 5-C). Because different cell types had abundant cytoplasm, the electron dense of bright germinal centre (GC) was less than the rest of the follicle (Fig 6-A). The nuclei of apoptotic lymphocytes were small and dark. Masses of heterochromatin filled the peripher of the nuclei (Fig 5-D). The FDC were only observed in GC. The nuclei of FDC were

roundish or oval shape, which were surroundly with euchromatin. Large nucleoli were occasional observed. Long cytoplasmic processes of FDC extend among the lymphoid cells in GC (Fig 6-B). Plasma cells, inter-digitating cells (IDC) and macrophages were associated with lymphocytes. The processes of IDC were different in length and thickness, extended among lymphocytes (Figs 6-C, D).

Immunohistochemistry

Secondary lymphoid follicles with the cytomorphological and immunophenotypic features of MALT were identified in different regions of the CALT from 4 camels. The positive cells were indicated by the presence of cytoplasmic and/or membrane brown staining in different areas of the secondary lymphoid follicles. There was no positive labelling in negative control sections. CD3 positive T lymphocytes were mainly localised in PFA (Fig 7-A). Most of CD20 positive B lymphocytes were identified in FA (Figs 7-B, C). CD68 positive macrophages were distributed predominantly in PFA and inter-follicular areas (Fig 7-D). The positive FDC were found only in GC (Fig 7-E, F). More details were summarised in table 1.

Discussion

Morphology and distribution characteristics of CALT in the bactrian camels

CALT is a part of the EALT (2000; Knop and Knop, 2002), although the quantity and distribution of discrete lymphoid follicles might be affected by individual and age of the animal or ocular health (Chodosh and Kennedy, 2002; Chodosh *et al,* 1998b; Fix and Arp, 1991a; Fix and Arp, 1991b). The camel's CALT was formed by solitary or aggregate lymphoid follicles, few scattered lymphoid cells, which were mainly restricted to fornical zones in the upper and lower eyelid. The results were compatible with the characteristics of MALT in different farm animal (Chodosh *et al,* 1998a). The present study confirmed that as a normal constituent, CALT appeared in bactrian camel's conjunctiva. The camel was exposed to the surrounding environment with a number of antigens (Zidan and Pabst, 2012). So, the development of numerous secondary lymphoid follicles was observed in the CALT. The distribution and concentration of antigen at the ocular surface can be conceivably reflected in topography of CALT follicles (Knop and Knop, 2005c). Iron oxide in the preocular tear film was taken up preferentially by conjunctival lymphoid tissue, supporting the hypothesis that mammalian conjunctival lymphoid follicles may participate in the acquired immune response to pathogens in the tear film (Astley and Chodosh, 2005). Although few bacillar bacteria were occasional bound to the surface of CALT M cells in the scatter or clusters, which were similar to those that have been reported for the healthy canine (Giuliano *et al,* 2002), there were no bacterial eye infections in the health camels. The phenomenon suggests that the function of the camel's CALT might account for immune response against antigens, especially at the ocular surface.

Morphologic and functional features of lymphoid follicles

The basic microstructure of the 2nd lymphoid follicle was in accordance with description in camel and other mammals (Yang and Wang, 2013b). The ultrastructural images of CALT revealed that the epithelial cells overlying the lymphoid follicles were often flattened and had short irregular microvilli. There were no goblet cells in the FAE. The term 'M' cells was used as a diminutive of microfold cell in human Pyer's patches (Owen and Jones, 1974). Kraehenbuhl and Neutra (1992) provided contradictory evidence for the development of M-cells criteria. Cells fulfill the morphologic of M-cells criteria was proved by the camel's conjunctival FAE. M cells of CALT were covered with blunt microvilli and microfolds, numerous small sized vesicles varied from supranuclear region to the luminal surface. Soluble and particulate antigens from the mucosal surface were sampled by the vesicles. After that, through basolateral membrane of M cells, these antigens were delivered to lymphoid tissue

Table 1. Semiquantification of positive cell types in different regions of the camel CALT.

Phenotype	Epithelium	Laminapropria mucosae	Inter-follicular area	Parafollicular area	Germinal centre
CD^{3+}	+	++	+	+++	++
CD^{20+}	+++	+	+	++	+++
CD^{68+}	+	++	++	+	+
FDC^{+}	–	–	–	–	++

Note: not detected; +, few; ++, moderately frequent; +++, very frequent.

beneath (Giuliano *et al,* 2002). The ultrastructural features of superficial cells of the camel CALT were fundamentally similar to those of M cells in MALT (Yang and Wang, 2013a; 2013b). The origin of M cells was different. In mouse, M cells were proved from the crypts of Peyer's patch (Bye *et al,* 1984). In rabbit, immature precursors distributed in the follicle periphery originated from the counterpoints of caecum (Jepson *et al,* 1993). The origin of M cells in camel CALT will require further study.

FDC were distributed in the GC of the lymphoid follicles to play an important role in generation of effective humoral antibody responses (Heinen *et al,* 1995). To support the differentiation of the proliferating lymphoblasts, prominent vesicles in proliferating lymphoblasts and FDC continuously provided antigens on their surface (Wolniak *et al,* 2004). In camel CALT, the apoptotic lymphocytes were found in GC. When differentiating lymphocytes exhibit inappropriate antigen specificity or binding affinity, they are induced to die (Knop and Knop, 2005c). In the lymphoid follicles, apoptotic lymphocytes might been ingested by macrophages presented (Zidan and Pabst, 2012). The morphological characteristic of HEV was similar to CALT and others lymphoid organs of camel, rabbit and human (Knop and Knop, 2005c). HEV were special post-capillary venules with adaptation in morphology and function, which efficiently regulated the migration of lymphocyte in lymphoid tissues (Knop and Knop, 2005c). Plasma cells, macrophages and IDC were observed among lymphocytes in the parafollicular area of the camel CALT follicles, compatible with descriptions of tonsil in horse and camel (Kumar and Timoney, 2005a; 2005b; Yang and Wang, 2013a).

Immunohistochemical characterisation of lymphocyte subsets

The immunohistochemistry demonstrated that most of CD20 positive B lymphocytes were identified in FA. CD3+ T lymphocytes were mainly localised in PFA. These results revealed that the distribution of B and T lymphocytes in camel CALT follicles mirrored the observation in PP of rabbit and baboon's CALT (Astley *et al,* 2003). Few CD68+ macrophages were identified in different areas which may phagocytose foreign antigen and apoptotic lymphocytes (Zidan and Pabst, 2012). The origin, movement and function of intraepithelial T and B lymphocytes and macrophages need elucidating (Kuper *et al,* 1992), such as in the tubal and nasopharyngeal tonsils in equine (Kumar and Timoney, 2005b; Kumar *et al,* 2001), and on the free surface of NALT of the rat. As one of the main composition of the lymphoid follicles, CD20 B lymphocytes were involved in the production of humoral antibodies in response to antigen stimulation (Zidan and Pabst, 2012). The FDC positive cells in GC of the camel CALT follicles were similar to the observation described previously in bovine tonsil. As an identification parameter for tonsils, FDC involved in forming a widespread three-dimensional mesh and functionally maintaining the lymphocytes in lymphoid follicles (Rebmann and Gasse, 2008). The distribution of B lymphocytes and FDC in GC of camel CALT follicles suggested that the interactions between B cells and FDC were essential for B cell survival and differentiation in GC (Park and Choi, 2005). The CD3 T lymphocytes distributed in the lymphoid follicles may act as regulatory cells in B cell dependent areas (Delverdier *et al,* 1996). These might be T helper cells in the bactrian camels, the one humped camel and other species (Zidan and Pabst, 2012).

In conclusion, The CALT of the camel was formed by solitary or aggregate lymphoid follicles, few scattered lymphoid cells, which were mainly restricted to fornical zones in the upper and lower eyelid, were compatible with the characteristics of MALT in different farm animal (Chodosh *et al,* 1998a). The structural and cellular architecture of CALT further supports the concept of an EALT in the camel (Knop and Knop, 2003; Knop and Knop, 2005a; Knop and Knop, 2005b; Knop and Knop, 2005c). The camel CALT had the cytologic basis of normal mucosa reaction, integrated into the MALT system. As an active component of the EALT in the camel, the CALT were served as inductive sites for the common mucosal immune system as well as important components in ocular defense. Ontogenesis of CALT topography will require further study.

Acknowledgements

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News

First cloned camel gives birth to first cloned offspring in Dubai

The first calf to come from a cloned camel has been born in Dubai. At the Reproductive Biotechnology Centre, her six-year-old mother proved that a cloned camel can be fertile and reproduce normally. Injaz, the world's first cloned camel, gave birth to a healthy female calf weighing about 38 kilos on November 2. Injaz, whose name means "achievement" in Arabic, was cloned in 2009 from the ovarian cells of a dead camel. Injaz conceived naturally and delivered after a normal gestation. This has proved that cloned camels are fertile and can reproduce the same as naturally produced camels, according to Dr Nisar Wani, Scientific Director of the Centre in Nad Al Sheba. Dr Wani said that many cloned camels have been created using cells from the skin of elite animals. He said that few more cloned camels are pregnant and we expect them to deliver next year.