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RESEARCH ARTICLE

Conjunctiva Associated Lymphoid Tissue in the Ostrich *(Struthio camelus)*

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Summary

This study was aimed at the investigation of the structure of conjunctiva-associated lymphoid tissue (CALT) in the ostrich (Struthio camelus). The upper and lower eyelids of 5 healthy adult ostriches obtained from a slaughterhouse constituted the material of the study. Aggregated lymphoid follicles occupied a large area beneath the fibrous flap in the lower palpebral conjunctiva, while their distribution in the upper palpebral conjunctiva was observed to be limited to regions near the nasolacrimal punctum. Light microscopic examination revealed the conjunctival epithelium to differ between non-lymphoid and lymphoid regions. Non-lymphoid regions had pseudostratified prismatic epithelium containing goblet cells. Follicle-associated epithelium (FAE) in lymphoid regions was composed of single-layered squamous epithelium without goblet cells. Aggregated and/or solitary lymphoid follicles were present in the subepithelium. Dome area, germinal centre, corona and interfollicular region were able to be distinguished in lymphoid follicles. Furthermore, interfollicular regions contained high endothelial venules, which are involved in lymphocyte migration. Electron microscopic examination demonstrated that FAE was closely associated with immune system cells. Cells, which resembled typical M cells in having short, thick apical micro-folds and vesicles, were present in the FAE. In the present study, the structure of CALT was demonstrated in the ostrich (Struthio camelus), based on macroscopic, microscopic and ultrastructural findings.

Keywords: *CALT, FAE, M cell, Ostrich*

Devekuşunda *(Struthio camelus)* Konjunktiva İlişkili Lenfoid Doku

Özet

Bu çalışma devekuşunda (Struthio camelus) konjunktiva ilişkili lenfoid doku (CALT)'nun yapısını ortaya koymak amacıyla yapıldı. Mezbahaneden sağlanan 5 adet sağlıklı erişkin devekuşu alt ve üst göz kapakları çalışma materyali olarak alındı. Agregat lenf foliküllerinin alt palpebral konjunktivada fibröz flap'ın altında geniş bir alana yayıldığı, üst palpebral konjunktivada ise dağılımlarının nazolakrimal punktuma yakın bölgelerde sınırlı olduğu görüldü. Işık mikroskobik incelemede konjunktiva epiteli, non-lenfoid ve lenfoid bölgeler arasında farklılık gösteriyordu. Non-lenfoid bölgeler kadeh hücresi içeren yalancı çok katlı prizmatik epitele sahipti. Lenfoid bölgelerde ise folikül ilişkili epitel (FAE) kadeh hücresi içermeyen ve tek katlı yassı epitelden oluşmaktaydı. Bu bölgelerde subepitelyumda agregat ve/veya soliter lenf foliküllerı bulunmaktaydı. Lenf foliküllerinde dome, germinal merkez, korona ve interfoliküler bölgeleri ayırt ediliyordu. Ayrıca interfoliküler alanlarda lenfosit göçünde görev alan yüksek endotelli venüller (HEV) görüldü. Elektron mikroskobik incelemelerde, FAE'nin immun sistem hücreleri ile yakın ilişkide olduğu gözlendi. FAE'de tipik membranöz hücrelere (M hücreleri) benzer şekilde kısa, kalın ve az sayıda mikrofoldlar dikkat çekti. CALT'ın makroskobik, mikroskobik ve ultrastruktural yapısı üzerinde elde edilen bulgular ile; bu dokunun devekuşundaki (Struthio camelus) yapısı ortaya konuldu.

Anahtar sözcükler: *CALT, FAE, M hücresi, Devekuşu*

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INTRODUCTION

The mucosal immune system protects ocular surfaces from antigenic stimulation [1,2]. Ocular mucosal defense involves both non-specific (orbital skeletal structure, eyelid architecture and closing reflex of the eyelids) and specific factors [3]. Conjunctiva-associated lymphoid tissue (CALT) [4-6], the Harderian gland [7-9] and the lacrimal glands [10] are factors involved in the specific response. These factors play a major role in the maintenance of sight, as well as conjunctival and corneal homeostasis [11].

Based on histological criteria, MALT and lymphocyte infiltrations are able to be distinguished. It is known that lymphocyte infiltrations lack compartments typical of lymphoid follicles and are composed mainly of T lymphocytes. Furthermore, lymphocyte infiltrations do not have a close association with the epithelium, which also means that they do not have any contact with lumen antigens. Lymphoid tissues in mucosa should bear certain morphological and functional characteristics in order to be classified as MALT. Accordingly, in order to be classified as MALT, lymphoid tissue must have very close association with the mucosal surface. Furthermore, solitary and aggregated lymphoid follicles should be composed of a dome area, germinal centre, corona and interfollicular region. The interfollicular regions should contain typical high endothelial venules (HEV) [12].

Histologically, mucosa-associated lymphoid tissue (MALT) is composed of aggregated and/or solitary lymphoid follicles. These follicles are reported to consist of a dome area, germinal centre, corona and interfollicular region [12,13]. A characteristic feature of MALT is the presence of HEV in interfollicular regions, which are involved in the tissue-specific migration of lymphocytes [12,14,15].

The epithelium lining the dome area is named as follicle-associated epithelium (FAE) or lympho-epithelium, due to its close association with lymphoid follicles. Regions lined with FAE do not contain goblet cells. Membranous cells (M cells), which are specialized in antigen uptake, exist in between FAE cells. A multitude of studies have been published on the presence of M cells in the MALT of various tissues [12,16-18]. M cells have an irregular apical surface, which is generally characterized with short micro-folds. Their cytoplasm contains abundant mitochondria, endoplasmic reticulum (ER), a distinct Golgi complex and a few lysosomes. Furthermore, connective complexes and lateral extensions exist between M cells and their neighbouring cells. The basolateral surface of M cells forms a deep and wide package [19-21]. One or more cells are reported to be densely packed within the deep basal invaginations [4,18]. The cells situated within these invaginations mostly include lymphocytes, lymphoblasts and macrophages. Antigen samples at macromolecular level are transferred by M cells to lymphocytes and macrophages. Following the stimulation of B lymphocytes in the germinal centres of lymphoid follicles by macrophage-processed antigens, lymphoblasts divide and transform into antibody-producing plasma cells [3,22,23].

CALT, which is the conjunctival component of MALT, has been investigated in various mammalian and avian species. In studies carried out in domestic avian species, it has been determined that a greater number of CALT follicles are located in the conjunctiva of the lower eyelid, when compared to that of the upper eyelid [8,24,25]. In the turkey and chick, CALT follicles have been determined to be greater in number beneath the smooth epithelial plateau in the lower conjunctiva, and to be found to a less extent in regions near the nasal margin of the upper conjunctiva. In these species, CALT has been determined to display similarity to other components of MALT localized in different parts of the body with respect to morphological localization, epithelial structure and vascular features [8,24].

The majority of CALT studies in avian species were carried out in species of economic value. Effective protection against major respiratory diseases by means of ocular vaccination is dependent on the presence of CALT in the conjunctiva [8]. The present study was aimed at the investigation of the structure of CALT in the ostrich (*Struthio camelus*) for the first time.

MATERIAL and METHODS

Ten eyeballs of 5 healthy adult ostriches slaughtered humane in compliance with animal welfare rules at the Sincan Slaughterhouse in Ankara constituted the study material. In order to determine the distribution of conjunctiva lymphoid follicles in eyelids, 3% acetic acid was injected into the eyeball within the first hour after death and was allowed to react for 24 h [6,8]. Palpebral conjunctiva samples were first fixed in Bouin's solution and 10% buffered formalin. After fixation samples were cut and collected and were dehydrated in graded series of alcohols, methylbenzoate and benzoles, and finally blocked in paraplast, trimmed and 6µm sections cut. Mounted sections were stained with Mallory's modified triple staining technique [26].

Electron microscopic tissue samples were first prefixed in glutaraldehyde-paraformaldehyde (pH 7.4), as described by Karnovsky [27], and subsequently were fixed for a second time in 1% osmic acid solution for 2 h. Following the second fixation, tissue samples were

maintained in 1% uranyl acetate for 2 h, dehydrated through an ascending series of graded alcohols and propylene oxide, and embedded in Araldite M. The semi-thin 1-µm-thick sections cut from these blocks were stained with toluidin blue, and following the marking of the targeted area, thin sections of 30-40 nm thickness were cut. These sections which were taken on grids stained with uranyl acetate and lead citrate as described by Veneable and Coggeshall [28], and were examined with the transmission electron microscope.

RESULTS

The examination performed using acetic acid revealed the presence of aggregated lymphoid follicles beneath the fibrous flap in the lower palpebral conjunctiva *(Fig. 1A)*. Lymphoid follicles were determined to be less in the upper palpebral conjunctiva, in comparison to the lower conjunctiva. Characteristically, it was clustered around the opening of the nasolacrimal duct into the eyeball *(Fig. 1B)*.

Histologically, the characteristics of the conjunctival epithelium differed between non-lymphoid and lymphoid regions. Accordingly, the non-lymphoid conjunctiva was covered with pseudostratified columnar epithelium containing goblet cells. In lymphoid regions, the epithelium associated with aggregated and solitary lymphoid follicles was simple squamous with no goblet cells. The follicles lay close to the epithelial basal lamina. Lymphoid follicles were composed of four regions, namely the dome area, corona, germinal centre and interfollicular region, which were able to be distinguished *(Fig. 2)*. The subepithelial area beneath the FAE, which is named as the dome, is the region where cells of the immune system establish a close association with the epithelium. The FAE lining the dome area was observed to flatten and become single-

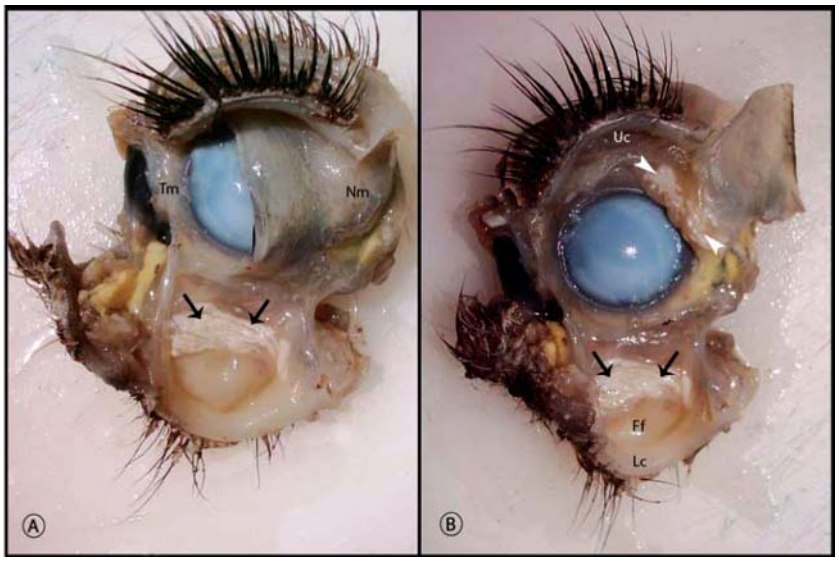

Fig 1. Macroscopic appearance of CALT after acetic acid 3% application of the ostrich conjunctivas, **(A)**: Aggregate lymphoid follicles (arrows), Tm: temporal margin, Nm: nasal margin, **(B)**: Aggregate lymphoid follicles (arrows) were seen a large area beneath the fibrous flap (Ff) in the lower conjunctiva (Lc), while their distribution in the upper palpebral conjunctiva (Uc) were found to be clustered to regions near the nasolacrimal punctum (arrowheads)

Şekil 1. Devekuşu konjunktivalarına %3 asetik asit uygulaması sonrası CALT'ın makroskobik görünümü, **(A)**: Agregat lenf folikülleri (oklar), Tm: temporal kenar, Nm: nazal kenar, **(B)**: Agregat lenf folikülleri (oklar) dağılımlarının alt palpebral konjunktivada (Lc) fibröz şişkinliğin (Ff) altında geniş bir alanda iken üst palpebral konjunktivada (Uc) nazolakrimal punktuma (ok başları) yakın bölgelerde kümelendiği görüldü

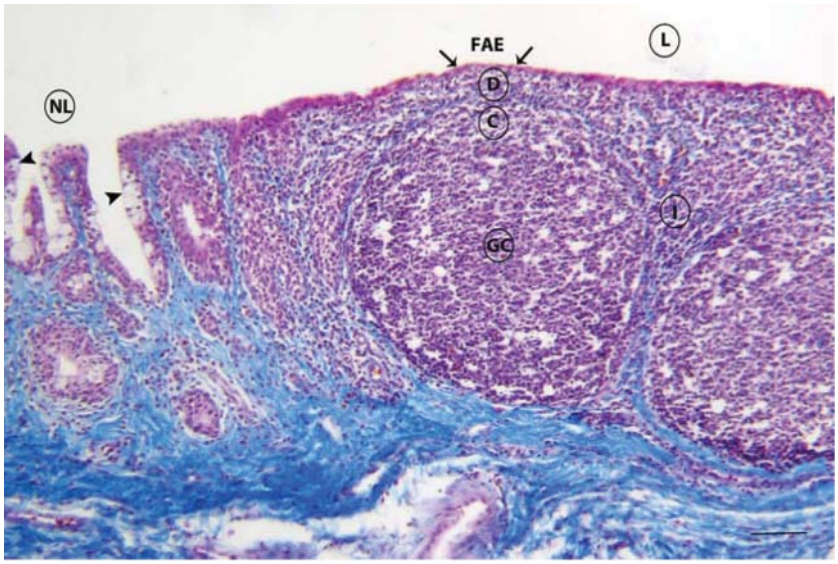

Fig 2. The epithelium of the palpebral conjunctiva, non-lymphoid regions (NL) were covered with pseudostratified columnar epithelium containing goblet cells (arrowheads). In lymphoid region (L), follicle-associated epithelium (FAE, arrows) was composed of single-layered squamous epithelium. Lymph follicles were observed four distinct areas; dome (D), corona (C), germinal centre (GC) and interfollicular region (I), Bar: 75 µm

Şekil 2. Palpebral konjunktiva epiteli, non-lenfoid bölgeler (NL) kadeh hücreleri (ok başları) içeren yalancı çok katlı prizmatik epitel ile örtülüydü. Lenfoid bölgede (L), folikül ilişkili epitel (FAE, oklar) tek katlı yassı epitelden oluşmaktaydı. Lenf foliküllerinde dört ayrı bölge gözlendi: dom bölgesi (D), korona (C), germinal merkez (GC), interfoliküler alan (I), Bar: 75 µm

layered squamous epithelium without goblet cells. The corona region was situated between the dome area and the germinal centre. The germinal centre was located in the centre of follicles and contained lymphoblasts with euchromatic nuclei. In both paraffin and semi-thin sections, interfollicular regions containing high endothelial venules (HEV) were observed in-between follicles *(Fig. 3)*.

Ultrastructurally, it was ascertained that some epithelial cells of FAE displayed structural features typical of M cells. Although M cells were in close association with the immune system cells situated beneath them, no cellular connection was determined to exist between them. However, tight junctions and desmosomal connections were observed between adjacent M cells. The apical part of M cells, which contained many pinocytic vesicles, had short, thick and irregular microfolds, while wide and deep invaginations were present in the basal part of the cells *(Fig. 4)*. Immune system cells, including lymphocytes and macrophages were present in these invaginations *(Fig. 5)*.

DISCUSSION

The conjunctiva and the cornea are the surfaces of the eye, which are exposed to pathogens transmitted by air and contact [29,30]. CALT, which is the conjunctival component of MALT, has been investigated in various animal species [6,8,20,24,25]. It is reported that CALT displays similarity to lymphoid tissues located in other mucosa of the body (such as, gut-associated lymphoid tissue and bronchus-associated lymphoid tissue) with respect to localization, epithelial structure, cell composition and vascular appearance in the turkey [24], chick [8], goat [4,14], cattle [6], and humans [31]. In the present study, the presence of any of the abovementioned characteristics of MALT

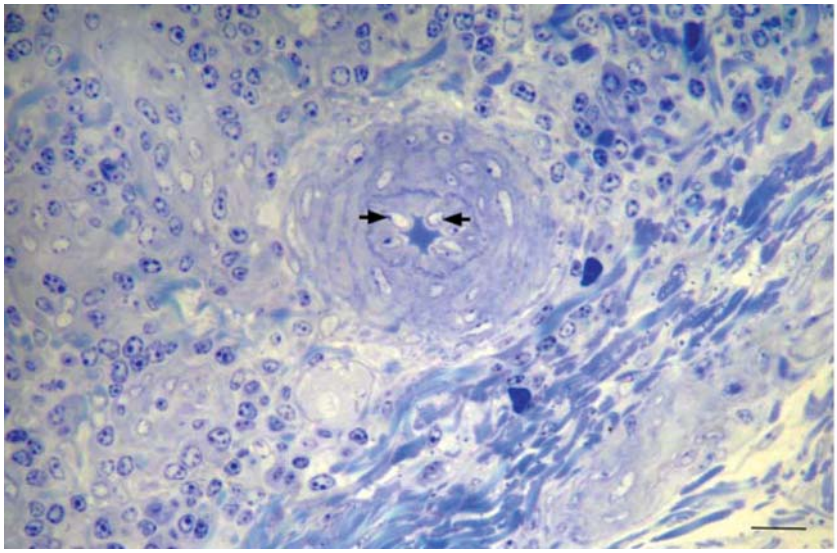

Fig 3. Semi-thin section microphotograph of the high endothelial (arrows) venules, Bar: 20 µm

Şekil 3. Yüksek endotelli venüllerin (oklar) yarı-ince kesit mikrofotoğrafı, Bar: 20 µm.

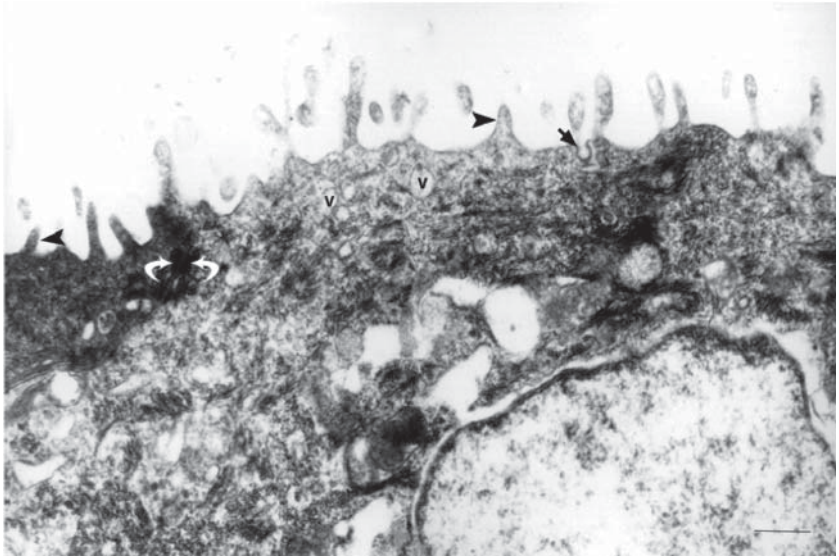

Fig 4. Electron microphotograph of follicle-associated epithelium (FAE) of the apical region, apical invagination (arrow) vesicles (v) and microfolds (arrowheads), terminal junctions (curved arrows), Bar: 0.03 µm

Şekil 4. Folikül ilişkili epitelin (FAE) apikal bölgesinin elektron mikrofotoğrafı, apikal invaginasyon (ok), veziküller (v) ve mikrofoldlar (ok başları), terminal bağlantılar (eğri oklar), Bar: 0.03 µm

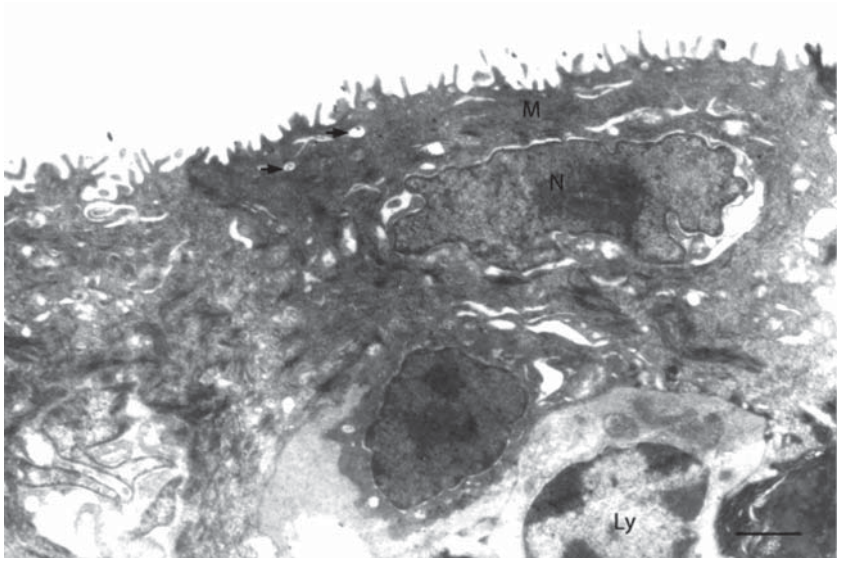

Fig 5. Electron microphotograph of the follicle-associated epithelium (FAE), M cell (M) with indented nucleus (N), numerous apical vesicles (arrows) and lymphocyte (Ly), Bar: 0.1 µm

Şekil 5. Folikül ilişkili epitelin (FAE) elektron mikrofotoğrafı, çok sayıda apikal vezikülü (oklar), çentikli çekirdeği (N) ile M hücresi (M) ve lenfosit (Ly), Bar: 0.1 µm

in the CALT of the ostrich was investigated.

Localization and Histological Structure of Lymphoid Follicles

The distribution of CALT in the upper and lower palpebral conjunctiva varies between species. Lymphoid follicles are reported to be dispersed in a large area in both the upper and lower palpebral conjunctiva in farm animals including cattle [6], pig and sheep [12]. The upper palpebral conjunctiva has a larger surface area in humans [31] and the monkey [32], and accordingly, it is reported that the upper eyelid has a greater amount of lymphoid tissue. The studies carried out in the rabbit [5], turkey [24,25] and chick [8] showed that CALT dominated the lower palpebral conjunctiva, and characteristically, it was clustered around the opening of the nasolacrimal duct into the eyeball in the upper palpebral conjunctiva. Similar to the rabbit, turkey and chick, the localization of CALT in the ostrich, as a result of the form-function relation, covered the sites of deposition and elimination of tears in the eyeball.

Epithelial Features

The mucosal immunity function requires an immune reaction and/or immune tolerance to be developed against microorganisms in the mucosa. In avian species, which are frequently vaccinated by ocular route, the development of an immune reaction is dependent on the production of antibodies specific to these antigens [11]. FAE, which lines lymphoid follicles that are closely associated with mucosa, bear the characteristic ultra-structural features of M cells, which are specialized in the transfer of antigens from the lumen to lymphoid follicles [16-18]. The presence of typical M cells in CALT has been reported in the dog [20], rabbit [5] and cattle [6]. The studies carried out in the turkey [24,25] and chick [8] have focused on the postnatal development of CALT and the uptake of particles from the lumen. In the ostrich, M cells with structural properties similar to those of M cells reported to exist in the FAE of the dog, rabbit and cattle were found to be present.

Vascular Appearance

High endothelial venules (HEV) play a key role in the development of a common reaction by the organized mucosal immune system. HEV, which are located in the interfollicular regions, are structures characteristic of MALT [33-35]. The studies carried out in the turkey [24,25] and chick [8] have shown the presence of HEV in the base of follicles, which were composed of cubic endothelial cells and frequently contained lymphocytes. Similarly, HEV was found to exist in the conjunctival follicles of the ostrich, in between lymphoid follicles and in the base of follicles.

In conclusion, it is suggested that CALT has a possible role as the ocular component of the mucosal immune system with respect to its localization, and epithelial and vascular characteristics in the ostrich.

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