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Case Report

Corneal Impression Cytology for the Diagnosis of Limbal Stem Cell Deficiency in a Dog

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Abstract

A 14-year old, castrated, female terrier was referred to Near East Animal Hospital with a complaint of red eye. Ophthalmoscopic examination revealed superficial corneal vascularization originating from conjunctiva and encompassing proximal one-third of cornea in 10-02 o'clock position together with photophobia and vision loss. Schirmer tear test was normal and fluorescein staining was negative for the affected eye. Since combined treatment with topical corticosteroid, antibiotic and cycloplegic did not improve the vascular regression, rather exacerbated the symptoms, blood and serum samples were collected for hematologic, biochemical and serologic analyses in order to investigate possible etiologic factors in human LSCD and to define its association with prevalent local blood disorders. As hematologic, biochemical and serologic test results were within the normal ranges, corneal impression cytology was conducted. Upon the observation of goblet cells the gold standard for the diagnosis of LSCD and the indicative of conjunctivalization in cornea the diagnosed of idiopathic partial limbal stem cell deficiency was confirmed in the dog in which it was the first case with ocular surface defect in veterinary medicine.

Keywords: *Limbal stem cell deficiency (LSCD), Corneal impression cytology, Dog*

Bir Köpekte Limbal Kök Hücre Yetmezliğinin Tanısında Korneal İmpresyon Sitolojisi: Olgu Sunumu

Özet

Yakın Doğu Hayvan Hastanesine kırmızı göz şikayeti ile getirilen 14 yaşlı kısırlaştırılmış, dişi, Terrier ırkı bir köpekte oftalmoskopik muayene sonucu sağ gözde, saat 10-02 pozisyonunda korneanın proksimal 1/3'ünü kaplayan konjunktivadan köken alan korneal yüzeyel vaskülarizasyon, fotofobi ve görmede azalma saptandı. Schirmer göz yaşı testi normal ve florescein boyama negatif olan gözde, damarların regresyonu için uygulanan topical kortikosteroit, antibiotik ve sikloplejik kombinasyonundan olumlu bir sonuç alınamadı ve semptomların artması ve gerilememesi üzerine olgunun kan ve serum örnekleri, insanlardaki olası limbal kök hücre yetmezliği (LKHY) etiyololojik faktörlerini irdelemek ve bölgesel yaygın kan hastalıkları ile ilişkilendirmek amacı ile hematolojik, biyokimyasal ve serolojik analizler için toplandı. Test sonuçları normal sınırlarda olduğu gözlenen olguda korneal impresyon sitolojisi gerçekleştirildi. LKHY tanısında altın standart olan korneada konjunktivalizasyonun göstergesi goblet hücrelerinin gözlenmesi ile veteriner hekimlikte ilk kez oküler yüzey bozukluğu olan bir olguda idiopatik parsiyel LKHY tanısı kondu.

Anahtar sözcükler: *Limbal kök hücre yetmezliği, Korneal impresyon sitoloji, Köpek*

INTRODUCTION

Tunica fibrosa bulbi, outer layer of bulbus oculi consists of sclera and cornea which lacks pigment, vessels and cells. Cornea maintains its transparency with stem cells in limbus that have unlimited proliferation feature. In order to maintain its transparency and integrity, limbal stem cells (LSC) prevent conjunctival invasion of corneal surface. Limbus prevents the growth and migration of conjunctival epithelial cells towards corneal surface and it consists of

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Langerhans cells and melanocytes unlike cornea, and as well as it lacks goblet cell layer unlike conjunctiva [1,2]. Corneal epithelium do not regenerate in limbal stem cell deficiency (LSCD) [3-5] and conjunctivalisation (growth of conjunctival epithelia upon the cornea), vascularization, chronic inflammation, calcification, ulceration or persistent epithelial defects (PED) develop consequently [2,6,7]. In LSCD cases, the corneal surface is covered by conjunctival epithelium and goblet cells are observed in cornea [8]. In humans LSCD results in corneal opacity, vision loss, chronic pain, and photophobia and results in failure of topical medical treatment and/or keratoplasty operations.

Investigations in human LSCD patients yielded the identification of several factors in primary (congenital) etiology of the disease. Aniridia associated with *PAX6* mutations [9], ectrodactyl-ectodermal-dysplasia-clefting syndrome, keratitis-ichthyosis-deafness syndrome, xeroderma pigmentosum, dominant congenital keratitis and dyskeratosis congenita result in primary LSCD. On the other hand, inflammatory features of Steven-Johnsons Syndrome [10], ocular cicatricial pemphigoid [11], graft versus host disease, vernal keratoconjunctivitis [12] are implicated in the etiology of secondary LSCD. Several etiologic factors were defined for human LSCD patients day by day and beside the factors enumerated above, it was shown that neurotrophic keratopathy, bullous keratopathy, radiotherapy and systemic chemotherapy, topical chemotherapeutics, benzalkonium chloride toxicity during medical treatment of glaucoma [13], ocular surface tumors and pterygium [14] result in LSCD as well. Furthermore, chemical-thermal injuries, multiple surgical interventions including limbal region, chronic mechanical microtrauma induced by contact lens and inflammatory diseases of ocular surface are reported to cause LSCD [3,5,6,10,12]. Additionally, diabetes and vitamin A deficiency have a role in the development of LSCD [9]. LSCD develops in albino rats with induced type 2 diabetes mellitus as a result of diabetic keratopathy. Idiopathic LSCD was observed in humans and stem cell loss was shown to originate from direct cell and/or cellular microenvironment damages [2].

Clinical manifestations of LSCD in humans are epiphora, photophobia, vision loss, and erosion of palisades of Vogt observed under slit-lamp biomicroscopy, corneal neovascularization and conjunctivalization. Among the clinical symptoms of human LSCD, stippled late staining pattern with fluorescein stems from the loss of tight junctions between the cells and results in staining of basement membrane. Stippled staining may be observed as swirling around the center and fluorescein stain tends to pool on the conjunctivalised area due to relative thinness of epithelium [3,5,6]. While vision loss and photophobia develop in patients at this stage, precorneal tear film and corneal erosion at variable levels might also be observed. Disarrayed palisades of Vogt and perilimbal vascular archades are reported as early anatomical changes in mild LSCD, but the absence of pallisades of Vogt, which is observed only via slit lamp biomicroscope, may not be a sign of LSCD alone [15].

Observation of PED and superficial vascularization was reported in human cases having mild deficiency, and PEDs yield to scarring, ulceration, stromal neovascularization, corneal thinning and perforation. PEDs cause pain, photophobia and vision loss, and keratinization may develop if tear deficiency accompanies these symptoms.

Total LSCD is characterized by the total loss of LSC population together with conjunctivalization of the entire corneal surface. Neovascularization is also observed frequently but not in all LSCD cases. Similarly, conjunctiva is detected in many disorders such as chemical burns, Stevens-Johnson syndrome, mucous membrane pemphigoid, all of which result in LSCD. Chronic inflammation frequently yields to fibrosis and continues to damage LSCs. Sub-conjunctival fibrosis leads to symblepharon formation and consequently to goblet cell deficiency. Subsequent poor tear function further worsens the ocular surface in such patients [5]. Although clinical diagnosis of LSCD can be established with symptoms mentioned above, some of those may also be observed in other conditions without LSCD component. Especially, symptoms of partial deficiency may remain subtle and subclinical. It is known that impression cytology of the cornea is the gold standard in the diagnosis of LSCD in human medicine [5,10]. This method employs the removal of cells by impressing the cellulose acetate paper over the corneal surface and conduction of cytological analyses to observe goblet cells of which are the signs of the deficiency [16]. The removal of 1-3 cell layers by impressing nitrocellulose acetate filter paper over ocular surface makes only superficial cells available for the analyses. Beside cytology, immuno-histologic and molecular analyses can also be conducted on cells collected on the membrane. Epithelial morphology and goblet cells are evaluated [5,17]. Goblet cells are the sign of conjunctivalization which confirms limbal deficiency. However, these cells may be absent in severe burns and Stevens-Johnson syndrome. It should be kept in mind that absence of goblet cells may yield to false negative results in chemical and thermal damages since 36% of the goblet cells are also damaged in such cases [5,16].

This case report will explore the current challenges and future research directions that will be required to increase our understanding of corneal diseases in dogs and cats and consider the diagnosis of LSCD to veterinary ocular surface patients. Studies have been focused on the human LSCD with little attention being paid to animals. Developing a deep understanding of the limbus and corneal cell turnover in all species is of paramount importance. The successful treatment of LSCDs in humans and animals, and validation of comparative studies between species depends on this knowledge.

CASE HISTORY

A 14-years old, castrated, female, terrier was referred to Near East Animal Hospital with a complaint of red eye. In the ophthalmoscopic examination, corneal surface vascularization encompassing proximal one-third of cornea was observed in 10-02 o'clock position in the right eye together with photophobia, epiphora and vision loss *(Fig. 1)*. Schirmer tear test was normal and corneal fluorescein staining was negative. Dexamethasone (0.1% Onadron®), Ciprofloxacin (0.3% Siprogut®) and a pain killer, Cyclopentonate HCl (1% Sikloplejin®), were administered and the progress was monitored every other day. However, the recovery could not be achieved and symptoms were exacerbated.

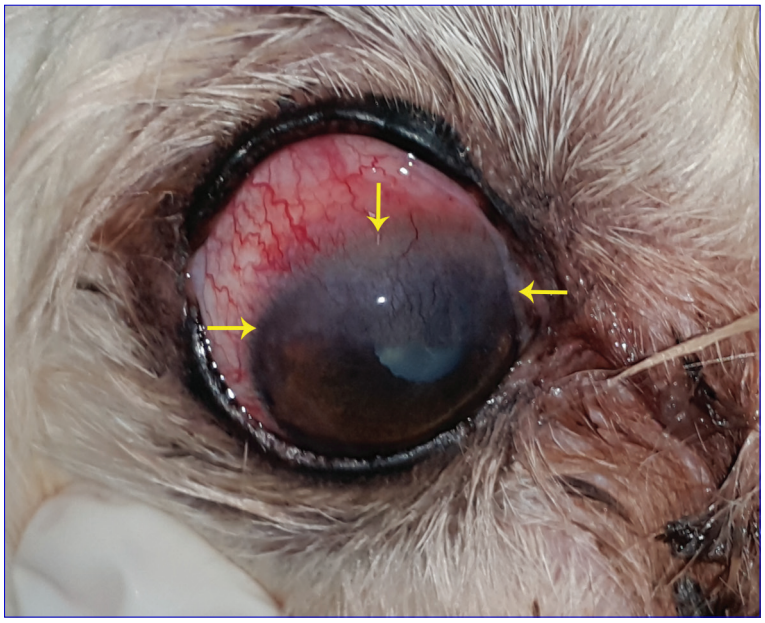

Fig 1. Conjunctivalization observed in 10-02 o'clock position of right eye of the dog and the region where corneal impression cytology was applied *(arrows)*

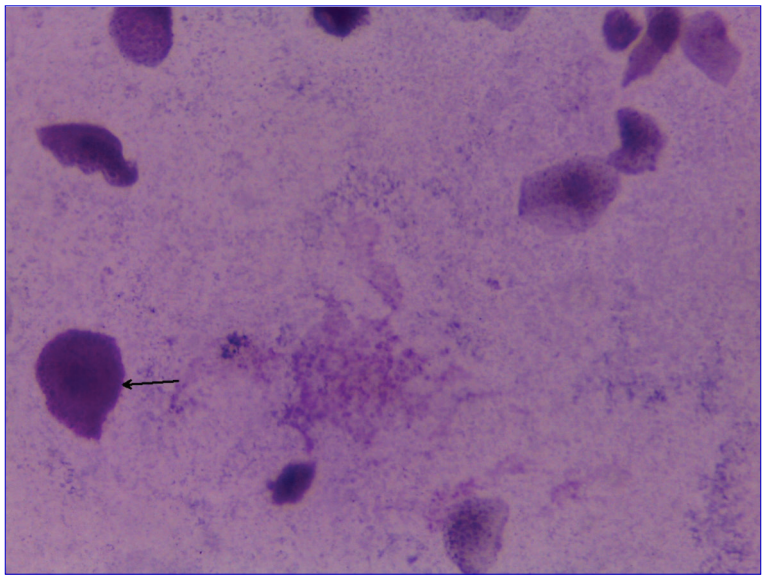

Fig 2. Observation of goblet cells in cornea as a result of corneal impression cytology *(arrow)*

The case was considered to be LSCD; therefore, blood and serum samples were collected in order to conduct hematologic, biochemical and serologic analyses for the evaluation of possible etiologic factors of human LSCD and local prevalent blood diseases (Leishmania, Ehrlichiosis). The haematological parameters were analyzed using an automatic analyzer (BC- 2800Vet, Mindray, Shenzen, China) and white blood cell (WBC) count, red blood cell (RBC) count, Hct, Hb, MCV, MCH, MCHC and RDW were recorded for this study. Serum biochemical analyses were measured by using commercial assay kits (Randox Laboratories Ltd., UK; Mindray Chemistry Reagents, Shenzen, China) and an automated blood chemistry analyzer (BS120, Mindray, Shenzen, China). The serum concentrations of albumin (bromocresol green method, in g/dL), creatinine (jaffe method, in mg/dL), total and direct bilirubin (vanadate oxidase method in mg/dL), phosphorus (P, phospho-molybdate method in mg/dL), glucose (glucose oxidase method in mg/dL), calcium (arsenozo III method in mg/dL), urea (urease method in mg/dL), total cholesterol (cholesterol oxidase-peroxidase method in mg/dL), magnesium (xylidyl method in mg/dL), total protein (biuret method in g/dL), triglycerides (glycerol kinase-peroxidase method in mg/dL), uric acid (uricase peroxidase method in mg/dL) and the enzyme activities of alanine amino-transferase (ALT, IFCC method in U/L), aspartate amino-transferase (AST, IFCC method in U/L), γ-glumatyltrans peptidase (γ-GT, IFCC method in U/L), alkaline phosphatase (ALP, IFCC method in U/L), creatine kinase (CK, IFCC method in U/L), lactate dehydrogenase (LDH, IFCC method in U/L), lipase (enzymatic colorimetric method in U/L) were noted for this study. Immunofluorescence assay (IFA) were performed for the detection of Leishmania infantum and Ehrlichia canis IgG antibodies. Standardized assay kits were used for this purpose which were supplied by MEGACOR Diagnostik GmbH, Austria. The results of hematological, biochemical and serological parameters were within normal limits.

Samples for corneal impression cytology were collected from right eye surface, suspected for partial LSCD, using cellulose acetate filter with 0.20 µm pore diameter under local ophthalmic anesthesia. Opaque surface of cellulose filter papers in 3x5 mm dimensions were placed on cornea using a forceps with a

smooth tip and removed after a soft impression for 5 sec. Collected samples were fixed with 95% ethanol. Flowingly, samples were stained with periodic acid Shiff (PAS) and Hemalun, and evaluated under light microscope. Goblet cells were observed as the indicator of conjunctivalization which is the gold standard of LSCD diagnosis *(Fig 2)*. Autologous limbal stem cell transplantation from healthy left eye to the damaged right eye was considered; however, pet owner did not approve the operation on the geriatric animal.

DISCUSSION

Unlike human medicine, limbal deficiency is defined under ocular surface defects but not further classified in veterinary medicine. However, eye diseases causing ocular surface defects in cats and dogs, such as Uberreiter's syndrome, keratoconjunctivitis sicca, feline corneal necrosis, and feline eosinophilic keratitis, disrupt the transparency of cornea and thus cells and pigments cover the surface of it. It is considered that these features show similarities with primary stem cell deficiency cases of humans. Similarly, corneal alkaline burns, frequently observed in humans, were shown to cause secondary stem cell deficiency in veterinary medicine [18].

In human and veterinary ophthalmology, topical medical treatment predominantly with corticosteroid and/or corneal transplantation, cornea-conjunctival transpositioning, conjunctival pedicle graft applications are routine procedures for the treatment of eyes with corneal transparency loss and without epithelial defect. It is known in human medicine that corneal epithelium cannot regenerate itself in LSCD condition, which consequently results in PEDs, corneal conjunctivalization, neovascularization, corneal scaring and chronic inflammation. This condition negatively influences both medical treatment and also corneal transplantation (keratoplasty) [7,12]. According to Sanchez and Daniels, comparatively to humans, what is known about the healthy limbus and corneal surface physiology of companion animals is still very little. Blinding corneal diseases in animals such as symblepharon in cats with Feline Herpes Virus-1 infections require a basic understanding of the functional companion animal limbus and corneal stem cells [19].

It is considered to evaluate the diseases causing corneal vascularization, conjunctivalization, pigmentation, keratinization, ulceration and erosion, and ultimately to loss of corneal transparency and vision in cats and dogs in terms of LSCD for veterinary medicine. It was reported for conditions in which the connection of corneal epithelial cells with basement membrane was weak, as in Boxer ulcus, events such as positive fluorescein staining, repeating epithelial erosions resulting in PEDs and consequently to corneal perforations may stem from LSCD. On the other hand, the development of fibrovascular pannus and predominant corneal scar formation were reported in cases with severe limbal deficiency [3,6]. Taking these facts into consideration, it was considered that in veterinary ophthalmology, definitive diagnosis of LSCD in cases irresponsive to traditional treatments was crucial and this study aimed to contribute to literature on this field. In human medicine, researches on LSCD contributed several factors of underlying etiology to literature. Similarly, further studies on the etiology of LSCD are highly recommended in veterinary medicine.

In present case, blood and serum samples were collected in order to conduct hematologic, biochemical and serologic analyses for the evaluation of possible etiologic factors of human LSCD and local prevalent blood diseases (Leishmania, Ehrlichiosis) and laboratory parameters were within the normal ranges. In this context, LSCD was diagnosed in a dog for the first time depending on the clinical signs, presence of goblet cells in corneal impression cytology and laboratory parameters.

Beside primary ocular surface disorders that are frequently observed in cats and dogs, that cause corneal vascularization, conjunctivalization, pigmentation, keratinization, ulceration and erosion, and ultimately to loss of corneal transparency and vision, association of ocular surface pathologies with LSCD should also be evaluated in cases of type-2 diabetes in which secondary corneal pathologies may also be observed, and in endemic systemic disorders such as Erchlisiosis and leishmaniosis. It should be kept in mind that the clinical signs of LSCD include epiphora, photophobia, vision loss, corneal vascularization and conjunctivalization, and corneal impression cytology should be performed in cases with ocular surface defect and irresponsive to medical treatment. Final results of cytology should be compared to laboratory analyses.

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