Veterinary Ophthalmology (2009) **12**, 4, 211–215

Conjunctival effects of canine distemper virus-induced keratoconjunctivitis sicca

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Abstract

Objective This study compared the histopathology of canine distemper virus (CDV)-induced keratoconjunctivitis sicca (KCS) to non-infectious KCS in conjunctival tissues.

Animals studied Forty mongrel dogs were assigned to three distinct groups: (i) non-infectious KCS (G1, $n = 10$), (ii) CDV-induced KCS (G2, $n = 20$), and (iii) healthy animals without any ocular alterations (G3, $n = 10$).

Procedure IgG titers and physical and ophthalmic examinations (e.g. Schirmer tear test [STT], tonometry, biomicroscopy, indirect biomicroscopy, and fluorescein test) were performed on all dogs. Conjunctival biopsies were collected and examined microscopically.

Results Non-infectious and CDV-induced KCS demonstrated similar histopathological changes. Both types of KCS correlated with low STT, conjunctival hyperemia, mucopurulent ocular discharge, predominant lymphoplasmacytic infiltration, and acantholysis and keratinization of the ocular surface. G1 had lower conjunctival goblet cell counts than G3. Inclusion bodies were sporadically found in conjunctival samples of dogs from G2. The severity of ocular lesions in G1 and G2 did not correlate with the histopathological findings.

Conclusions Dogs with non-infectious and CDV-induced KCS had very similar conjunctival histopathology. Our findings suggest that the pathophysiology of CDV-induced KCS is likely to be the same as non-infectious KCS, that is, a result of lacrimal deficiency and inflammation of the ocular surface.

Key Words: conjunctivae, distemper, dogs, inflammation, keratoconjunctivitis sicca, morbillivirus

INTRODUCTION

Keratoconjunctivitis sicca (KCS) is a chronic ocular disease characterized by inadequate secretion (quantitative tear film deficiency) or excessive evaporation (qualitative tear film deficiency) of the tear film. Ultimately, KCS culminates in damage to corneal and conjunctival surfaces[1–6] and vision loss.[7,8] Clinical signs of KCS include conjunctival hyperemia, ocular irritation, chemosis, blepharospasm, and photophobia.[4,8] Corneal pigmentation, vascularization, keratinization and dense corneal scar formation are often seen with KCS.[9] Ocular lesions presented in KCS have been related not only to a lacrimal deficiency but also to an immune-mediated inflammation that affects either the lacrimal glands or the ocular surface; however, the exact KCS pathogenesis remains obscure.[5,10,11] Recent histological studies on the immunological mechanisms involved in the pathogenesis of KCS revealed high numbers of $CD4^+$ T cells in conjunctival inflammation consistent with conjunctival samples from humans presenting with KCS.[12] Infiltration of inflammatory cells, predominantly T lymphocytes,[4,6,13–17] can result in squamous metaplasia, where the conjunctivae becomes a keratinized stratified squamous epithelium,[18] accompanied by epithelial stratification, metaplasia of superficial epithelial cells and loss of goblet cells.[4–6,14,17,19,20] Cytokines released by these $CD4^+$ T cells contribute to the development of ocular surface lesions.[21] Additionally, in dogs with KCS, apoptotic mediators, such as p53, fas, and fasL were highly

expressed in the ocular surface, suggesting a role of apoptosis besides or in association with immune-mediated inflammation in the pathophysiology of dry eye.[22]

There are numerous etiologies of canine KCS and some cases may be deemed idiopathic. Conditions that are known to cause bilateral KCS include immune-mediated disorders (e.g. similar to Sjögren syndrome),[9,23–25] endocrinopathies (e.g. diabetes mellitus, hypothyroidism and Cushing's disease),[26] medical toxicity (e.g. sulfonamides),[8] parasitic[27] and viral infections.[18]

Canine distemper virus (CDV) is one virus frequently associated with transient or permanent KCS. CDV is a morbillivirus-induced disease characterized by immune-mediated cytolysis in the epithelial and nervous cells. CDV leads to a multitude neurological, respiratory, gastrointestinal pathological signs that can be fatal.[28–30] Morbilliviral inclusion bodies are characterized by cytoplasmic aggregates and represent the sites of viral replication within the cells. Gröne *et al*. described the presence of inclusion bodies in the central nervous system leading to an up-regulation of pro-inflammatory cytokines.[31] Inclusion bodies have been identified in conjunctival epithelium of dogs.[32] Although vaccines against CDV are available and effective, the incidence of CDV-induced KCS remains high in animal shelters and in some geographic regions of many countries, such as Brazil.

Based on the presence of inclusion bodies in the conjunctivae of dogs with CDV infection, we hypothesize that CDV-induced KCS promotes more severe clinical and histophatological ocular changes than non-infectious KCS. To address our hypothesis, we performed a battery of clinical and histopathological examinations in conjunctival tissues of dogs with non-infectious and CDV-induced KCS.

MATERIALS AND METHODS

This study was conducted in accordance with the guidelines of the Association for Research in Vision and Ophthalmology. It was also approved by our Institution's Ethics Committee. Forty mongrel dogs (28 males and 12 females) were assigned to three groups (G1, G2, and G3) based on physical, laboratorial (e.g. IgG titers and blood work) and ophthalmologic examinations that included: (i). Schirmer tear test (STT, Ophthalmos, São Paulo, Brazil), (ii) slit-lamp biomicroscopy (model SL 14, Kowa, Tokyo, Japan), (iii) applanation tonometry (Tono-pen XL, Mentor O & O, Norwell, MA), (iv) indirect binocular biomicroscopy (Omega 180, Heine Optotechnik), and (v) fluorescein staining (Ophthalmos, São Paulo, Brazil). Animals with non-infectious KCS were assigned to G1 (n = 10) and those with infectious CDV-induced KCS were assigned to G2 (G2 = 20). Healthy dogs without any clinical abnormalities were assigned to G3 (n = 10). The diagnosis of KCS was based on low STT values (< 8 mm/min) and conjunctival and corneal changes associated with lacrimal deficiency (e.g. conjunctival hyperemia, ocular secretion, ocular surface lesions and corneal ulcers) (Table 1).[29] The diagnosis of CDV was based on the presence of high CDV titers and abnormal clinical and neurological abnormalities including seizures, paresis, ataxia, myoclonus, and nystagmus. IgG titers were measured in sera using a commercially available ELISA kit (Immunocomb, Biogal Laboratories) that is based on a color-coded scale from 0 to 6. Overall, all dogs from G2 met the following criteria: high antibody titers, at least one neurological sign associated with CDV, and no history of vaccinations.

Table 1. Ophthalmic alterations shown by animals from the non-infectious KCS (G1), CDV-induced KCS (G2) and healthy groups (G3)*

	Ophthalmologic alterations			
Groups	STT values (mm/min)	Conjunctival hyperemia (%)	Ocular discharge (%)	Positive fluorescein test (%)
G1 (n = 10)	2.15 ± 0.49*	48.0*	70.0*	33.0*
G2 (n = 20)	2.50 ± 0.30*	45.0*	70.0*	35.0*
G3 (n = 10)	21.8 ± 0.82	0.0	0.0	0.0

*Statistically significant values (P < 0.05).

The severity of ocular inflammation (e.g. conjunctival hyperemia, ocular secretion and corneal lesions) was characterized subjectively using a scoring system developed by our laboratory: (0) absence, (+) mild, (+ +) moderate, and (+ + +) severe inflammation. Histopathological examinations were performed of the conjunctival biopsies that were collected from the most severely affected eye of dogs from G1 and G2 and from the OS of dogs from G3. Biopsies were performed using aseptic technique and local anesthesia (0.5% proparacaine chloridrate; Anestalcon®, Alcon, Brazil). Specimens were obtained (3 × 4 mm) from the superotemporal quadrant of the bulbar conjunctivae using a curved 115 mm iris scissor and a 135-mm forceps at 3–5 mm posterior to the limbus.[4,19] Conjunctival tissues were fixed in 10% buffered formalin, and after 24 h, stored in 70% alcohol for no more than 48 h at room temperature. Samples were then embedded in paraffin, subjected to 5 μm sectioning, and stained with H&E, and with periodic acid-Schiff (PAS).[32–34] Samples were then rehydrated in distilled water for 5 min, immersed in 0.5% periodic acid for 5 min, washed in distilled water for another 5 min, immersed in Schiff's reagent for 5 min, washed for 5 min in running water, immersed in sodium metabisulfite (three successive immersions for 5 min), washed for 10 min in running water, counterstained with Harris' hematoxylin for 1 min and washed in running water for 5 min. After these steps samples were dehydrated in 95% and 100% ethyl alcohol (two successive immersions for 5 min each). Finally, samples were mounted on slides, and cover-slipped using synthetic resin. Slides were examined, selected and photographed under the light microscope.[34] Goblet cells were identified by the presence of crescent-shaped nuclei associated with PAS-positive intracellular material. They were quantified by scanning each sample in a sinuous and continuous pattern (zigzag)[9,34] until 500 cells were counted.

Statistical analysis was performed using the computer software Statistical Analysis System (SAS institute Inc. 1999–2001, for Windows Version 8.2, Cary, NC). The differences

Table 2. Histopathological alterations shown by animals from the non-infectious KCS (G1), CDV-induced KCS (G2) and healthy groups (G3)

	HISTOPATHOLOGICAL ALTERATIONS						
	Inflammation						
Groups	Mononuclear cells' infiltration (%)	Mononuclear + polymorphonuclear cells' infiltration (%)	Polymorphonuclear cells' infiltration (%)	Goblet cells (number of cells/slide)	Acantholysis and degeneration	Hyperkeratosis	Inclusion bodies
G1 (n = 10)	60.0*	40	0.0	65	63*	30*	0
G2 (n = 20)	75.0*	20	5.0	10#	60*	25*	25¥
G3 (n = 10)	0.0	15.0	20.0	104	0	0	0

*#¥Statistically significant values ($P < 0.05$).

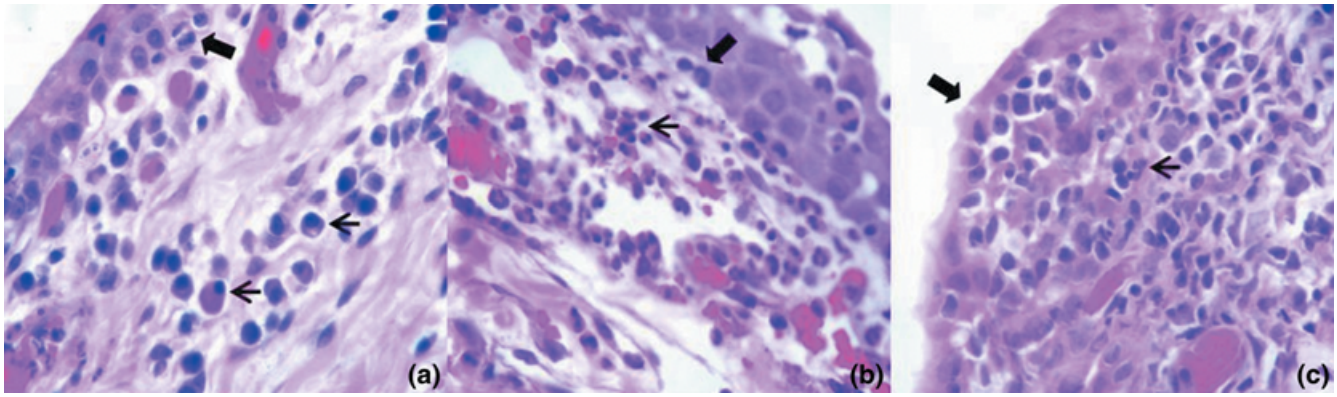

Figure 1. (a) G1-Lymphoplasmocitary inflammatory infiltration present in the epithelium (thick arrow) and adjacent stroma (arrow), H&E, 40×; (b) G3-Polymorphonuclear inflammatory infiltration present in the epithelium (thick arrow) and adjacent stroma (arrow), H&E, 40×; (c) G2-Mono and polymorphonuclear inflammatory infiltration present in the epithelium (thick arrow) and in the adjacent stroma (arrow), H&E, 40×; 266 × 77 mm (96 × 96 DPI).

between groups were investigated using χ^2 followed by the Fisher test. To determine differences in the severity of the ocular inflammation between groups, Kruskal–Wallis followed by Student Neumann–Keuls were used. The Spearman correlation test was used to evaluate potential correlations between clinical and histopathological signs. $P < 0.05$ was considered to be statistically significant.

RESULTS

Ophthalmic alterations

No differences in mean STT, conjunctival hyperemia, ocular discharge, or the fluorescein staining were observed between G1 and G2. Mean STT was significantly lower in G1 (2.15 ± 0.49 mm/min) and G2 (2.50 ± 0.30 mm/min) compared to G3 (21.8 ± 0.82 mm/min) ($P < 0.05$) (Table 1). Conjunctival hyperemia was observed in 48% and 45% of dogs from G1 and G2, respectively. Ocular discharge (muco-purulent nature) was present in 70% of dogs from G1 and G2 (Table 1). Overall, incidence of conjunctival hyperemia and ocular discharge was significantly higher in G1 and G2 when compared to G3 ($P < 0.05$). Fluorescein staining was positive in 33% of dogs from G1, 35% from G2, and 0% from G3 (Table 1). Incidence of corneal ulcers was significantly higher in G1 (33%) and G2 (35%) compared to G3 (0%) ($P = 0.0083$).

Histopathological alterations

Inflammatory infiltrates were observed in epithelial, sub-epithelial and perivascular regions of the conjunctivae for all dogs in all groups. Mononuclear cells (predominantly lymphocytes and plasmacytes) were most prominent ($P = 0.001$) in dogs from G1 (60%) and G2 (75%), suggesting that KCS promotes chronic conjunctival inflammation (Table 2; Fig. 1a). Polymorphonuclear cell infiltrates (predominantly neutrophils) were also found but to a lesser extent (40% in G1 and 20% in G2) (Table 2; Fig. 1b,c). These infiltrates had mild, moderate or accentuated intensities and their distribution was variable (focal, multifocal or diffuse). Interestingly, inflammatory cell infiltrates were also detected in G3 but to a significantly lesser extent than G1 and G2 ($P = 0.003$).

Goblet cells were identified in conjunctival tissues stained with H&E and PAS. H&E stain was able to illustrate clear morphological differences between goblet and epithelial cells. Goblet cells had a round and large shape while epithelial cells had flat and elongated nuclei, and abundant cytoplasm. PAS stain illustrated goblet cells with an intense purple hue filled with mucous content. Overall, G2 showed a significantly lower number of goblet cells than G3 ($P < 0.05$) (Table 2).

G1 (63%) and G2 (60%) had similar incidence of acantholysis and conjunctival degeneration of epithelial cells. Hyperkeratosis of mild to severe intensities was found in dogs from G1 (30%) and G2 (25%) (Table 2; Fig. 2a). Acantholysis ($P < 0.0001$),

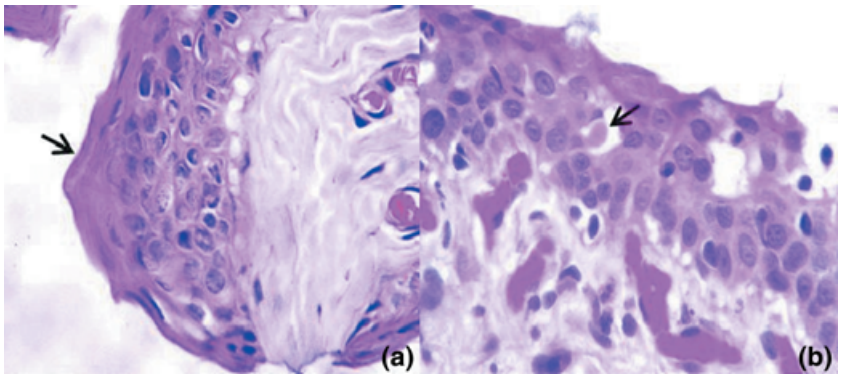

Figure 2. (a) G2- Epithelial hyperkeratosis (arrow), HE, 40×; (b) G2- Lenz viral inclusion corpuscle adjacent to the conjunctiva epithelial cells (arrow), H&E, 40×; 176 × 78 mm (96 × 96 DPI).

conjunctival degeneration ($P < 0.0001$) and keratinization ($P = 0.0471$) were predominantly found in dogs from G1 and G2. Inclusion bodies with acidophilic appearance, and round shape and edges that were free of pigmentation, were predominantly found in 25% of dogs from G2 ($P < 0.05$) (Table 2; Fig. 2b). No correlation was found between ophthalmologic and histopathological findings.

DISCUSSION

This study intended to shed light on the pathophysiology of canine CDV-induced KCS by determining histopathological and clinical changes in the conjunctivae of dogs with CDV-induced KCS and compare them to those with non-infectious KCS.

The low STT values found in dogs with CDV-induced KCS support findings by others[28–30,35] who suggest that the morbillivirus induces damage to lacrimal glands causing lacrimal deficiency. Similarly, the increased conjunctival hyperemia and mucopurulent ocular discharge found with non-infectious and CDV-induced KCS are likely due to the tear deficiency and inflammation of the ocular surface.[4,8,9] Xerophthalmia is well described to occur due to poor lubrification of the ocular surface[1,5,6] leading to ocular discomfort and pain.[2–4,15]

Immune cell infiltrates constitute the conjunctivae of humans and dogs, and were observed in the animals from G1, G2, and G3. These infiltrates are likely part of the resident population of immune cells that normally surveys the conjunctivae and builds immunity against harmful antigens.[36] Otherwise extended lymphocytic infiltrates found in the conjunctivae of dogs from G1 and G2 likely contribute to the histopathological changes found in the ocular surface and to the pathological signs of KCS.[4–6,13–15,37] Pro-inflammatory cytokines and chemokines secreted by lymphocytes and known to induce and/or exacerbate inflammation may, therefore, play a role in the ocular surface lesions noted in this study.

The presence of keratinization, acantholysis, and conjunctival degeneration observed in dogs from non-infectious and CDV-induced KCS groups is consistent with squamous metaplasia[4–6,14,17,19,20] secondary to tear film deficiency and chronic inflammation.[5,6,14,15,17,19,20] Goblet cell loss has also been associated with squamous metaplasia[1,4,6,14,17,19,20] and the results from our study support this premise. Goblet cells are responsible for the production of mucin that is an important component of the tear film.[38] Mucin deficiency is known to play an important role in the pathophysiology of KCS[14] and, therefore, goblet cell density has been suggested to be an indicator of the severity of ocular disease in KCS patients.[17] The lack of correlation between goblet cell density and STT values, acantholysis, keratinization and the degree of inflammation, however, contradict the association of goblet cell numbers with KCS severity. Similarly, although a strong correlation between inflammatory infiltrates and squamous metaplasia of the bulbar conjunctivae has been reported,[14] such correlation was not found in our study. The corneal ulcers detected in dogs from both groups are due to the tear film deficiency and chronic inflammation on the ocular surface.[18]

In summary, our study shows that CDV-induced KCS has a very similar histopathological profile to non-infectious KCS, suggesting that these two conditions may share a common pathophysiology. The lesions present at the ocular surface in the CDV-induced KCS group are consistent with lacrimal deficiency and subsequently ocular surface inflammation. Furthermore, the alterations seen in the ocular surface of dogs with CDV-induced KCS may not be due to the morbillivirus per se but to the xerophthalmia and inflammation that occurs within the ocular surface. To confirm this, further research is needed to establish the direct effects of morbillivirus on the eye.

ACKNOWLEDGMENTS

We thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (Process n°04/04700-8) for sponsoring this research. The Veterinary Pathology Laboratory at the Universidade Estadual Paulista (FCAV-UNESP), and the Universidade Federal Rural de Pernambuco (UFRPE) for technical support. We also thank Drs Patricia E. de Almeida and Erin Boyd for their review of this manuscript.

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