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Characterization of lacrimal gland lesions and possible pathogenic mechanisms of keratoconjunctivitis sicca in dogs with leishmaniosis

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Abstract

In a previous study, it was found that 2.8% of dogs with leishmaniosis had keratoconjunctivitis sicca (KCS). The aim of this study was to characterize the lesions present in the lacrimal glands of dogs with leishmaniosis and to determine the presence of the parasite by means of immunohistochemistry. The inflammatory infiltrate was described as granulomatouos or pyogranulomatous and was located around the ductal component of the glands. Immunoperoxidase staining localized the parasites following the same pattern. Samples from eyes that had clinical signs compatible with KCS presented inflammatory infiltrate and parasite more commonly than those from eyes without clinical signs. One of the mechanisms of KCS in dogs with leishmaniosis may be the inflammatory infiltrate located around the ducts of lacrimal glands, producing retrograde accumulation and retention of secretion. Meibomian gland was the most commonly affected by the infiltrate, highlighting the possibility of a qualitative KCS in these dogs.

Keywords: Keratoconjunctivitis sicca; Lacrimal glands; Immunohistochemistry; *Leishmania* spp.; Dog

1. Introduction

Canine leishmaniosis (CL) is endemic in the Mediterranean basin. In this region, it is caused by the parasite *Leishmania infantum* and it is transmitted by sand flies of the genus *Phlebotomus* (Slappendel, 1988; Ferrer, 1992; Ciaramella et al., 1997; Slappendel and Ferrer, 1998; Fisa et al., 1999; Koutinas et al., 1999; Solano-Gallego et al., 2001). CL is a systemic disease with widely variable clinical signs including skin disorders, generalized lymphadenomegaly, weight loss, lameness, ocular lesions, renal failure, epistaxis and diarrhea (Slappendel, 1988; Ciaramella et al., 1997; Koutinas et al.,

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1999). Ocular manifestations are reported to range between 16 and 80.49% of the affected animals (Slappendel, 1988; Molleda et al., 1993; Ciaramella et al., 1997; Koutinas et al., 1999; Peña et al., 2000). These signs can represent between 3.48 and 7% of presenting complaints in dogs with CL and they can be the only clinical manifestation in a percentage of cases that varies from 3.72 to 16%. The prevalence of keratoconjunctivitis sicca (KCS) in dogs suffering from leishmaniosis with ocular signs varies between 2.8 and 26.83% (Molleda et al., 1993; Ciaramella et al., 1997; Koutinas et al., 1999; Peña et al., 2000; Ciaramella and Corona, 2003).

The pathogenic mechanisms by which *Leishmania* parasites cause lesions in different organs include non-suppurative inflammatory infiltrate and the production of circulating immune complexes that deposit in various tissues (Ferrer, 1992). Often, very few amastigotes are present within the inflammatory infiltrate and, in these cases, immunoperoxidase staining has proved a sensitive and specific method to detect the parasite in tissue samples (Ferrer et al., 1988; Bourdoiseau et al., 1997). Roze (1986) cited three theories that could explain tear deficiency in dogs with leishmaniosis. Firstly, it could result from the direct destructive action of the parasite accompanied with intense inflammation of the lacrimal glands. Secondly, it could also be due to obstruction of the secretory ducts due to inflammation of the adjacent structures or finally, it could derive from reduced reflex secretion following hypoaesthesia of the damaged cornea.

Tear film is a trilaminar fluid consisting of lipid, aqueous and mucin components. The outer lipid layer is secreted by Meibomian glands (MG), the intermediate aqueous fluid is produced by main lacrimal gland (MLG) and nictitating membrane gland (NMG), and the innermost mucus layer is secreted by goblet cells of the conjunctiva. When there is a deficit of the aqueous component, quantitative KCS develops, whereas qualitative tear film disease occurs when there is a deficiency of one of the other two components of tears (Moore, 1990, 1999).

To the best of the authors' knowledge, no histopathological or parasitological studies have tried to describe the pathogenic mechanisms of KCS in dogs with leishmaniosis. The purpose of this study was to describe the histopathological findings present in lacrimal glands of dogs affected with leishmaniosis and to assess the presence of the parasite in these tissues by means of immunohistochemical techniques, in order to determine the pathogenic mechanism of keratoconjunctivitis sicca in CL.

2. Materials and methods

2.1. Animals

Twenty-eight dogs were included in the study, 25 of which were clinical cases affected with leishmaniosis and the remaining 3 were used as controls. Twenty-two of the ill dogs suffered from natural infection and three had been inoculated with *Leishmania infantum* promastigotes. Dogs with experimental infection and control dogs belonged to a research group of the Universitat Autonoma de Barcelona (UAB).

The diseased dogs were diagnosed at the Hospital Clínic Veterinari (HCV) from UAB. They all had clinical signs, complete blood count and serum biochemistry results compatible with CL. Diagnosis was confirmed with serology (Riera et al., 1999) and in some of them the parasite was detected by direct observation or amplification of DNA in bone marrow, lymph nodes or biopsies.

The three control dogs were from an endemic area and had no signs of CL. They had negative serology titers and negative delayed hypersensitivity test to leishmanin. Eighteen of the dogs with CL had been treated against *Leishmania* during a range of time that varied from 3 days to 10 years. The remaining seven dogs had not received any specific treatment.

Ophthalmic signs (Table 1) were detected in 11 of the cases by the attending clinician, four of which were evaluated by a certified ophthalmologist. Six dogs (12 eyes) had blepharitis, four dogs (8 eyes) had periocular alopecia, three dogs (5 eyes) had conjunctivitis, six dogs (11 eyes) had keratoconjunctivitis and two dogs (4 eyes) had anterior uveitis. The 22 dogs that suffered from natural infection died or were euthanized at the HCV from UAB after progressive worsening of the dogs' condition, due to non-responsiveness to treatment or because of the owner decision. The three inoculated dogs and the three control dogs were euthanized at the end of their study period.

Table 1
Clinical signs, histological and immunohistochemical results of lacrimal glands lesions from dogs with leishmaniosis

Case	Eye	Clinical signs	Meibomian glands		Main lacrimal gland		Nictitating membrane gland	
			HE	IH	HE	IH	HE	IH
1	OD	C, K	G	+	0	+	G	+
	OS	C, K	G	+	0	+	G	+
2	OD	A, C, K	G	+	0	+	G	+
	OS	A	G	+	0	+	G	+
3	OD		0	–	0	–	0	–
	OS		LP	–	0	–	LP	–
4	OD	B, C, K	G	–	0	–	0	–
	OS	B, C, K	G	–	0	–	0	–
5	OD		0	–	0	–	LP	–
	OS		0	–	0	–	LP	–
6	OD		G	–	0	–	LP	–
	OS		G	–	0	–	G	–
7	OD		0	–	0	–	G	+
	OS		0	+	/	/	G	+
8	OD	B	G	–	0	–	LP	–
	OS	B	G	–	0	–	LP	–
9	OD		LP	–	0	–	LP	–
	OS		0	–	0	–	G	–
10	OD	A, C	G	–	LP	–	0	+
	OS	A, C	G	–	LP	–	G	–
11	OD		LP	–	0	–	LP	–
	OS		0	–	0	–	LP	–
12	OD	C, K	G	+	LP	+	G	+
	OS	C, K	G	+	LP	+	G	+
13	OD		0	–	0	–	0	–
	OS		0	–	0	–	G	–
14	OD		0	–	0	–	LP	–
	OS		G	–	0	–	0	–
15	OD		0	–	0	–	G	–
	OS		0	–	0	–	0	–
16	OD		G	–	LP	–	G	–
	OS		G	+	0	–	LP	–
17	OD	A, B, C	G	–	0	–	G	–
	OS	A, B	G	–	LP	–	G	–
18	OD		G	–	/	/	G	–
	OS		G	–	0	–	G	–
19	OD	C, K	0	–	0	–	LP	–
	OS	C, K	0	–	LP	–	LP	–
20	OD		G	–	0	–	0	–
	OS		0	–	0	–	0	–
21	OD	B	G	+	LP	–	G	–

Table 1 (*Continued*)

Case	Eye	Clinical signs	Meibomian glands		Main lacrimal gland		Nictitating membrane gland	
			HE	IH	HE	IH	HE	IH
	OS	B	G	+	LP	–	G	–
22	OD		G	+	LP	+	0	–
	OS		0	+	LP	–	G	–
23	OD	B, C	G	+	0	+	G	+
	OS	B, C	G	+	LP	–	G	+
24	OD	A, B, C, K	G	+	G	+	G	+
	OS	A, B, C, K	G	+	G	+	G	+
25	OD		G	–	0	–	G	–
	OS		G	–	0	–	G	–

OD, right eye; OS, left eye; HE, hematoxilin and eosin; IH, immunohistochemistry; A, alopecia; B, blepharitis; C, conjunctivitis; K, keratoconjunctivitis; G, granulomatous to pyogranulomatous infiltrate; LP, lymphoplasmacytic infiltrate; 0, absence of infiltrate; +, presence of parasite; –, absence of parasite; /, sample that cannot be evaluated.

2.2. Collection and processing of samples

For each animal, samples of MG (eyelids), MLG and NMG of both eyes were collected. That is, three samples from each eye (six samples per animal) were obtained. MLG could not be retrieved from two eyes (left eye from case 7 and right eye from case 18) due to problems with the dissection (Table 1).

These samples were fixed in 10% neutral buffered formalin and paraffin embedded. Sections of the tissue were cut and stained for routine histological and immunohistochemical examination as described by Ferrer et al. (1988).

2.3. Statistical analysis

For statistical analysis, SPSS® software was used. In order to establish relationships between variables, contingence table analysis was performed and statistical significance was set at $P < 0.05$.

3. Results

3.1. Histopathology

No histopathologic alteration was found in the samples from control dogs (Figs. 1A and 2A) (Table 1). Occasional infiltration of lymphocytes and plasma cells were seen in the interstitium of the glands of control dogs (Figs. 1B and 2B).

Inflammatory infiltrate seen in samples from ill dogs was classified as granulomatous or pyogranulomatous. It was located mainly around the ductal component (Figs. 1C and 2C) of the MG, MLG and NMG, and occasionally around the acinar component. Only in one case this infiltrate was also seen destroying the acinar component in the three glands of both eyes (case 24). Macrophages were predominant (Figs. 1D and 2D), with scattered lymphocytes and plasma cells. Isolated neutrophils could also be seen in some sections. This periductal infiltrate, that in some cases was even destroying duct walls, provoked retrograde dilation of the ducts and retention of secretion (Figs. 2C and 5A).

In sections of the nictitating membrane in which conjunctiva could be seen, the same kind of infiltrate could also be detected in the subepithelial region of the bulbar surface, surrounding lymphoid follicles and gland secretory ducts.

This infiltrate was present in 60 out of 148 total samples (40.54%) that belonged to 37 eyes (74%) from 21 dogs (84%). No significant differences between left and right eye were found ($P = 0.738$).

Inflammatory infiltrate presence in each of the glands is represented in Fig. 3. MG was the most commonly affected gland, but there was no statistical significance in infiltrate presence between this structure and NMG ($P = 0.418$). Only two MLG had this infiltrate, and they were from the same dog (case 24). Differences were statistically significant between MG and MLG ($P < 0.001$) and between

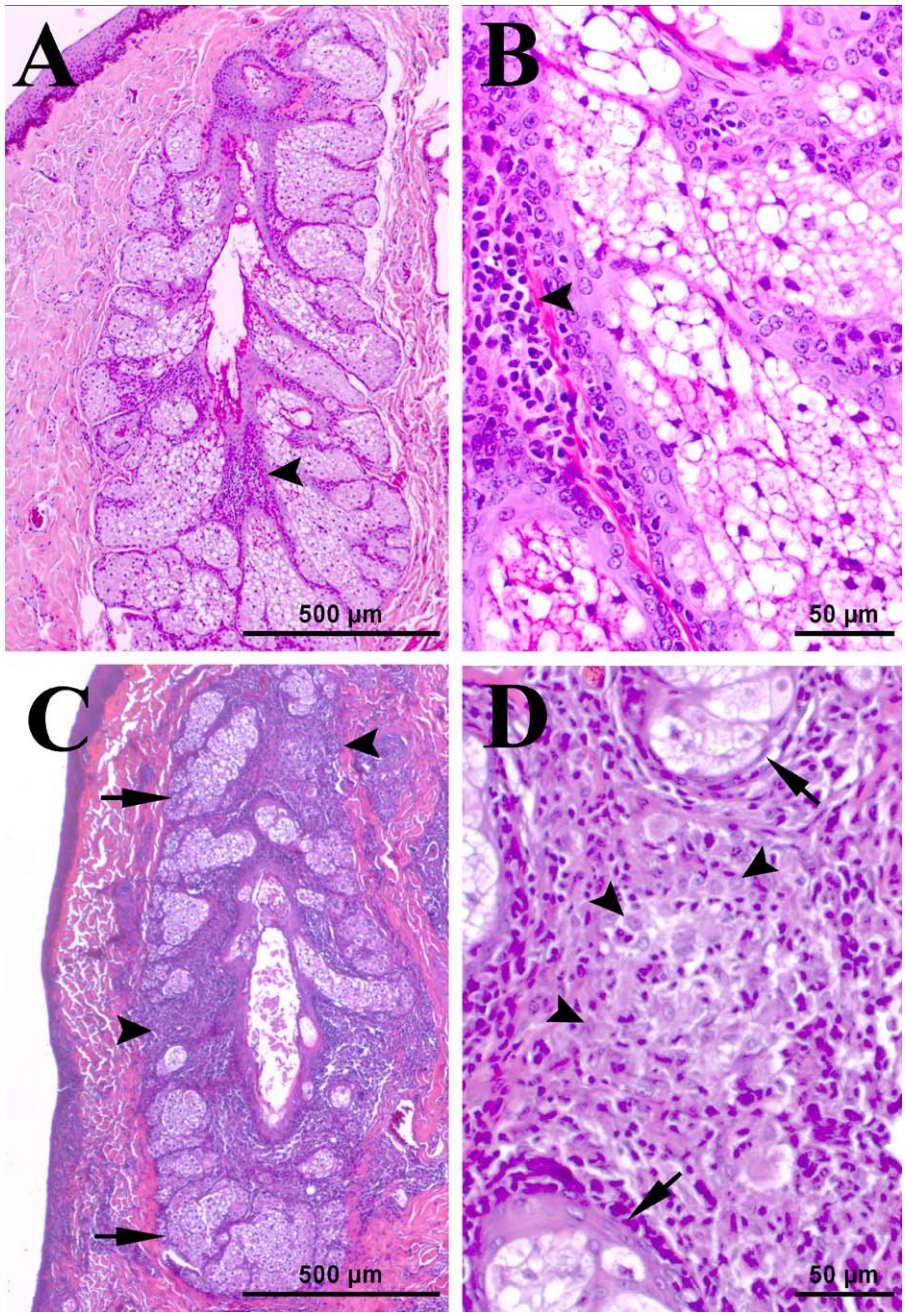

Fig. 1. Meibomian gland. (A and B) Control dog. (A) Focal periductal infiltrate (arrowhead). (B) Scant periductal infiltrate composed of lymphocytes and plasma cells (arrowhead). (C and D) Dog with leishmaniosis. (C) Granulomatous to pyogranulomatorus periductal inflammatory infiltrate (arrowheads). Acinar component is preserved (arrows). (D) Macrophages (arrowheads) were predominant, with scattered lymphocytes, plasma cells and neutrophils. H&E.

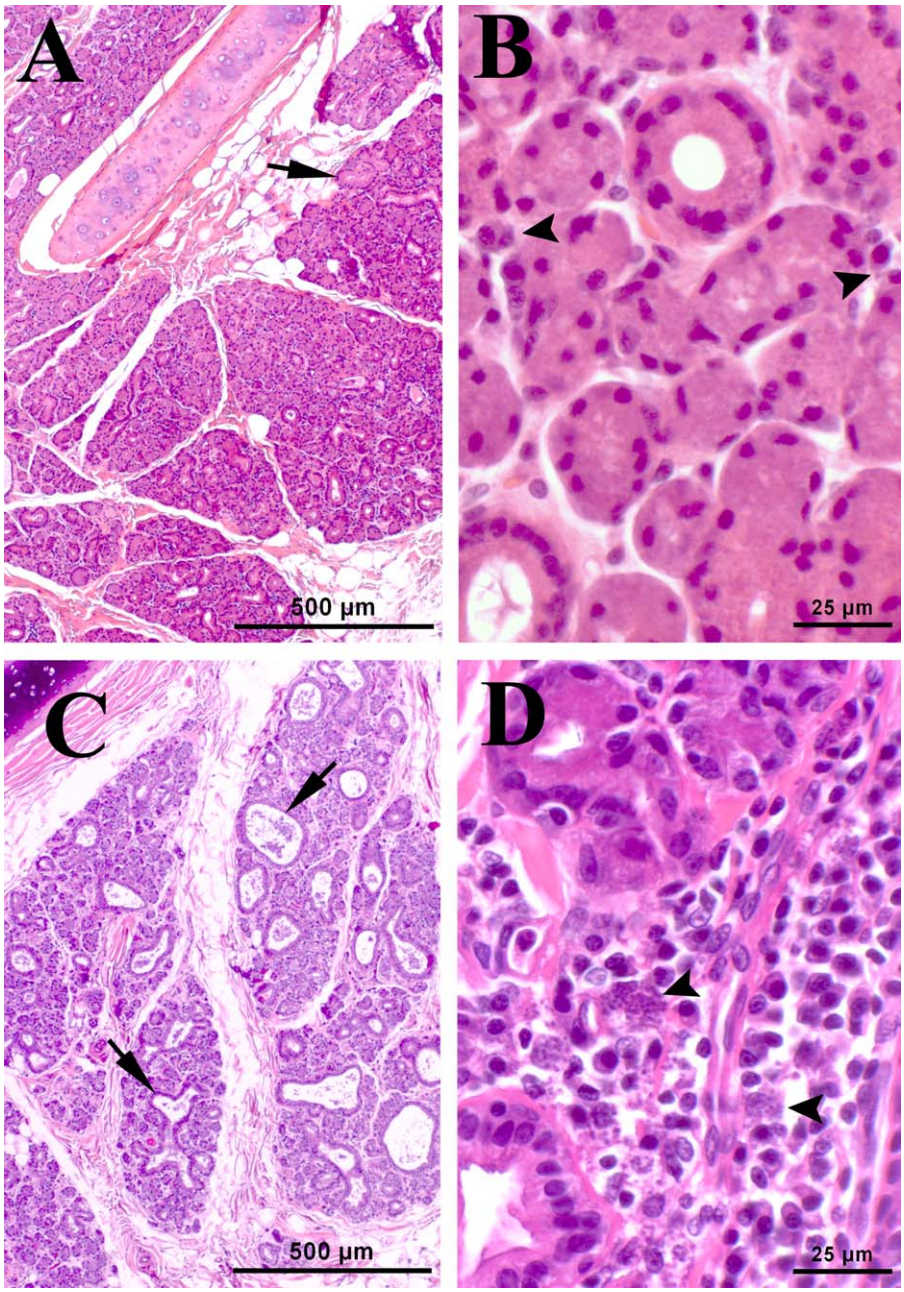

Fig. 2. Nictitating membrane. (A and B) Control dog. (A) Normal gland architecture with non-dilated ducts (arrow). (B) Acinar component with scarce interstitial lymphocytes and plasma cells (arrowhead). (C and D) Dogs with leishmaniosis. (C) Dilation of ducts with retention of secretion (arrows) and granulomatous to pyogranulomatous periductal inflammatory infiltrate. (D) Macrophages with intracytoplasmic amastigotes (arrowheads), lymphocytes and plasma cells. H&E.

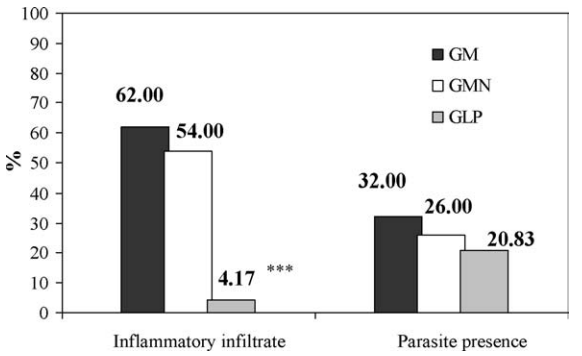

Fig. 3. Percentage of granulomatous/pyogranulomatous inflammatory infiltrate and parasite presence in lacrimal glands of dogs with leishmaniosis. Number above bar is the percentage of cases represented by the bar (50 MG and NMG and 48 MLG were examined). MG, Meibomian gland; MLG, main lacrimal gland; NMG, nictitating membrane gland. ***Significant differences between MG and MLG ($P < 0.001$) and between NMG and MLG ($P < 0.001$).

NMG and MLG ($P < 0.001$). Of the 37 eyes affected, only in 2 of them (5.4%) the infiltrate was present in the three glands. These two eyes were from the same dog (case 24), which suffered from natural infection. Nineteen eyes (51.35%) had two of the three glands affected, and these glands were always MG and NMG. Sixteen eyes (43.24%) had only one gland affected. In 10 of these eyes, this structure was MG and, in the remaining 6 eyes, NMG was the only one affected.

Samples belonging to eyes with clinical signs compatible with KCS were more commonly affected by the inflammatory infiltrate described than those from eyes without clinical signs ($P < 0.001$) (Fig. 4). When evaluating ocular clinical signs separately, MG

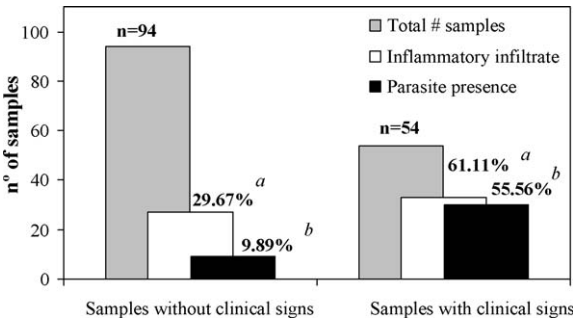

Fig. 4. Inflammatory infiltrate and parasite presence in samples from dogs without clinical signs related to KCS (left) or with clinical signs related to KCS (right). [a]Significant differences in inflammatory infiltrate $P < 0.001$. [b]Significant differences in parasite presence $P < 0.001$.

from eyes with periocular alopecia, blepharitis and conjunctivitis were more commonly affected by the inflammatory infiltrate ($P = 0.018$, 0.002 and 0.011). There was also relationship between MLG infiltration and occurrence of keratoconjunctivis ($P = 0.049$). There was no association between MG affection and keratoconjunctivitis ($P = 0.125$), MLG and conjunctivitis ($P = 0.106$), NMG and conjunctivitis ($P = 0.151$) and NMG and keratoconjuntvitis ($P = 0.468$).

Clinical signs compatible with KCS were more commonly related to those eyes that had two or three glands affected by the infiltrate than to those eyes in which no lacrimal gland or only one of the three glands was affected by the inflammatory infiltrate ($P = 0.001$).

3.2. Immunohistochemistry

Parasite was not observed in any of the samples from the control dogs (Table 1).

In the samples from ill dogs, macrophages with immunolabeled parasites (Fig. 5A and B) were located around the ductal component of the glands studied, following the same pattern as the inflammatory infiltrate.

Parasite was visualized in 39 out of 148 samples (26.35%) that belonged to 18 eyes (36%) from 10 animals (40%). Differences between left and right eyes were not statistically significant ($P = 0.351$).

Parasite presence in the different types of glands is represented in Fig. 3. No statistical significance was found between the different tissues ($P = 0.454$), but in the 10 MLG in which parasite was found, only one or two organisms were detected in each sample.

Within the 18 eyes that had the parasite detected, it was present in the three glands in 9 of them (50%). In three eyes (16.6%) parasite was seen in two of the three glands. One of these two glands was MG in the three eyes, and the other gland that contained the parasite was the NMG in two eyes and MLG in one eye. Six eyes (33.3%) had the parasite in only one gland. This gland was MG in four eyes and NMG in two eyes.

Parasite was more frequently detected in samples obtained from eyes that had clinical signs than in those from eyes that had not ($P < 0.001$) (Fig. 4). Those eyes in which more than one gland contained the parasite had clinical signs more commonly than those

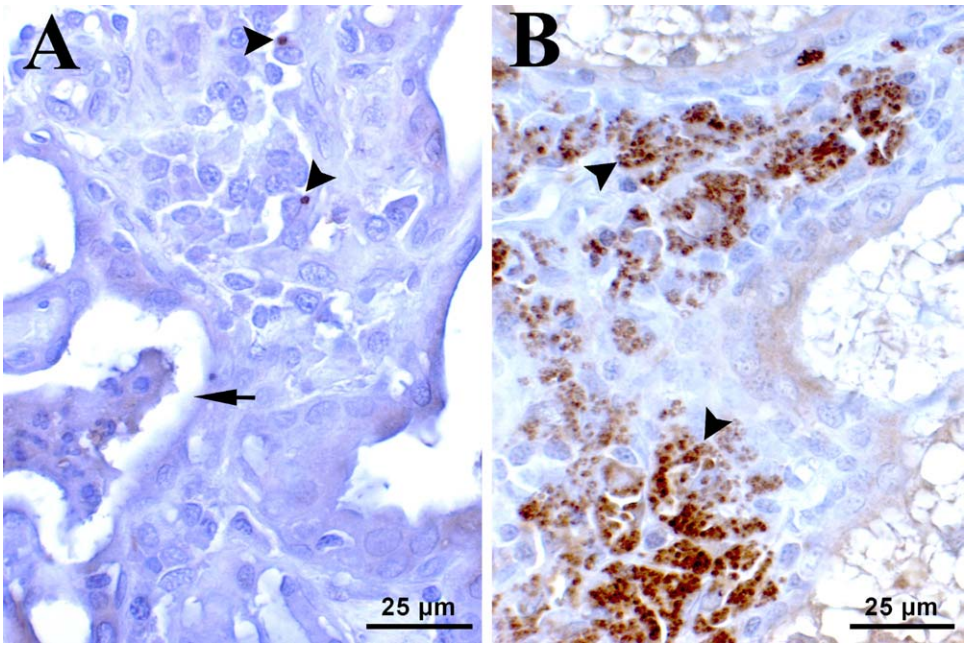

Fig. 5. Immunohistochemistry to *Leishmania.* (A) Nictitating membrane. Dilation of ducts with retention of secretion (arrow). Macrophages with immunolabeled parasites within the cytoplasm (arrowheads). (B) Meibomian gland. Granulomatous periductal inflammatory infiltrate composed of macrophages packed with immunolabeled amastigotes (arrowheads).

in which only one gland or no gland had the parasite detected ($P = 0.002$). There was an association between eyes that had conjunctivitis and parasite presence in the three glands ($P = 0.012$ for MG and $P < 0.001$ for MLG and NMG) and eyes that had keratoconjunctivitis and parasite presence in the three glands ($P = 0.011$ for MG, $P < 0.001$ for MLG and $P = 0.001$ for NMG). There was no relationship between parasite presence in MG and alopecia ($P = 0.234$) or blepharitis ($P = 0.125$).

Presence of granulomatous infiltrate was related to presence of parasite when all samples were considered together, regardless of the tissue considered ($P < 0.001$), and also when every gland was considered separately ($P = 0.012$, 0.005 and 0.001 for the MG, MLG and NMG, respectively).

4. Discussion

Although KCS is a potentially blinding disease, no attempt had been made to determine the etiology of this disease in dogs suffering from leishmaniosis. Results obtained in this study allow us to propose a pathogenic mechanism by which KCS in dogs with leishmaniosis may develop. Granulomatous infiltrate that accumulates around lacrimal glands ducts provokes retrograde dilation with accumulation of secretion. These findings are in agreement with the second theory postulated by Roze (1986). We have not seen destruction of the acinar component of the glands in any of the samples, not even in the case in which we detected the inflammatory cells and the parasite around the acinis.

In our study we have not evaluated the contribution of corneal hypoaesthesia to the decrease in tear secretion. Inflammation in the cornea, specially when it becomes edematous and hyperpigmented, can diminish corneal sensation and thus decrease reflex component of tears (Xu et al., 1996; Mathers, 2000). Due to the design of our study, in which we obtained the samples just after death or euthanasia of the dogs, we could not determine neither corneal sensitivity nor Schirmer Tear Tests (I and II). Anyway, if a dog would have been examined for evaluation of corneal disease, it would have been impossible to determine what had

occurred first: primary keratitis due to leishmaniosis with subsequent decrease in Schirmer Tear Test or corneal alteration following the development of KCS.

In another study (García-Alonso et al., 1996), histopathological findings in two dogs affected with CL revealed intense inflammatory reaction with granulation around lacrimal ducts, composed of lymphocytes, plasma cells and macrophages. These authors also described epithelial necrosis and partial loss of epithelium, but they did not discuss about the possible implications on tear deficiency. We have not seen granulation tissue or necrosis in our samples.

Inflammatory infiltrate detected in samples from control dogs, composed of scattered lymphocytes and plasma cells, is the one described in normal lacrimal glands from healthy dogs (Martin et al., 1988). These plasma cells are responsible for the production of IgA that tears contain (Martin et al., 1988; Schlegel et al., 2003). So, we have not considered it as pathologic when we have seen it either in the samples from control or diseased dogs.

MG was the most commonly affected by the inflammatory infiltrate in diseased dogs, although differences with NMG were not statistically significant. MG produce the most external layer of the lacrimal film. When these glands are damaged, lipid secretion gains polarity and therefore early evaporation of tears occurs. These altered lipids may also be directly toxic to corneal surface. This combination of insults leave the cornea unprotected and they can lead to surface disease. Qualitative tear film disease can evolve with a Schirmer Tear Test of over 15 mm, due to reflex tearing that tries to compensate for the early evaporation and dilute abnormal lipids secreted (Moore, 1990, 1999). So, it is possible that dogs with leishmaniosis might suffer from a qualitative tear film disease, although it has not been clinically evaluated in this study nor cited in other publications.

NMG was more frequently affected than MLG. In addition, we have seen inflammatory infiltrate not only in the ductal component of the gland, but also subepithelially in the bulbar surface of nictitating membrane, where secretory ducts exit (Moore et al., 1996). This can create even more retention of secretion in these glands. NMG can produce between 30 and 50% of aqueous component of tears, depending on the individual dog (Helper et al., 1974; Gelatt et al., 1975). So it is possible that, when this gland is massively infiltrated in dogs whose NMG synthesizes a high proportion of the tears, KCS can develop, even when MLG remains unaffected. Conversely, in those dogs whose aqueous component of tears is mainly secreted by MLG, lesions in NMG can have no clinical consequences. This also occurs in animals in which NMG is removed, as in some of them MLG can compensate increasing its production (Helper et al., 1974; Gelatt et al., 1975; Dugan et al., 1992).

In addition, although we have not evaluated goblet cells systematically, subepithelial infiltration of macrophages and parasites in nictitating membrane sections in which bulbar surface of conjunctiva could be seen suggests that mucous secretion can be also affected in these dogs.

The fact that MLG has been the least frequently affected may be due to its location. Whereas MG and NMG are located near tissues presumed to have high concentrations of parasites, MLG is situated within orbital tissue and it can be more difficult to reach for the parasite. MG are located in the eyelid, the external part of which is a prolongation of the skin, and they are modified sebaceous glands (Moore, 1990, 1999). Eyelids are highly vascularized tissues, and in other studies on leishmaniosis (Koutinas et al., 1992; Solano-Gallego et al., 2004), skin sebaceous glands have been observed totally obliterated by perifollicular granulomatous inflammation. NMG is located in the nictitating membrane, which is a fold of the conjunctiva. Conjunctival biopsies or scrapings have been used in many studies to diagnose leishmaniosis (Berrahal et al., 1996; Solano-Gallego et al., 2001; Strauss-Ayali et al., 2004), as it is a tissue that usually harbors high amounts of parasites.

Samples with inflammatory infiltrate and parasite presence were associated to eyes with clinical signs compatible with KCS. This is in disagreement with what has been reported in another study about KCS from other causes, in which histopathologic lesions in lacrimal glands were not correlated to severity of clinical signs (Kaswan et al., 1984), although we have not graded clinical nor microscopical severity. Despite this relationship we have found, there were samples with infiltrate and parasite that belonged to eyes without clinical signs. This discordance may be due to an underestimation of clinical signs in our study, given the fact that not all the dogs were submitted to a systematic ophthalmic examination. It is also possible

that a certain degree of inflammation or parasite presence is required in order to achieve the retention of secretion necessary to produce KCS. That means that minimal inflammation or parasite presence can be associated with subclinical degrees of KCS.

Another possible explanation is that the affection of more than one gland may be necessary in order to produce KCS. This hypothesis is supported by the association that we have found between eyes in which there are two or three glands affected, either by the inflammatory infiltrate and/or the parasite, and incidence of clinical signs. This is in agreement with the fact that in autoimmune KCS, it is believed that MLG and NMG are affected simultaneously when they produce the disease (Helper, 1976; Kaswan et al., 1984).

Although statistical relationships have been found between some specific ocular signs and each of the glands, numbers are too low to interpret these results. Many of the dogs included in the study had been treated for leishmaniosis during varying amounts of time. This fact could also have influenced the disagreements between microscopic findings and presence of clinical signs. The treatment could have improved some of the ophthalmologic signs and it could also have diminished inflammatory infiltrate or even make the parasite disappear from the tissue. It can be discussed that lesions we have seen in these lacrimal glands can be due to immune mediated KCS, as many dogs included in the study were from breeds predisposed to this condition (ACVO, 1999). Histopathologic lesions in spontaneous KCS have been described elsewhere (Kaswan et al., 1984; Izci et al., 2002), and they include massive lymphoplasmacytic infiltrate with acinar atrophy and fibrosis in advanced cases. We have not seen any of these alterations, and inflammatory infiltrate observed in our study is typical of leishmaniosis and is related to parasite presence. In conclusion, one mechanism by which KCS in dogs with leishmaniosis occurs is the dilation of the ducts of lacrimal glands due to pyogranulomatous inflammation surrounding these ducts, induced by the presence of amastigotes leading to accumulation and retention of secretion.

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