PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
DOUTORADO EM FISIOPATOLOGIA E SAÚDE ANIMAL

MARCOS ROGERIO SGRIGNOLI

USO TÓPICO DE CÉLULAS-TRONCO MESENQUIMAIS HETERÓLOGAS DE TECIDO ADIPOSO CANINO NO TRATAMENTO DA CERATOCONJUNTIVITE SECA EM CÃES

Presidente Prudente - SP
2018

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Tese, apresentada a Pró-Reitoria de Pesquisa e Pós-Graduação, Doutorado em Fisiopatologia e Saúde Animal, Universidade do Oeste Paulista, como parte dos requisitos para obtenção do título de Doutor em Fisiopatologia e Saúde animal.

Orientador: Dra. Silvia Maria Caldeira Franco Andrade

Presidente Prudente - SP
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Presidente Prudente, 20 de novembro de 2018

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“Sem a presença de Deus nosso criador, nada tem sentido”.

RESUMO

Uso tópico de células-tronco mesenquimais heterólogas de tecido adiposo canino no tratamento da ceratoconjuntivite seca em cães

A ceratoconjuntivite seca (CCS) é uma doença ocular inflamatória crônica, principalmente imunomediada em cães e homens. Estudos descrevem o uso de células tronco mesenquimais (CTM) injetável na glândula lacrimal canina no tratamento da CCS. Este é o primeiro estudo com uso tópico de CTM que seria uma técnica mais prática. O objetivo desta pesquisa foi analisar a eficácia do uso tópico de CTM heterólogas de tecido adiposo canino no tratamento da CCS em cães. Foram utilizados 22 cães com diagnóstico bilateral para CCS avaliados pelos sinais oculares, Teste Lacrimal de Schirmer 1 (TLS-1), Teste de Ruptura do Filme Lacrimal (TRFL), Teste de Fluoresceína (TF) e Teste de Lissamina Verde (TLV). Para análise citológica foi realizado citologia esfoliativa conjuntival e para análise histopatológica foi realizado biopsia conjuntival e punção aspirativa por agulha fina da glândula da terceira pálpebra. Os animais foram tratados topicamente com 50µl ($1x10^6$ CTM), em ambos os olhos, em 4 aplicações com intervalo de 7 dias, e colírio lubrificante à base de propilenoglicol, 1 gota, 3 vezes ao dia, em ambos os olhos, somente por 30 dias, e observados por 180 dias com exames oculares periódicos. Houve uma melhora significativa ($p<0.05$) nos sinais clínicos oculares e na resolução das úlceras de córnea, e aumento significativo ($p<0.05$) no TRFL e TLS-1, até o final do estudo. Houve retorno de secreção ocular e conjuntivite em 7 animais (31,8%) até o final do estudo. Tanto na avaliação citológica quanto histopatológica houve aumento significativo ($p<0.05$) do número das células caliciformes e diminuição significativa ($p<0.05$) das células inflamatórias polimorfonucleares e mononucleares, metaplásicas e escama córnea. Houve redução de todos os marcadores (CD4, IL-6, IL-1, TNFα) em ambas as técnicas histopatológicas avaliadas (imunohistoquímica e imunocitoquímica). O uso tópico de CTM e o protocolo terapêutico utilizado demonstraram grande potencial no tratamento adjuvante da CCS. Houve diminuição significativa dos marcadores inflamatórios. Além disso, esses marcadores podem ser excelentes ferramentas para o diagnóstico e progressão da CCS, porém, alguns animais apresentaram retorno dos sinais clínicos da CCS, possivelmente devido à uma reativação da agressão imunomediada às glândulas lacrimais. Desta maneira, mais estudos devem ser realizados para minimizar a agressão imunomediada, possivelmente com o uso de imunossupressores após a aplicação tópica de CTM.

Palavras-chave: Células-tronco mesenquimais. Tópico. Ceratoconjuntivite seca. Marcadores Imunológicos. Olho seco. Citologia. Histopatologia, Cães.

ABSTRACT

Topical use of heterologous mesenchymal stem cells of canine adipose tissue in the treatment of dry keratoconjunctivitis sicca in dogs

Dry keratoconjunctivitis (CCS) is a chronic inflammatory ocular disease, mainly immunomediated in dogs and men. Studies describe the use of injectable mesenchymal stem cells (CTM) in the canine lacrimal gland in the treatment of CCS. This is the first study with topical use of CTM that would be a more practical technique. The objective of this research was to analyze the efficacy of topical use of heterologous CTMs of canine adipose tissue in the treatment of CCS in dogs. Twenty-two dogs with bilateral diagnosis for CCS evaluated by ocular signs, Schirmer's Lacrimal Test 1 (TLS-1), Lacrimal Film Rupture Test (TRFL), Fluorescein Test (TF) and Green Lissamina Test (TLV) were used. For cytological analysis, conjunctival exfoliative cytology was performed and histopathological analysis was performed conjunctival biopsy and fine needle aspiration of the gland of the third eyelid. The animals were topically treated with 50µl (1x106 CTM) in both eyes in 4 applications at 7 day intervals, and propylene glycol lubricant eye drops, 1 drop, 3 times a day, in both eyes, for only 30 days, and observed for 180 days with periodic ocular exams. There was a significant improvement ($p < 0.05$) in ocular clinical signs and resolution of corneal ulcers, and a significant ($p < 0.05$) increase in TRFL and TLS-1 by the end of the study. There was a return of ocular secretion and conjunctivitis in 7 animals (31.8%) until the end of the study. In the cytological and histopathological evaluation, there was a significant increase ($p < 0.05$) in the number of goblet cells and a significant decrease ($p < 0.05$) in polymorphonuclear and mononuclear, metaplastic, and corneal scales. All markers (CD4, IL-6, IL-1, TNFα) were reduced in both histopathological techniques evaluated (immunohistochemistry and immunocytochemistry). The topical use of CTM and the therapeutic protocol used demonstrated great potential in the adjuvant treatment of CCS. There was a significant decrease in inflammatory markers. In addition, these markers may be excellent tools for the diagnosis and progression of CCS, but some animals showed a return of clinical signs of CCS, possibly due to a reactivation of immune-mediated aggression to the lacrimal glands. In this way, more studies should be performed to minimize the immune-mediated aggression, possibly with the use of immunosuppressants after the topical application of CTM.

Key words: Mesenchymal stem cells. Topic. Dry keratoconjunctivitis. Immunological Markers. Dry eye. Cytology. Histopathology, Dogs.

SUMÁRIO

1 ARTIGO 1

Topical mesenchymal stem cells improve clinical signs and ocular exams in animal model with keratoconjuntivitis sicca

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Topical mesenchymal stem cells improve clinical signs and ocular exams in animal model with keratoconjunctivitis sicca

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Abstract

Background: Keratoconjunctivitis sicca (KCS) is a chronic inflammatory ocular disease that is mainly immune-mediated in dogs and humans. Studies have described the use of injectable mesenchymal stem cells (MSC) in the canine lacrimal gland in the treatment of KCS. This is the first study to report the topical use of MSC as a more practical technique. The objective of this research was to analyze the efficacy of the topical use of heterologous MSC of canine adipose tissue in the treatment of KCS in dogs.

Methods: The ophthalmic signals of twenty-two dogs with a bilateral positive diagnosis of KCS were evaluated with a slit lamp (ocular secretion, conjunctivitis, corneal opacity, corneal pigmentation and neovascularization) and the Schirmer tear test-1 (STT-1), fluorescein test (FT), lissamine green test (LGT) and tear film break-up time (TBUT), and cytological and histopathological analysis was conducted. In the conjunctival sac, 50 µl ($1x10^6$ MSC) was used topically in 4 applications with intervals of 7 days, and 1 drop of lubricant eye solution was applied 3 times a day for 1 month. The dogs were then followed monthly for 6 months.

Results: There was a significant improvement ($p<0.05$) in the clinical ocular signs and resolution of corneal ulcers and a significant ($p<0.05$) increase in STT-1 and BUT values by

the end of the study. There was a return of ocular secretion and conjunctivitis in 7 animals (31.8%) until the end of the study. Cytological and histopathological evaluation showed that there was a significant decrease ($p<0.05$) in the number of polymorphonuclear, mononuclear, metaplastic, and squamous cells and a significant increase ($p<0.05$) in the number of goblet cells.

Conclusions: The topical use of MSC and the therapeutic protocol utilized demonstrated great potential in the adjuvant treatment of KCS; however, some animals showed a return of the clinical signs of KCS, possibly due to a reactivation of immune-mediated aggression to the lacrimal glands. Therefore, more studies should be performed to avoid the return of immune-mediated reactions after the use of MSC.

Keywords: Mesenchymal Stem Cells, Topical, Dry Keratoconjunctivitis, Dry Eye, Cytology, Histopathology, Dogs.

Background

Keratoconjunctivitis sicca (KCS), or dry eye syndrome, is a pathology of social, economic and emotional importance because it is a chronic ocular degenerative inflammatory disease that directly affects the quality of life of the patients. It is also one of the major causes of ocular morbidity. KCS decreases the quantity of tears, modifies their quality and/or decreases their stability, leading to greater evaporation. Ocular signs include mucopurulent secretion, conjunctivitis, keratitis, pigmentation and corneal ulcer, leading to ocular discomfort and, in more severe cases, blindness [1-4].

Immune-mediated reactions in the lacrimal glands are the most important causes of KCS in humans and dogs [5-7]. Therefore, dogs are an excellent animal model for the study of this disease. Studies in patients with KCS attribute as the cause of chronic inflammation of the ocular surface and lacrimal glands to increased levels of CD4 (T helper), cytokines, tumor necrosis factor-α (TNFα), interleukins and interferon (IFN)-γ [5-8]. The immuno-mediated reaction mainly involves a decrease or alteration in tear film quality, a decrease in mucin secretion and an increase in metalloprotease level [1-4]. Conventional KCS treatment mainly consists of topical immunosuppressants, such as cyclosporine, tacrolimus, pimecrolimus, and, more recently, lifitegrast. These immunosuppressants are associated with lubricants or artificial tears, and one of their weaknesses is that they are palliative and not curative, with the cost of topical immunosuppressive treatments ranging from medium to high, and many KCS patients, after being treated for some time, no longer respond to conventional therapy [1-3,9-15].

In this context, there is the possibility of using stem cells that have the capacity for tissue regeneration and have shown therapeutic potential in the treatment of degenerative ocular diseases such as dry eye [16-22]. Mononuclear stem cells can be multipotential (hematopoietic stem cells) or pluripotent (mesenchymal stem cells - MSC). They have the ability to differentiate into a variety of cell types, such as osteogenic, adipogenic, chondrogenic, and myogenic cells and many other secretory cells, depending on

physiological or experimental conditions [18-20]. The use of mononuclear stem cells is viable because they can be cultivated and isolated, thus maintaining their potential [16-19].

The use of stem cells in immune-mediated diseases is explained by their regenerative capacity that restores tissues destroyed by the action of autoantibodies and by their immunomodulatory action caused by the release of bioactive molecules. These molecules are immunosuppressive, especially for T helper CD4 lymphocytes and B lymphocytes, thus inhibiting the release of interleukin as IL-6 present in KCS, in addition to stimulating angiogenesis and cellular mitosis, inhibiting apoptosis and the release of proinflammatory mediators [16-22].

Studies have described the efficacy of MSC in the treatment of KCS in laboratory animals and dogs: injections in the lacrimal gland in mice with experimentally induced KCS [18], topical use in rats with experimentally induced KCS by benzalkonium chloride (BAC) [19], injections in the lacrimal gland in dogs with KCS refractory to conventional topical treatments [20], injections into the dorsal lacrimal gland and third eyelid in dogs [21] and injections around the nictitant and orbital gland in dogs with KCS [22].

Due to the importance of KCS in humans and dogs and the need for new therapies, the objective of the present study was to analyzing the efficacy of the therapeutic protocol, the route of administration and the use of heterologous MSC derived from the adipose tissue of dogs.

Methods

Animals

Twenty-two dogs with bilateral positive diagnoses for KCS from the ophthalmology service of the Veterinary Hospital of UNOESTE, Presidente Prudente, SP, Brazil, were used.

The animals included in the study presented a Schirmer tear test-1 (STT-1) score of 5-10 mm/min or a tear film break-up time (TBUT) of ≤ 15 seconds, in addition to presenting ocular signs of KCS such as opacity, ocular secretion, conjunctivitis, pigmentation, and neovascularization and ulcer of the cornea. Animals with STT-1 of 0-4 mm/min were excluded from the study (due to the severity of KCS with the possibility of low presence of viable cells in the conjunctiva, lacrimal gland and third eyelid that respond to MSC treatment), cancer patients (due to the risk of neoplastic cell enlargement) and use of corticosteroids and/or immunosuppressants for at least 1 month (due to possible interference with the viability of the MSC). Animals that were not successfully treated during the first 30 days were directed to conventional treatment for KCS.

Experimental design

The animals were followed for 180 days (D0, D7, D14, D21, D30, D60, D90, D120, D150 and D180). The ocular signs were evaluated with a slit lamp (ocular secretion, conjunctivitis, opacity, and corneal pigmentation). The ophthalmic exams consisted of an Schirmer tear

test-1 (STT-1) without anesthetic, fluorescein test (FT), lissamine green test (LGT), TBUT, cytological analysis by conjunctival cytology (CC), and histopathological analysis by conjunctival biopsy (CB). The experimental design with topical MSC treatment, with the time in days, and the tests performed at each time are described in Table 1.

Table 1. Scheme of the experimental design of dogs (n=22) with bilateral keratoconjunctivitis sicca and treated with heterologous mesenchymal stem cells (MSC) from canine adipose tissue.

Time (days)	**Treatment**		**Specific Tests***
	MSC	**Ocular Lubricant**	
0	No	Treatment with lubricant eye drops begins at day 0 and remains for only 30 days in both eyes 3x daily	Clinical signs, STT-1, TBUT, FT, LGT, CC, CB
7	Yes		Clinical signs and STT-1
14	Yes		Clinical signs and STT-1
21	Yes		Clinical signs and STT-1
30	Yes		Clinical signs and STT-1
60	No	No	Clinical signs, STT-1, FT, TBUT, LGT
90	No	No	Clinical signs, STT-1, FT, TBUT, LGT, CC
120	No	No	Clinical signs, STT-1, FT, TBUT, LGT
180	No	No	Clinical signs, STT-1, TBUT, FT, LGT, CC, CB

*(STT-1) Schirmer tear test-1, (FT) fluorescein test, (LGT) lissamine green test, (TBUT) tear film break-up time, (CC) conjunctival cytology, (CB) conjunctival biopsy.

Ophthalmic exams

The ocular signs (opacity, pigmentation and neovascularization of the cornea, ocular secretion and conjunctivitis) were evaluated with a portable slit lamp (Kowa, Japan) using the following scores: (1) negative; (2) mild; (3) moderate; (4) severe. In addition, the dogs' eyes were photographed at all times for macroscopic comparative analysis of disease progression.

The STT-1 (Teste de Schirmer®- Laboratory Ophthalmos, São Paulo, Brazil) was performed without anesthetic eye drops to quantitatively evaluate the portion of the tear by introducing 0.5 cm of the strip into the conjunctival sac for one minute; values ≤ 10 mm/min were considered positive. TBUT was used to qualitatively evaluate the portion of tears: two measurements were made consecutively, and the mean was calculated. After instilling one

drop of 1% fluorescein eye drops (Allergan, São Paulo, Brazil) with the slit lamp (Kowa, Japan), the time between the last blink and the first appearance of spots or dark spots on the lacrimal film was considered positive for TBUT if the values were ≤ 15 seconds.

The FT was performed using one drop of 1% fluorescein eye drops (Allergan, São Paulo, Brazil). The eyes were washed with a physiological solution, and then the presence or absence of corneal ulcers was evaluated and graded (1 - none, 2 - small superficial ulcer, 3 - medium superficial ulcer, 4 - extensive superficial ulcer, 5 - small stromal ulcer, 6 - medium stromal ulcer, 7 - extensive stromal ulcer, 8 – descemetocele, and 9 - keratomalacia or melt ulcer).

The LGT was used to evaluate the presence of devitalized corneal and conjunctival cells with a green lissamine strip (Laboratory Ophthalmos, São Paulo, Brazil); the strip was placed in contact with the tear meniscus of the fundus of the lower bag, and the analysis was carried out after 2 minutes. The following scoring system was used: (1) no staining, (2) only the conjunctiva was stained, (3) only the cornea was stained, (4) both conjunctiva and cornea were stained.

Cytological and histopathological analysis

Cytology was evaluated after the eye had been cleaned with a physiological saline solution. Cells were obtained from the lower conjunctiva with a sterile swab that had been moistened with the physiological solution. Staining was performed with the MGG technique (May-Grunwald-Giemsa). Lymphocytes, neutrophils, metaplastic cells, and squamous cells in 10 fields were counted under an optical microscope with a 40x objective lens. The histopathological examination was performed with a withdrawal of 1-3 mm after one drop of Anestésico® eye solution (1% tetracaine hydrochloride + 0.1% phenylephrine hydrochloride, Allergan, São Paulo, Brazil) was instilled in the fornix of the medial inferior conjunctiva using tweezers and conjunctive scissors.

The histological section was placed on standardized paper 1 × 1 cm in size, fixed in formaldehyde and embedded in paraffin (Dynamics Analytical Reagents, São Paulo, Brazil). With the help of a rotating microtome, 5-µm-thick sections of the conjunctiva were obtained, stained with hematoxylin and eosin (HE) (Dolles, São Paulo, Brazil) and PAS (Merck, USA) and subsequently evaluated for the following parameters: polymorphonuclear and mononuclear counts, presence of squamous metaplasia and edema using HE staining, and goblet cell density in 10 fields using PAS counting with a 40x objective lens.

Mesenchymal Stem Cells

The MSC were supplied by the Regenera Laboratory of Campinas, Brazil and enrolled in the Regional Council of Veterinary Medicine of the State of São Paulo under CRMV-SP 33715-PJ (patent filing no. 860130011774). All MSC were tested to confirm their ability to differentiate into osteoblasts, adipocytes, and chondrocytes and were adequately induced under medium conditions. The expression of CD44, CD73, CD90, CD105 typical C-TM markers, vimentin, nestin, and OCT3/4 and the absence of CD14, hematopoietic and CD45 markers were evaluated using an immunofluorescence assay. The cells were also screened

for the elimination of pathogens and contaminants (e.g., bacteria, fungi, viruses, mycoplasma and endotoxins). MSC were supplied in cryotube vials with $1x10^6$ MSC unit doses per animal, together with a defrosting solution, washing solutions 1 and 2 and the 3 Pasteur pipettes that were required in the MSC preparation procedure.

The MSC were prepared as follows: a cryotube was thawed for 2 minutes in a water bath at 37°C; then, the contents of the cryotube were immediately transferred to a centrifugal thawing solution and centrifuged for 5 minutes at low speed (1100 rpm; relative centrifugal force (RCF) = 216 g) (Fanem Centrifuge, Model 206BL). After centrifugation, the supernatant was discarded, washing solution 1 was added, and the cells were homogenized gently until the precipitate dissolved. The cells were centrifuged again at 216 ×g and 1100 RPM for 5 minutes. After centrifugation, the supernatant was discarded, and washing solution 2 was added. The cells were homogenized gently until the precipitate dissolved and were centrifuged again at 216 ×g and 1100 RPM for 5 minutes. The supernatant was discarded, and 50 µl of saline was added. The cells were then gently homogenized with a Pasteur pipette until the precipitate dissolved. The MSC could not remain in the physiological solution for more than 10 minutes because after this time, they are impracticable for use.

The animals were topically treated in both eyes with MSC using a 50 µl/millimetric pipette; 4 applications were performed every 7 days, and 1 drop of propylene glycol-based lubricant eye drops was applied 3 times daily in both eyes, for a total of 30 days. Lubricating eye drops were used only to promote ocular comfort during the MSC therapy because the use of lubricant alone in the treatment of KCS is not considered a specific treatment but rather a coadjuvant. The animals were followed for 180 days, weekly for the first 30 days and monthly for the remainder of the period.

Statistical analysis

For the variables of STT-1, TBUT, goblet cell density, and numbers of squamous, metaplastic, mononuclear and polymorphonuclear cells, we used two analyses of variance (ANOVA) paired with contrast by Tukey's method. For the FT and LGT variables, we used Friedman's nonparametric test with Dunn method contrasts. For binomial variables (clinical signs), we used the McNemar chi-square test to compare moments. Significance was adopted for $P < 0.05$. The software used for statistical analysis was R version 3.2.2 (The R Foundation for Statistical Computing, 2015).

Results

There was a significant improvement ($p<0.05$) in the clinical signs evaluated (opacity, pigmentation and neovascularization of the cornea, secretion and conjunctivitis) after 180 days of treatment with topical application of heterologous MSC of canine adipose tissue (Table 2). In the first 30 days of treatment, all the animals showed improvement. There was a return of ocular secretion and conjunctivitis until the end of the study in 7 animals (31.8%), which were later directed to conventional treatment with 0.03% topical tacrolimus.

Table 2. Percentage of clinical signs observed in 44 eyes of 22 dogs with KCS before and after topical treatment with heterologous MSC of canine adipose tissue ($1x10^6$ MSC/50 µL).

Clinical signs	Before Treatment (Day 0)	After Treatment (Day 180)
Corneal opacity	45.5% (1)	84.0% (1)*
	36.4% (2)	13.7% (2)*
	11.3% (3)	2.1% (3)*
	6.8% (4)	0% (4)*
Corneal pigmentation	47.7% (1)	63.7 (1)*
	38.6% (2)	35.3% (2)
	4.5% (3)	1.0% (3)
	9.0% (4)	0% (4)*
Corneal neovascularization	61.4% (1)	79.6% (1)
	22.7% (2)	11.4% (2)*
	15.9% (3)	9.0% (3)*
	0% (4)	0% (4)
Conjunctival secretion	15.9% (1)	59.1% (1)*
	65.9% (2)	36.4% (2)*
	11.3% (3)	4.5% (3)*
	6.8% (4)	0% (4)*
Conjunctivitis	29.5% (1)	63.6% (1)*
	43.3% (2)	27.3% (2)*
	22.7% (3)	9.0% (3)*
	4.5% (4)	0% (4)*

(1) negative; (2) mild; (3) moderate; and (4) severe.

*$p < 0.05$ (McNemar chi-square test compared to zero moment)

There was a significant ($p<0.05$) increase in STT-1 from D14 to D180 (Fig. 4A). In TBUT, the increase was significant ($p<0.05$) from D60 until the end of the study on D180 (Fig. 4B).

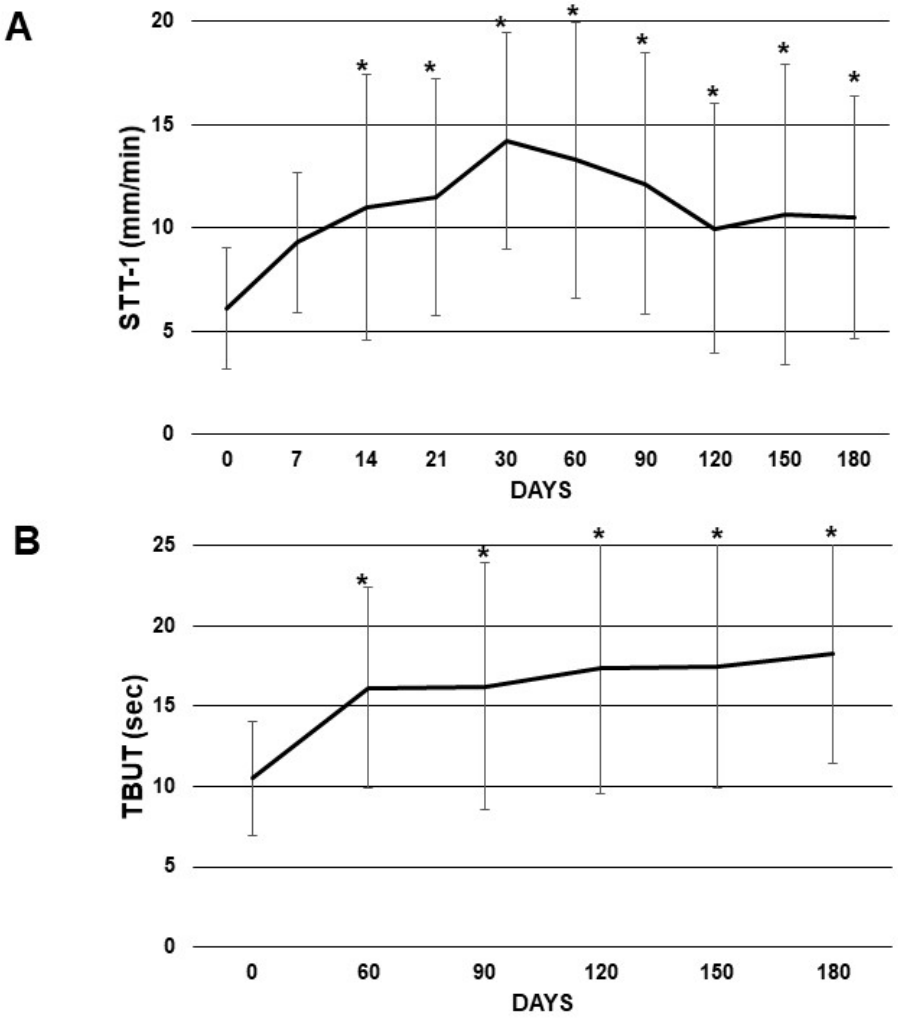

Fig. 1 (A) Mean and SD of STT-1 (mm/min) and (B) mean and SD of TBUT (seconds) of 44 eyes of 22 dogs with KCS before and after topical treatment with MSC heterologous canine adipose tissue ($1x10^6$ MSC/50 µL).

*$p<0.05$ (Tukey test compared to zero moment)

According to the FT values of the 44 evaluated eyes of the 22 dogs with topical MSC treatment, on D0, 81.8% (36 eyes) were negative for ulcers, and 18.2% (8 eyes) were positive, 5 eyes had small superficial ulcers, 2 eyes had small stromal ulcers, and 1 eye had descemetocele. After 30 days (D30), all eyes positive for FT stained negative for FT. The most severe case of ulcer was animal n. 22, with descemetocele at time zero and with complete healing sixty days after MSC treatment (Fig. 2).

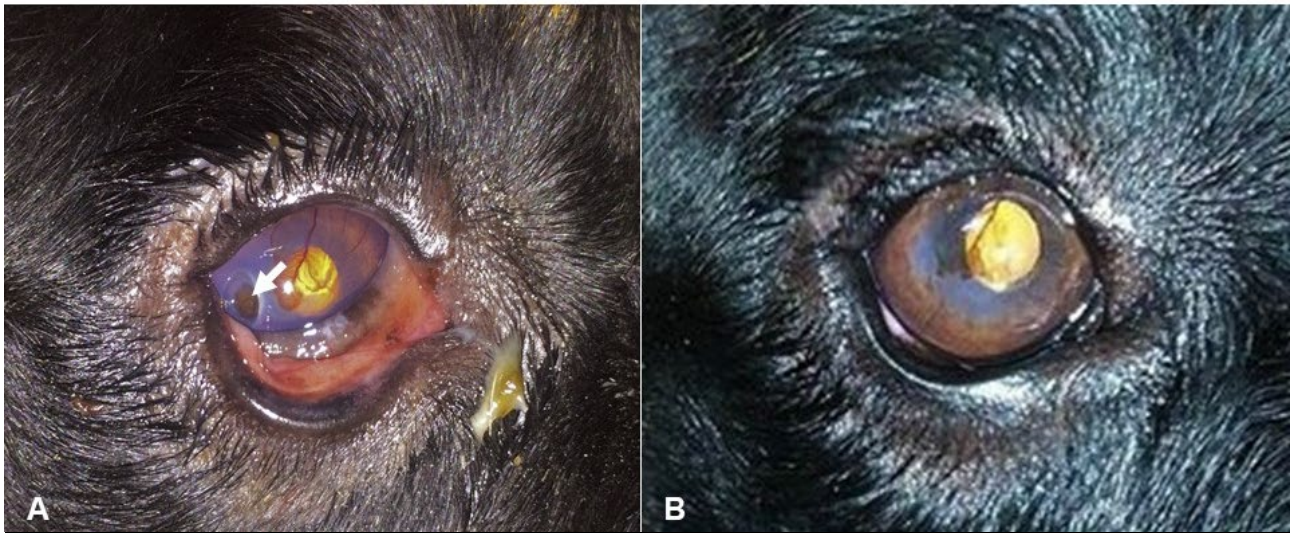

Fig. 2 Right eye (OD) of animal n. 22 before (A) and after (B) topical treatment with heterologous MSC from canine adipose tissue ($1x10^6$ MSC/50 µL). (A) OD on D0 presenting descemetocele (arrow). (B) OD on D60 with completely healed cornea.

According to the LGT values, on D0, of the 44 eyes analyzed, 52.2% (23 eyes) stained negative, only the conjunctiva was stained in 6.8% (3 eyes), only the cornea was stained in 13.7% (6 eyes), and both conjunctiva and cornea were stained in 27.3% (12 eyes). On D180, 100% of the eyes stained negative for LGT.

The evaluation of the inflammatory and desquamative cells from conjunctival cytology (Fig. 3) showed that there was a significant ($p<0.05$) decrease in the number of polymorphonuclear, mononuclear and squamous cells on D90 and D180 and of metaplastic cells on D90. The histopathological evaluation of the conjunctiva (Fig. 4) showed that there was a significant decrease ($p<0.05$) in the number of polymorphonuclear and mononuclear inflammatory cells on D180.

The evaluation of the goblet cell density from conjunctival cytology (Fig. 3) showed that the values had a significant increase ($p<0.05$) on D90 and D180. The assessment of goblet cell density from conjunctival histopathology (Fig. 4) showed that there was also a significant increase ($p<0.05$) on D0 and D180.

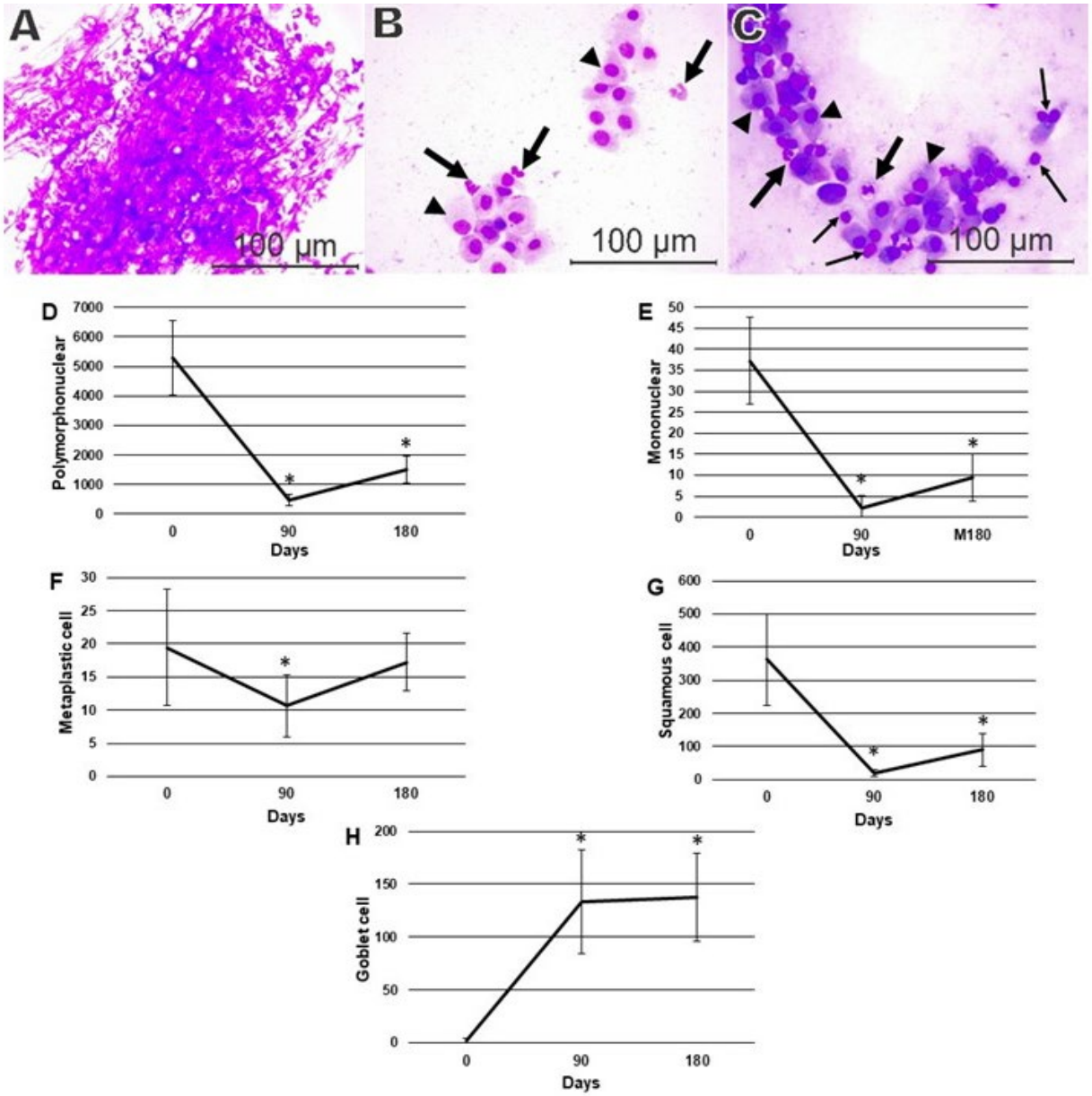

Fig. 3 Conjunctival cytology of 44 eyes of dogs (n=22) with KCS before and after topical treatment with MSC heterologous canine adipose tissue ($1x10^6$ MSC/50 µL). Photographs of slides with MGG staining at 400x magnification: (A) large number of polymorphonuclear cells in the middle of dense mucus (D0), (B) a large number of goblet cells (arrow) in the middle of rare polymorphonuclear cells (arrowhead) (D90), (C) large number of goblet cells (arrowhead) in the middle of rare polymorphonuclear cells (arrow), mononuclear cells (thin arrow) and fluid mucus (D180). The mean and SD of the cell counts from the conjunctival cytology analysis: (D) polymorphonuclear, (E) mononuclear, (F) metaplastic, (G) squamous and (H) goblet cells at 0 (D0), 90 (D90) and 180 (D180) days after topical treatment with heterologous MSC from canine adipose tissue ($1x10^6$ MSC/50 µL).

*$p<0.05$ (Tukey test in relation to M0).

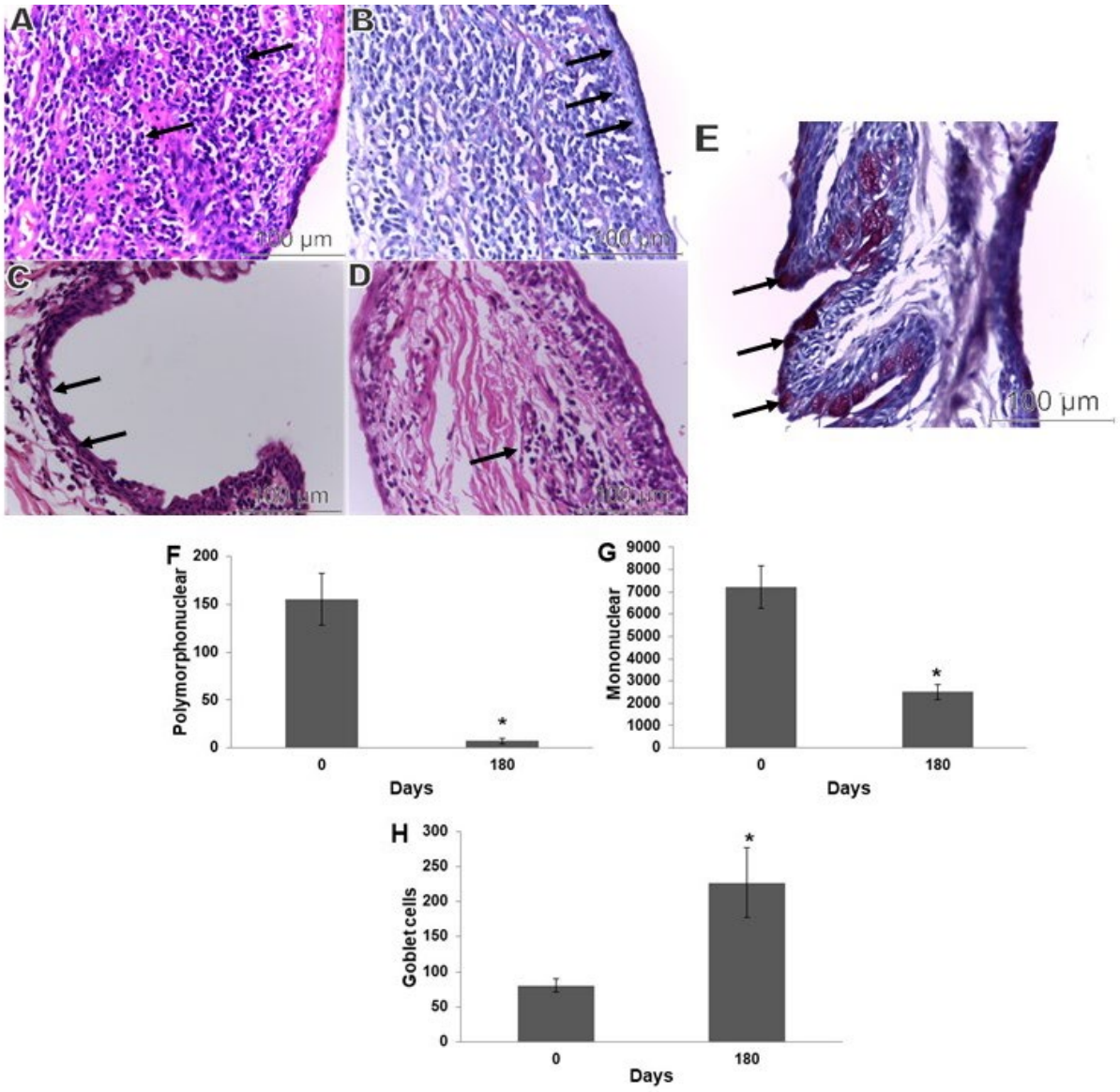

Fig. 4 Conjunctival histopathology of 44 eyes of dogs (n=22) with KCS before (D0) and after (D180) topical treatment with MSC heterologous canine adipose tissue ($1x10^6$ MSC/50 µL). Photomicrograph at a magnification of 400x of conjunctiva in A, C, D stained with hematoxylin-eosin (HE) and of B and E in PAS. (A) Intense mononuclear infiltration in the stroma (arrow) on D0, (B) absence of goblet cells (arrow) on D0, (C) area of squamous metaplasia of the epithelium (arrow) on D0, (D) image of mononuclear infiltration in the stroma (arrow) on D180, and (E) image of a large number of goblet cells (arrow) on D180. Mean and SD of the cell counts from conjunctival histopathology: (F) polymorphonuclear, (G) mononuclear, and (H) goblet cells.

*$p<0.05$ (Tukey's test) in relation to M0.

Discussion

This is the first study of the topical use of MSC in the treatment of dogs naturally affected with KCS. The present study presents a simpler technique of MSC administration, using a topical and noninjectable route and a larger number of eyes as an animal model for understanding this disease.

The use of topical MSC significantly improved the symptoms observed in the 22 dogs with KCS (Table 2). Most of the animals continued to show improved symptoms until the end of the 6-month observation period, and only 7 animals had a recurrence of ocular secretion and conjunctivitis until the end of the study. The return of these symptoms in some animals may be due to the return of immune-mediated attack on lacrimal gland cells [5-7]. In a study in which three dogs were treated with MSC inoculated around the nictitans and orbital glands, the symptoms were reported to worsen in the first 3 months of appearance and in subsequent months, depending on the stage of KCS [22].

From the evaluation of the aqueous portion of the tear with STT-1, we can observe a significant increase up to 90 days with a decrease in the values in 120 days; however, these values were still higher than those observed initially and increased again from 120 to 180 days. This increase may be related to the return of the symptoms in some animals, especially of immune-mediated reactions to the lacrimal glands, the main producers of the aqueous portion of the precorneal tear film. Other studies have also shown an increase in STT values with topical use in rats [19] and with injections of MSC in the lacrimal gland in mice [18] and dogs [20,21,22]. In contrast to STT-1 values, TBUT values, which indirectly reflects the quality of the precorneal tear film produced by the Tarsal or Meibomian and Zeis glands located in the palpebral margin [23, 24], showed a significant increase from 60 days until the end of the study (180 days).

An improvement in the healing of corneal ulcers was observed with the FT in all positive animals, including in those with severe ulcers such as descemetocele; this phenomenon has also been described in other studies with the use of MSC [18,20,21]. The results of the LGT, which was used to evaluate the presence of devitalized corneal and conjunctival cells at the beginning of the study, showed that 47.8% of the eyes had some marking on the conjunctiva or cornea; in the end, no marking was observed, showing an improvement in the ocular surface after topical treatment with MSC.

Both cytological and histopathological analysis of the conjunctiva showed a significant decrease in the number of inflammatory cells and a significant increase in the number of goblet cells until the end of the study (180 days). The cytologic analysis of the conjunctiva showed a significant decrease in the number of metaplastic cells at 90 days. These results may be due to the regenerative capacity of MSC that restores tissues destroyed by the action of autoantibodies. MSC also have an immunomodulatory action caused by the release of bioactive molecules that are immunosuppressive, especially for CD4 T helper and B-lymphocytes, thus inhibiting the release of interleukin-like IL-6 present in keratoconjunctivitis sicca [16-18], in addition to stimulating angiogenesis and cellular mitosis, inhibiting apoptosis and the release of proinflammatory mediators [17].

The increase in goblet cell density observed with the use of topical MSC was extremely relevant. The observed increase in histopathology was approximately 3 times higher than that at the beginning of the study. Goblet cells are responsible for the production of mucin in the prelacrimal film, which helps lubrication and maintains the quality of the ocular surface [24]. Lee et al. also observed a significant increase in goblet cell density and an improvement in ocular surface quality in MSC-treated mice [18]. Of all the studies already published by our research group on several types of KCS treatments using immunosuppressants with or without being associated with omegas in rabbits [25-27] and dogs [29,30], the use of topical MSC as a treatment of KCS presented the greatest increase in goblet cell density at the end of the study. This significant increase in the number of goblet cells with the use of MSC is directly related to the action potential of MSC to increase the number of cells of a tissue, in this specific case the conjunctival goblet cells, thus improving the quality of the prelacrimal film and the ocular surface [17,18].

Conclusion

The topical use of MSC and the therapeutic protocol utilized demonstrated great potential in the adjuvant treatment of MSC due to significant improvement in clinical signs, significant increase in the quantitative and qualitative portion of tear, significant decrease in inflammation and significant increase in the number of goblet cells. However, some animals showed a return of the clinical signs of KCS, possibly due to a reactivation of immune-mediated aggression to the lacrimal glands. Therefore, more studies should be performed to minimize immune-mediated aggression after the topical application of MSC.

Abbreviations

BAC: Benzalkonium chloride; CB: Conjunctival biopsy; CC: Conjunctival cytology; D: Days; FT: Fluorescein test; GLT: Green lissamine test; KCS: Keratoconjunctivitis sicca; HE: Hematoxylin and eosin; IL-1: Interleukin 1; IL-6: Interleukin 6; MGG: May-Grunwald-Giemsa; MSC: Mesenchymal stem cells; OD: Right eye; PAS: Periodic-Schiff acid; STT-1: Schirmer tear test-1; TBUT: Tear film break-up time; TNF-α: Tumor necrosis factor alpha.

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Availability of data and materials

All data generated and/or analyzed during this study are included in this published article.

Authors´ contributions

MRS and SFA conceived this study, performed the experiments, collected the data, performed the data analysis, and prepared the manuscript. DAS, FFN, DAMS, HRD, BTDF, WBC, and ECF helped collect the samples. GAN and MGS helped perform cytology and histopathology. MAB and MKWB helped prepare the mesenchymal stem cells and provided instruction on their use. All authors read and approved the final manuscript.

Ethics approval

The study was conducted according to the standards of animal experimentation of the Commission of Ethics in the Use of Animals (CEUA - Protocol: no. 2954) of UNOESTE, Presidente Prudente, SP, Brazil.

Consent of publication

All authors gave consent for publication.

Competing interests

The authors declare that they have no competing interests.

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2 ARTIGO 2

Reduction in the inflammatory markers CD4, IL-1, IL-6 and TNFα in dogs with keratoconjunctivitis sicca treated topically with mesenchymal stem cells

Revista: **Stem Cell Research**

Tipo: **Short communication**

Reduction in the inflammatory markers CD4, IL-1, IL-6 and TNFα in dogs with keratoconjunctivitis sicca treated topically with mesenchymal stem cells

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ABSTRACT

Keratoconjunctivitis sicca (KCS) is of predominantly immune-mediated origin. Dogs are an excellent model for understanding this disease, as the origin of KCS in dogs is similar to that in humans. The objective of this study was to localize and quantify immunological markers such as CD4 lymphocytes, interleukin (IL)-1, IL-6 and tumor necrosis factor alpha (TNFα) before and after topical treatment with mesenchymal stem cells (MSC). Twenty-two dogs positive for KCS were topically treated with 50 µl ($1x10^6$ MSC) in the conjunctival sac and evaluated for 6 months. The levels of the markers CD4, IL-6, IL-1 and TNFα were analyzed in conjunctival biopsy and cytology of the third eyelid gland by immunohistochemistry and immunocytochemistry techniques. The results showed that before treatment, there was marked expression of all the markers (CD4, IL-6, IL-1 and TNFα), and after 6 months, there was a significant ($p<0.05$) reduction in the expression of all the markers. The results demonstrated that topical MSC treatment promotes a significant decrease in the expression of these inflammatory markers and could be used as adjuvant therapy in the treatment of KCS in dogs and humans. In addition, these markers can be excellent tools for diagnosing and analyzing the progression of KCS.

Key words: Mesenchymal stem cells, Topical, Immunological markers, Keratoconjunctivitis sicca, Dry eye, Dogs.

1. Introduction

Keratoconjunctivitis sicca (KCS), or dry eye syndrome, a mainly immune-mediated and degenerative disease that directly affects the vision and quality of life of patients, is one of the major causes of ocular morbidity in dogs and humans, and dogs are excellent animal models to understand this disease (Williams, 2008; McGinnigle et al., 2012; Stevenson et al., 2012; Stern et al., 2013; Messmer, 2015).

Immune-mediated reactions that affect the lacrimal glands are the main causes of KCS, affecting tear quality and/or quantity and causing inflammation of the ocular surface with signs including mucopurulent ocular secretion, conjunctivitis, keratitis, ulcers and corneal pigmentation (Williams, 2008; Barabino et al., 2012; Stevenson et al., 2012; Stern et al., 2013; Messmer, 2015; Dodi, 2015). In humans, another autoimmune disease that significantly affects tear quality is Sjögren's syndrome, which affects mainly women (Chen et al., 2012; Mavragani and Moutsopoulos, 2014).

Immunohistochemical studies have demonstrated the accumulation of various inflammatory markers on the ocular surface in immune-mediated KCS, such as CD4 T lymphocytes (T helper cells) (Tang-Liu and Acheampong, 2005; Zhang et al., 2011; Stevenson et al., 2012; Coursey et al., 2013; Stern et al., 2013; Vijmasi et al., 2014; Villatoro et al., 2015), interleukin (IL)-1 and IL-6 (Kwok et al., 2012), tumor necrosis factor alpha (TNFα) (Dong et al., 2010) and interferon gamma (IFNγ) (Chen et al., 2012; Zhang et al., 2014).

Some researchers have studied the use of stem cells in the treatment of degenerative eye diseases (Beyazyildiz et al., 2014, Lee et al., 2015; Villatoro et al., 2015). Stem cells (hematopoietic and mesenchymal) can be extracted from adipose tissue (Villatoro et al., 2015) and from bone marrow (Caplan, 2007; Beyazyildiz et al., 2014; Lee et al., 2015), and they are used for the treatment of immune-mediated diseases because of their ability to restore tissues destroyed by the action of autoantibodies. Another important therapeutic action of stem cells is immunomodulation through the release of bioactive molecules. These molecules have immunosuppressive action, especially for CD4 T helper lymphocytes and B lymphocytes. This immunosuppressive effect decreases the release

of ILs, such as the IL-6 present in KCS, and stimulates angiogenesis and cellular mitosis in addition to inhibiting apoptosis and the release of proinflammatory mediators (Caplan, 2007; Lee et al., 2015; Villatoro et al., 2015).

Treatment for immune-mediated KCS is palliative and continuous and consists mainly of the use of topical immunosuppressants, such as cyclosporine, pimecrolimus, tacrolimus and, recently, lifitegrast, in addition to topical ocular lubricants (Izci et al., 2002; Tang-Liu and Acheampong, 2005; Berdoulay et al., 2005; Nell et al., 2005; Messmer, 2015; Pflugfelder et al., 2017). Research on the use of mesenchymal stem cells (MSC) in the treatment of KCS includes studies on topical use in rats (Beyazyildiz et al., 2014) and injectable use in the lacrimal glands of mice (Lee et al., 2015) and dogs (Villatoro et al., 2015; Bittencourt et al., 2016; Giménez et al., 2017).

To date, there have been no studies on topical MSC administration, which is a simpler method of administration than injection, in the treatment of KCS in dogs nor even on the immunological markers present before and after treatment. The aim of this study was to compare the expression of CD4 lymphocytes, IL-6, IL-1 and TNFα before and after topical treatment with MSC in dogs with naturally occurring immune-mediated KCS.

2. Materials and methods

2.1 Animals

This experiment was carried out at the Veterinary Hospital of Universidade do Oeste Paulista (UNOESTE), Presidente Prudente, SP. The study was conducted according to the animal experimentation standards of the Committee on Ethics in Animal Use (CEUA; Protocol: 2954) of UNOESTE and the ARVO (*Association for Research in Vision and Ophthalmology*) *Statement for the Use of Animals in Ophthalmic and Visual Research*.

Twenty-two dogs with bilateral positive diagnosis for KCS from the ophthalmology service at the Veterinary Hospital of UNOESTE were used, with no breed, age or sexual preference. To be included in the research, the animals had to present a Schirmer tear test-1 (STT-1) values of 5-10 mm/min or a tear film break-up time (TBUT) ≤ 15 seconds in addition to presenting KCS ocular signs such as opacity, ocular secretion, conjunctivitis, pigmentation, neovascularization, and corneal ulcers. Animals with STT-1 values ≤4 mm/min were excluded from the study (due to the severity of KCS with the possibility of a low presence of viable cells in the conjunctiva, lacrimal gland and third eyelid that

could respond to MSC treatment), as were animals that presented with neoplasia (due to the risk of an increase in neoplastic cells) and those that had been treated with corticosteroids and/or immunosuppressants for 1 month or more (due to possible interference with the viability of MSC).

2.2. Mesenchymal stem cells

Heterologous MSCs derived from canine adipose tissue were supplied by the biotechnology laboratory Regenera in Campinas, SP, Brazil. The MSCs were tested to confirm their ability to differentiate into osteoblasts, adipocytes, and chondrocytes under induction conditions in appropriate media. The expression of the typical MSC markers CD44, CD73, CD90, CD105, vimentin, nestin, and OCT3/4 and the absence of CD14 and the hematopoietic markers CD34 and CD45 were evaluated using immunofluorescence assays. The cells were also screened for the absence of pathogens and contaminants (e.g., bacteria, fungi, viruses, mycoplasma and endotoxins). The MSCs were supplied in single-dose cryotube vials along with defrosting solution, wash solutions 1 and 2 and the 3 Pasteur pipettes required to prepare the MSCs.

The KCS-positive dogs were treated topically in both eyes with 50 µl of MSCs ($1x10^6$) in the conjunctival sac (with a milliliter pipet) in 4 applications every 7 days and with propylene glycol-based lubricating eye drops (1 drop per eye) 3 times a day for 30 days. The lubricating eye drops were used only to promote ocular comfort during the MSC therapy, since the use of lubricant alone is not considered a specific treatment for KCS but only a coadjuvant. The KCS-positive dogs were followed for a period of 180 days (6 months). Animals for which treatment was unsuccessful during the first 30 days were directed to conventional treatment for KCS.

2.3. Experimental design and ophthalmic exams

Dogs with KCS were evaluated for 180 days; the day of diagnosis was considered day zero (D0). Clinical signs (conjunctival secretion, conjunctivitis, opacity, pigmentation and neovascularization of the cornea) were evaluated with a slit lamp on D7, D14, D21, D30, D60, D90, D120, D150 and D180. Ophthalmic tests were performed, including an STT-1 to evaluate tear quantity (D0, D30, D90 and D180) and a TBUT test to evaluate tear quality (D0, D60, D120 and D180). On D0 and D180, a fluorescein test (FT) was performed to evaluate the presence of corneal ulcers, a lissamine green test (LGT) was performed to evaluate the presence of devitalized corneal and conjunctival cells, conjunctival biopsy (CB) was performed to obtain material for immunohistochemistry (IHC), and fine

needle aspiration (FNA) of the third eyelid gland was performed to obtain material for immunocytochemistry (ICC).

2.4. Histopathological analysis

Conjunctival histopathology was performed after instillation of anesthetic eye drops (1% tetracaine hydrochloride + 0.1% phenylephrine hydrochloride, Allergan, São Paulo, Brazil) and removal of 1-3 mm sections from the fornix of the medial inferior conjunctiva with the aid of forceps and conjunctival scissors. The histological sections were placed on standardized 1x1 cm pieces of paper, fixed in formaldehyde and embedded in paraffin (Dynamics Analytical Reagents, São Paulo, Brazil). With the help of a rotating microtome, 5 µm thick sections of the conjunctiva were obtained, which were then stained with hematoxylin and eosin (HE) (Dolles, São Paulo, Brazil) for quantification of polymorphonuclear cells (cells/0.5 mm^2) and periodic acid-Schiff (PAS) for analysis of goblet cell density (cells/0.5 mm^2) under an optical microscope with a 40x objective.

2.5. Immunohistochemistry and immunocytochemistry

Biological materials from CB and FNA were processed and labeled with IHC and ICC techniques, respectively, using an anti-CD4 antibody (GeneTex, Irvine, CA, USA, GTX84720, 1:25 dilution), an anti-IL-1 antibody (GeneTex, Irvine, CA, USA, GTX22105, 1:100 dilution) in the presence of an anti-IL-6 antibody (Biorbyt, Cambridge, United Kingdom, ORB101390, 1:100 dilution), and an anti-TNFα antibody (LSBio, Seattle, WA, USA, LS-C391068/109263, 2 µg/ml). Antigen retrieval was performed with Trilogy 920P-07 (Cell Marque, Rocklin, CA, USA), endogenous peroxidase was blocked with 10 volumes of hydrogen peroxide, and nonspecific binding was blocked with an ImmPRESS HRP MP-7500 reagent kit (Vector Laboratories, Burlingame, CA, USA). The antibodies were diluted with a universal antibody diluent from EasyPath (EP-12205512). The secondary antibody used was the Vector Laboratories ImmPRESS Universal Reagent Anti-Mouse/Rabbit Ig (MP-7500). The developer used was ImmPACT DAB (Vector, Burlingame, CA, USA, SK-4105). The total areas of the cells marked by the antibodies were observed and quantified. Five photos of each slide stained with the IHC and ICC techniques were obtained with a camera coupled to a microscope. The brown-toned labeled area of each slide and the labeled area for each antibody (CD4, IL-6, IL-1, and TNFα) were analyzed using the ImageJ program (https://imagej.nih.gov/ij/) using the "Color Threshold/Measure" analysis in 5 fields, and the arithmetic means of the obtained areas were calculated.

2.6. Statistical analysis

The obtained STT-1 test and TBUT test values; the density of polymorphonuclear, mononuclear, and goblet cells; and the values from CD4, IL-6, IL-1, TNFα antibody staining were analyzed by paired t-test. Binomial variables (clinical signs) were compared with the McNemar chi-square test. $P<0.05$ was adopted as the level of significance. The software used for analysis was BioStat 5.3.

3. Results

There was a significant improvement ($p<0.05$) in ocular clinical signs in 44 eyes after MSC treatment. From before treatment (D0) to after MSC treatment (D180), the occurrence of conjunctival secretion decreased from 84.1% to 40.9%, that of conjunctivitis decreased from 70.5% to 36.4%, that of corneal opacity decreased from 55.5% to 16.0%, that of corneal neovascularization decreased from 38.6% to 20.4%, and that of corneal pigmentation decreased from 52.3% to 36.3%. On D0, 8 eyes were positive in the FT, indicating the presence of corneal ulcers, but at the end of the study, on D180, all were negative. In the LGT, on D0, 32 eyes were positive, demonstrating some degree of involvement of the corneal and conjunctival surface cells, but at the end of the study on D180, all eyes were negative.

There was a significant ($p<0.05$) increase in tear quantity (SST-1) (Figure 1A) and tear quality (TBUT) (Figure 1B) until the end of the study. There was also a significant ($p<0.05$) reduction in polymorphonuclear inflammatory cells (Figure 1C) and a significant increase ($p<0.05$) in goblet cells (Figure 1D) until the end of the study.

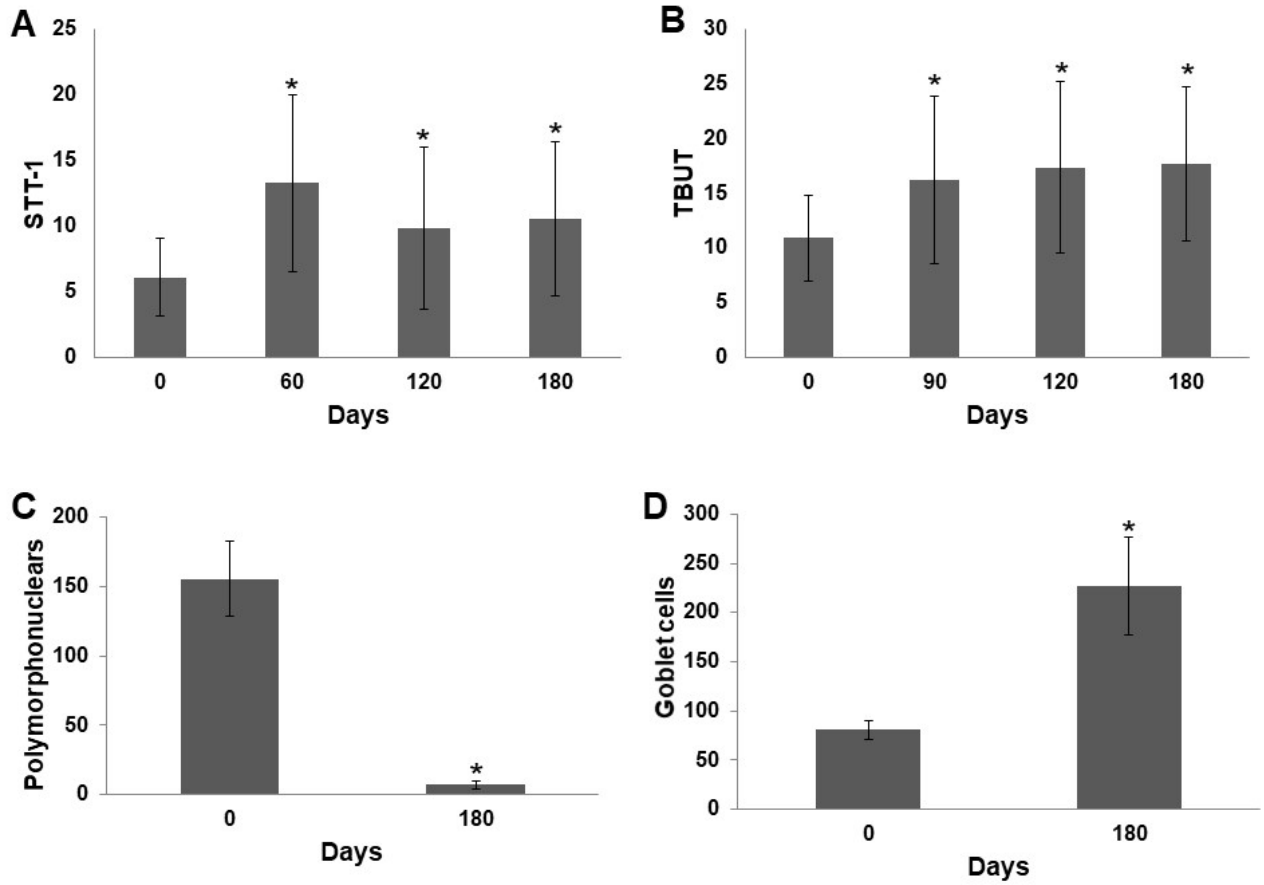

Figure 1. Mean and SD of the STT-1 values (mm/min) (A), TBUT values (sec) (B), polymorphonuclear cell density (cells/0.5 mm^2) (C), and goblet cell density (cells/0.5 mm^2) (D).

*p <0.05 (paired t-test).

There was a significant ($p<0.05$) reduction in immunostaining from D0 to D180 for all inflammatory markers (CD4, IL-6, IL-1 and TNFα) in both the IHC (Figure 2) and ICC (Figure 3) analyses.

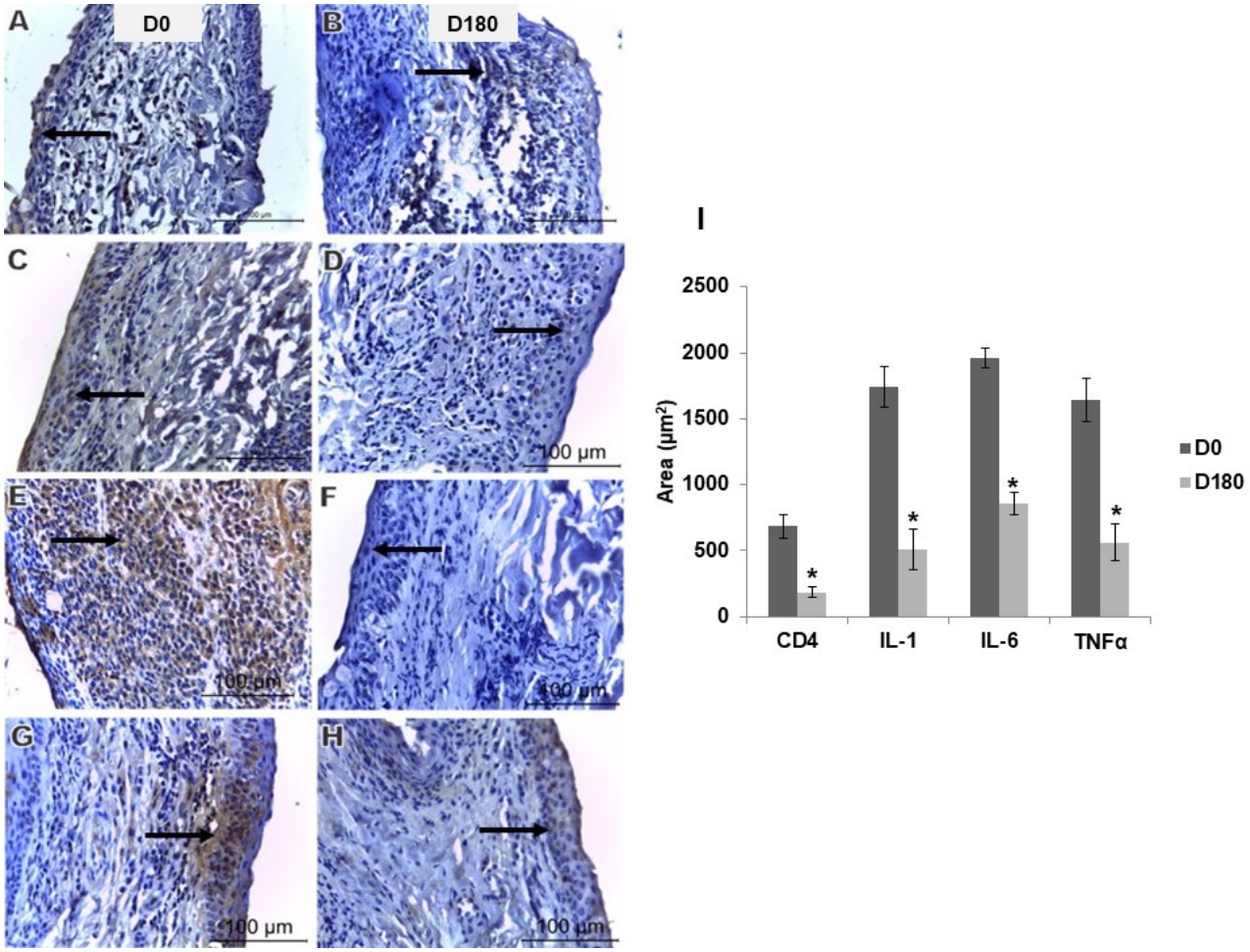

Figure 2. Microscopy images of conjunctival biopsies immunostained for CD4, IL-1, IL-6 and TNFα with the immunohistochemistry (IHC) technique at 400x magnification. The images in the left column are from D0 (A, C, E, G), and the images in the right column are from D180 (B, D, F, H). A and B: Anti-CD4 antibody staining with arrows showing strong diffuse cytoplasmic labeling in the epithelial and stromal cells in A and moderate labeling in B. C and D: Anti-IL-6 antibody staining with arrows showing strong cytoplasmic labeling in C and scarce labeling in D. E and F: Anti-IL-1 antibody staining with arrows showing strong cytoplasmic labeling in E and scarce labeling in H. G and H: Anti-TNFα antibody staining with arrows showing strong cytoplasmic labeling in G and scarce labeling in H. I: Mean and SD of the marked areas (μm^2) of the inflammatory markers CD4, IL-6, IL-1 and TNFα at D0 (beginning) and D180 (180 days after treatment with topical MSC).

*p <0.05 (paired t-test).

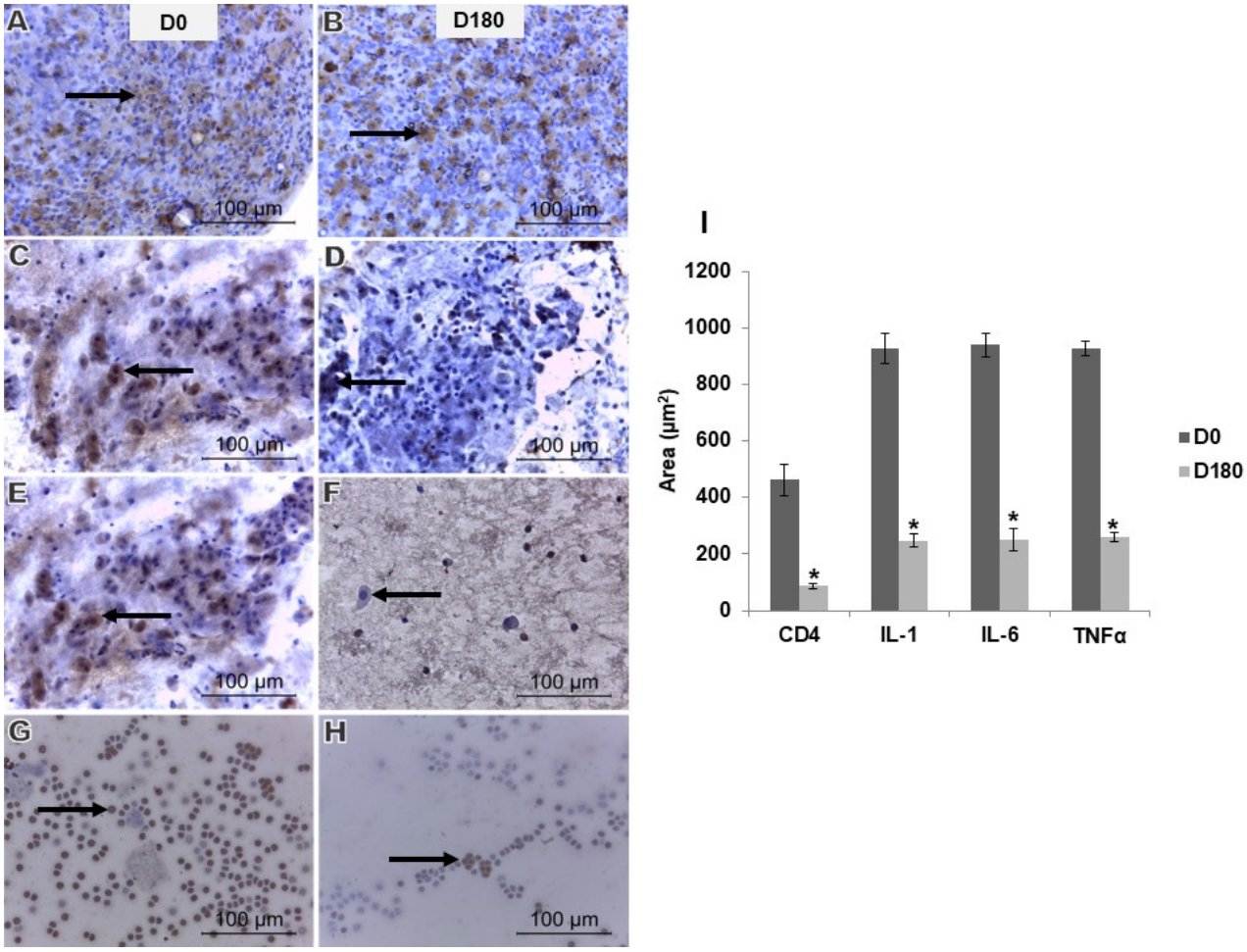

Figure 3. Microscopy images of the fine needle aspirate of the third eyelid gland immunostained with the immunocytochemistry (ICC) technique at 400x magnification. The images in the left column are from D0 (A, C, E, G), and the images in the right column are from D180 (B, D, F, H). A and B: Anti-CD4 antibody staining with arrows showing strong diffuse cytoplasmic labeling in A and moderate labeling in B. C and D: Anti-IL-6 antibody staining with arrows demonstrating strong cytoplasmic labeling in C and scarce labeling in D. E and F: Anti-IL-1 antibody staining with arrows showing strong cytoplasmic labeling in E and scarce labeling in F. G and H: Anti-TNFα antibody staining with arrows showing strong cytoplasmic labeling in G and scarce labeling in H. I: Mean and SD of the marked areas (μm^2) of the inflammatory markers CD4, IL-6, IL-1 and TNFα at D0 (beginning) and D180 (180 days after treatment with topical MSC).

*$p<0.05$ (paired t-test).

The IHC images in Figure 2 show predominantly diffuse marker expression mainly located in the cytoplasm of epithelial cells and some stromal cells, except for TNFα expression, which was

located only in epithelial cells (Figure 2G and H). ICC analysis also showed cytoplasmic staining for all the cytokines evaluated.

4. Discussion

The use of topical MSC significantly improved the clinical signs and tear quantity and quality; reduced the density of polymorphonuclear inflammatory cells in the palpebral conjunctiva; reduced the expression of the immunological markers CD4, IL-1, IL-6 and TNFα in the palpebral conjunctiva and third eyelid gland; and increased the goblet cell density, which resulted in reduced inflammatory process and subsequent tissue aggression to the ocular surface and lacrimal glands. The increased tear production or quantity can be explained by the decrease in CD4 T cells, ILs and TNFα observed in this study, since the increased presence of these mediators is closely related to decreases in tear production in patients with KCS (Reksten et al., 2009; Coursey et al., 2013). Another relevant factor is the significant increase in the density of conjunctival goblet cells, which are responsible for the production of mucin that improves tear quality (Davidson and Kuonen, 2004).

KCS is a significant degenerative ocular disease, and clinical studies have demonstrated elevated levels of CD4 T cells (Elan et al., 2009; Stern et al., 2002) and cytokines on the ocular surface of patients with KCS (Cannon, 2000) and in the conjunctival epithelium (Massingale et al., 2009; Pflugfelder et al., 1999); these findings coincide with our results, wherein both biological materials evaluated (conjunctival tissue and third eyelid gland cells) presented a considerable initial presence of CD4- and cytokine-positive cells that was significantly decreased after treatment with topical MSC.

The inflammatory process is one of the most important immune-mediated responses in the aggravation of KCS symptoms (Stern et al., 2013; Stern et al., 2002; Barabino et al., 2012). Cytokines are signaling molecules that mediate intercellular communication. Proinflammatory cytokine production is regulated by osmotic damage to the ocular surface. Cytokines such as IL-6, IL-1, IL-10, TNFα, and IFNγ can be produced both by lymphocytic cells infiltrating the ocular surface and by the ocular surface cells themselves (Reksten et al., 2009; Vijmasi et al. 2013). Clinical studies have reported elevated levels of these cytokines in KCS (Cannon, 2000) and in the conjunctival epithelium (Massingale et al., 2009; Pflugfelder et al., 1999), and IL-2 and TNFα have been found to promote conjunctival squamous metaplasia and the induction of apoptosis in conjunctival cells (Zhang, et al., 2011; De Paiva, et al., 2007). There is also evidence that TNFα and IL-1 produce metalloproteinases (MMp-3 and MMp-9) that increase early rupture of the tear film (De Paiva et al., 2009).

Increased IFNγ leads to increased CXCL9, which is one of the cytokines increased in the ocular surface tear film in patients with KCS; its increase is related to CD4 T cell infiltration of the ocular surface (El Annan et al., 2010). CD4 T cells associated with IFNγ and IL-17 secrete TH17 cells, which are suggested to be primary effector T cells in degenerative ocular diseases such as KCS (El Annan et al., 2009). Although part of the mechanism of TH1 and TH17 cells remains unclear, recent findings suggest that TH17 cells play a prominent role in the pathogenesis of KCS (De Paiva et al., 2009; Chauhan et al., 2009). It is believed that TH1 and TH17 cells are involved in the migration of lymph node CD4 cells from the lymph nodes to the ocular surface (Groom and Luster, 2011), which explains the strong CD4 immunostaining in the IHC and ICC analyses prior to the initiation of topical MSC treatment.

In KCS, the inflammatory mechanism of the proinflammatory cytokines IL-1, IL-6, TNFα and CD4, which can be produced both by lymphocytes infiltrating the ocular surface and by the ocular surface cells themselves, is one of the main targets in the treatment of KCS. MSC treatment reduces the levels of proinflammatory mediators associated with immunomodulatory action by promoting the release of bioactive molecules that suppress CD4, IL-1, IL-6 and TNFα (Reksten et al., Lee et al. 2015, Villatoro et al., 2015), explaining the lower immunoreactivity observed in IHC and ICC at the end of this study.

5. Conclusion

The use of topical MSC in the conjunctival sac has been shown to be a promising adjuvant therapy for the treatment of KCS in dogs, as demonstrated by improvements in clinical signs and tear quantity and quality, an increase in goblet cells, and significant reductions in polymorphonuclear inflammatory cells and the immunological markers CD4, IL-6, IL-1 and TNFα. Future studies may show a relationship between the stage of KCS and the levels of CD4, IL-6, IL-1 and TNFα, enabling more accurate assessment of the complexity, phase and severity of the disease and contributing to the understanding of KCS in dogs and humans.

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ANEXO 1 – NORMAS DA RESVISTA STEM CELL RESEARCH & THERAPY (ARTIGO 1)

Research

Criteria

Research articles should report on original primary research.

Stem Cell Research & Therapy strongly encourages that all datasets on which the conclusions of the paper rely should be available to readers. We encourage authors to ensure that their datasets are either deposited in publicly available repositories (where available and appropriate) or presented in the main manuscript or additional supporting files whenever possible. Please see Springer Nature's information on recommended repositories. Where a widely established research community expectation for data archiving in public repositories exists, submission to a community-endorsed, public repository is mandatory. A list of data where deposition is required, with the appropriate repositories, can be found on the Editorial Policies Page.

Preparing your manuscript

The information below details the section headings that you should include in your manuscript and what information should be within each section.

Please note that your manuscript must include a 'Declarations' section including all of the subheadings (please see below for more information).

Title page

The title page should:

- present a title that includes, if appropriate, the study design e.g.:
    - "A versus B in the treatment of C: a randomized controlled trial", "X is a risk factor for Y: a case control study", "What is the impact of factor X on subject Y: A systematic review"
    - or for non-clinical or non-research studies a description of what the article reports
- list the full names, institutional addresses and email addresses for all authors
    - if a collaboration group should be listed as an author, please list the Group name as an author. If you would like the names of the individual members of the Group to be searchable through their individual

PubMed records, please include this information in the "Acknowledgements" section in accordance with the instructions below

- indicate the corresponding author

Abstract

The Abstract should not exceed 350 words. Please minimize the use of abbreviations and do not cite references in the abstract. Reports of randomized controlled trials should follow the CONSORT extension for abstracts. The abstract must include the following separate sections:

- **Background:** the context and purpose of the study
- **Methods:** how the study was performed and statistical tests used
- **Results:** the main findings
- **Conclusions:** brief summary and potential implications
- **Trial registration:** If your article reports the results of a health care intervention on human participants, it must be registered in an appropriate registry and the registration number and date of registration should be in stated in this section. If it was not registered prospectively (before enrollment of the first participant), you should include the words 'retrospectively registered'. See our editorial policies for more information on trial registration

Keywords

Three to ten keywords representing the main content of the article.

Background

The Background section should explain the background to the study, its aims, a summary of the existing literature and why this study was necessary or its contribution to the field.

Methods

The methods section should include:

- the aim, design and setting of the study
- the characteristics of participants or description of materials
- a clear description of all processes, interventions and comparisons. Generic drug names should generally be used. When proprietary brands are used in research, include the brand names in parentheses
- the type of statistical analysis used, including a power calculation if appropriate

Results

This should include the findings of the study including, if appropriate, results of statistical analysis which must be included either in the text or as tables and figures.

Discussion

This section should discuss the implications of the findings in context of existing research and highlight limitations of the study.

Conclusions

This should state clearly the main conclusions and provide an explanation of the importance and relevance of the study reported.

List of abbreviations

If abbreviations are used in the text they should be defined in the text at first use, and a list of abbreviations should be provided.

Declarations

All manuscripts must contain the following sections under the heading 'Declarations':

- Ethics approval and consent to participate
- Consent for publication
- Availability of data and material
- Competing interests
- Funding
- Authors' contributions
- Acknowledgements
- Authors' information (optional)

Please see below for details on the information to be included in these sections.

If any of the sections are not relevant to your manuscript, please include the heading and write 'Not applicable' for that section.

Ethics approval and consent to participate

Manuscripts reporting studies involving human participants, human data or human tissue must:

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- include the name of the ethics committee that approved the study and the committee's reference number if appropriate

Studies involving animals must include a statement on ethics approval.

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If your manuscript does not report on or involve the use of any animal or human data or tissue, please state "Not applicable" in this section.

Consent for publication

If your manuscript contains any individual person's data in any form (including individual details, images or videos), consent for publication must be obtained from that person, or in the case of children, their parent or legal guardian. All presentations of case reports must have consent for publication.

You can use your institutional consent form or our consent form if you prefer. You should not send the form to us on submission, but we may request to see a copy at any stage (including after publication).

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ANEXO 2- NORMAS DA REVISTA STEM CELL RESEARCH (ARTIGO 2)

STEM CELL RESEARCH

AUTHOR INFORMATION PACK

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