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

USO DO ÔMEGA 3 ORAL EM DIFERENTES PROPORÇÕES DE EPA E DHA ASSOCIADO COM ANTIOXIDANTES COMO ADJUVANTE NO TRATAMENTO DE CERATOCONJUNTIVITE SECA EM CÃES

DANIELLE ALVES SILVA

Presidente Prudente – SP
2018

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

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DANIELLE ALVES SILVA

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Área de Concentração: Fisiopatologia e Saúde Animal

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RESUMO

Uso do ômega 3 por via oral com diferentes proporções de EPA e DHA associado com antioxidantes como adjuvante no tratamento de ceratoconjuntivite seca em cães

O objetivo deste estudo foi comparar a eficácia de duas formulações de ômega 3 por via oral, com diferentes proporções de EPA, DHA e antioxidantes, como adjuvante no tratamento de cães acometidos por CCS e avaliar por meio da análise fractal a conjuntiva palpebral dos cães tratados. Quarenta e cinco cães atendidos no HV da UNOESTE portadores de CCS foram avaliados mensalmente por 6 meses pelo Teste Lacrimal de Schirmer (TLS), Teste de Fluoresceína (TF), Tempo de Ruptura do Filme Lacrimal (TRFL), Teste de Rosa Bengala, citologia da conjuntiva no início, meio e fim do projeto, biopsia e análise fractal da conjuntiva no início e final do projeto. Os cães foram distribuídos aleatoriamente em 3 grupos (n=15): grupo T (tacrolimus 0.03% tópico), grupo TO (tacrolimus + ômegas EPA/DHA oral) e grupo TOA (tacrolimus + ômegas EPA/DHA + antioxidantes oral). Os resultados demonstraram que houve uma melhora significativa nos sinais clínicos em ambos os grupos. No TRFL todos os grupos apresentaram aumento no decorrer do tratamento, sendo que o grupo TO foi o que apresentou melhor resultado em todos momentos quando comparado aos demais grupos. Ao final do experimento, os grupos T, TO e TOA apresentaram na análise citológica, diminuição de linfócitos, neutrófilos, células metaplásicas e escamosas, e na análise histopatológica, diminuição de linfócitos e neutrófilos e aumento das células caliciformes, ressaltando o melhor desempenho ao TO. Na análise fractal, ao final do experimento, o grupo TO que apresentou melhor resultado e os valores próximos aos valores encontrados nos parâmetros fractais de olhos sadios. A diferença entre o grupo TO e os demais grupos está na concentração de EPA maior, sendo um anti-inflamatório natural, o que pode ser uma das causas de seu melhor desempenho. Concluímos que o ômega 3 oral que contém maior proporção de EPA do que DHA trouxe maior benefício quanto a melhora dos sinais clínicos e do processo inflamatório no tratamento de CCS em cães.

Palavras-chave: Ceratoconjuntivite seca, ômega 3, EPA, DHA, antioxidantes, cães, análise fractal

ABSTRACT

Use of oral omega 3 in different proportions of EPA and DHA associated with antioxidants as adjuvant in the treatment of keratoconjunctivitis sicca in dogs

The objective of this study was to compare the efficacy of two omega 3 oral formulations with different ratios of EPA, DHA and antioxidants, as an adjuvant in the treatment of dogs affected by KCS and to evaluate by fractal analysis the palpebral conjunctiva of the treated dogs. Forty-five dogs with KCS were evaluated monthly for 6 months by the Schirmer Tear Test (TLS), Fluorescein Test (TF), Tear Film Break-up Time (TBUT), Lissamine Green Test (LGT), cytology of the conjunctiva at the beginning, middle and end of the study, biopsy and fractal analysis of the conjunctiva at the beginning and end of the study. The dogs were randomly assigned into 3 groups (n = 15): T group (tacrolimus 0.03% topical), TO group (tacrolimus + omegas EPA/DHA) and TOA group (tacrolimus + omegas EPA/DHA + Antioxidants). The result demonstrated that there was a significant improvement in clinical signs in both groups. In TBUT, all groups presented increase during the treatment, and the TO group presented the best result at all times when compared to the other groups. At the end of the experiment, the groups T, TO and TOA presented cytological analysis, reduction of lymphocytes, neutrophils, metaplastic and squamous cells, and histopathological analysis, reduction of lymphocytes and neutrophils and increase of goblet cells highlighting the best performance at TO. In the fractal analysis, at the end of the experiment, the TO group presented the best result and the values close to the values found in the fractals of healthy eyes. The difference between the TO group and the other groups is in the higher EPA concentration, being a natural anti-inflammatory, which may be one of the causes of its better performance. We concluded that oral omega 3, which contains a higher proportion of EPA than DHA, has shown greater benefit in terms of the improvement of clinical signs and the inflammatory process in the treatment of KCS in dogs.

Key-words: dry eye, tacrolimus, omega, EPA, DHA, dogs, fractal analysis

FIGURE 5- (A) T group, moment T0 left eye, mucoid secretion, opacity and corneal pigmentation (B) Evolution of the patient and persistence of pigmentation (C) Photomicroscopy of T0 conjunctival cytology, presence of neutrophils, squamous cell (thick arrow), metaplastic cell (fine arrow), MGG coloration, 400x magnification (D) T6 moment, presence of squamous cells (thick arrow), MGG coloration, 400x magnification (E) Histopathology at T0, inflammatory infiltrate (*), MGG coloration, 400x magnification (F) T0, reduction of inflammatory process (G) T0, absence of goblet cells, PAS staining, increase of 400x (H) PAS at T6 moment, presence of goblet cells (fine arrow) (I) TO group, T0 moment left eye, mucoid secretion, descemetocele (J) Ulcer healing (K) Photomicroscopy of T0 conjunctival cytology, presence of neutrophils, globet cell (fine arrow), MGG, 400x magnification (L) T6 moment, presence of squamous cell (thick arrow), MGG coloration, 400x magnification (M) Histopathology at T0, inflammatory infiltrate (*), coloration HE, increase of 400x (N) T0, reduction of the inflammatory process (O) T0, absence of goblet cells, PAS staining, increase of 400x (P) PAS, T6, large amount of goblet cells. (Q)TOA group, moment T0 right eye, mucopurulent secretion (R) clinical improvement of patient (S) Photomicroscopy of T0 conjunctival cytology, presence of neutrophils, metaplastic cell (fine arrow), MGG coloration, 400x magnification (T) T6, presence of globet cell (fine arrow), MGG coloration, 400x magnification (U) Histopathology at T0, inflammatory infiltrate (*), HE staining, 400x magnification (V) T0, reduction of inflammatory process (X) T0, absence of goblet cells, PAS staining, increase of 400x (W) PAS at T6 moment, presence of goblet cells. 36

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LIST OF ABBREVIATIONS

KCS - Keratoconjunctivitissicca
FO - Fish oil
T - Tacrolimus 0.03% topical
TO - Tacrolimus topical + omegas 1.5 EPA : 1 DHA
TOA - Tacrolimus topical + omegas 1 EPA : 4.5 DHA + antioxidants C - Control
STT - Schirmer Tear Test LGT - Lissamine Green Test FT - Fluorescein Test
TBUT - Tear Film Break-up Time EFAs - Essential fatty acids
ω -3 - Omega-3
ω -6 - Omega-6
ALA - β-linolenic acid
EPA - Eicosapentaenoic acid DHA - Docosahexaenoic acid RvE1 – Resolvin E1
RvE2 - Resolvin E2
ARVO - Association for Research in Vision and Ophthalmology HE - Hematoxylin and eosin
MGG - May-Grunwald-Giemsa
CEUA - Ethical Committee on Animal Use
TE - Tacrolimus topical + omegas 1.5 EPA : 1 DHA
TD - Tacrolimus topical + omegas 1 EPA : 4.5 DHA + antioxidants

SUMARY

I SCIENTIFIC ARTICLE

Oral omega 3 in different proportions of EPA, DHA and antioxidants as an adjuvant in the treatment of keratoconjunctivitis sicca in dogs

Ômega 3 oral em diferentes proporções de EPA, DHA e antioxidantes como adjuvante no tratamento de ceratoconjuntivite seca em cães

Running head: omega 3 EPA DHA antioxidants adjuvant treatment KCS dogs

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ABSTRACT

Objective: To compare the efficacy of 0.03% topical tacrolimus associated with oral omega 3 with different ratios of EPA, DHA and antioxidants as an adjuvant in the treatment of KCS in dogs.

Procedure: Forty-five dogs with KCS were evaluated monthly for 6 months by Schirmer Tear Test (STT); Fluorescein Test (FT); Tear Film Break-up Time (TBUT); Lissamine Green Test (LGT); cytology of the conjunctiva at the beginning, middle and end of the study; and biopsy of the conjunctiva at the beginning and end of the study. The dogs were randomly assigned into 3 groups (n = 15): T group (tacrolimus 0.03% topical), TO group (tacrolimus topical + omegas 1.5 EPA : 1 DHA) and TOA group (tacrolimus topical + omegas 1 EPA : 4.5 DHA + antioxidants)

Results There was a significant improvement in clinical signs in both groups. In TBUT, all groups presented an increase during the treatment, and the TO group presented the best result at all times compared to the other groups. At the end of the experiment, the groups T, TO and TOA presented a reduction of lymphocytes, neutrophils, and metaplastic and squamous cells in a cytological analysis and a reduction of lymphocytes and neutrophils with an increase of goblet cells in a histopathological analysis. The best performance was found in the TO group.

Conclusion: Oral omega 3 with a higher proportion of EPA than DHA brought greater benefit to the treatment of KCS in dogs associated with 0.03% topical tacrolimus, possibly because EPA is a precursor of anti-inflammatory and immunomodulatory mediators.

Key-words: dry eye, tacrolimus, EPA, DHA, dogs

RESUMO

Objetivo: Comparar a eficácia do tacrolimus 0,03% tópico associado ao ômega 3 oral, com diferentes proporções de EPA, DHA e antioxidantes, como adjuvante no tratamento de cães acometidos por CCS.

Procedimento: Quarenta e cinco cães atendidos no HV da UNOESTE portadores de CCS foram avaliados mensalmente por 6 meses pelo Teste Lacrimal de Schirmer (TLS), Teste de Fluoresceína (TF), Tempo de Ruptura do Filme Lacrimal (TRFL), Teste de Rosa Bengala, citologia da conjuntiva no início, meio e fim do projeto e biopsia da conjuntiva no início e final do projeto. Os cães foram distribuídos aleatoriamente em 3 grupos (n=15): grupo T (tacrolimus 0.03% tópico), grupo TO (tacrolimus + ômegas 1.5EPA/1DHA oral) e grupo TOA (tacrolimus + ômegas 1 EPA/4.5DHA + antioxidantes oral).

Resultados: Houve uma melhora significativa nos sinais clínicos em ambos os grupos. No TRFL todos os grupos apresentaram aumento no decorrer do tratamento, sendo que o grupo TO foi o que apresentou melhor resultado em todos momentos quando comparado aos demais grupos. Ao final do experimento, os grupos T, TO e TOA apresentaram na análise citológica, diminuição de linfócitos, neutrófilos, células metaplásicas e escamosas, e na análise histopatológica, diminuição de linfócitos e neutrófilos e aumento das células caliciformes, com o grupo TO com melhor desempenho.

Conclusão: O ômega 3 oral com maior proporção de EPA do que DHA, trouxe maior benefício ao tratamento de KCS em cães associado ao tacrolimus 0,03% tópico possivelmente porque o EPA é um precursor de mediadores anti-inflamatórios e imunomoduladores.

Descritores: Ceratoconjuntivite seca, tacrolimus, EPA, DHA, cães

INTRODUCTION

Keratoconjunctivitis sicca (KCS), also known as dry eye syndrome, is an ocular disease resulting from inflammation of the lacrimal gland and decreased lacrimal film. KCS can occur due to lack of production of the aqueous portion of the tear film (quantitative deficiency) and/ or excessive evaporation of the tear (qualitative deficiency), thus rendering the protective function of the tear deficient. KCS mainly affects structures of the cornea and conjunctiva[1,2]. Among the causes of KCS in dogs are racial predisposition, hypothyroidism, medications (atropine, sulfonamides), surgical excision of the third eyelid gland, distemper and, mainly, autoimmune factors[3,4,5].

The main clinical signs observed are mucoid or mucopurulent secretion, conjunctival hyperemia, pigmentation, vascularization and opacity of the cornea, blepharitis, blepharospasm and, in cases of greater severity, presence of corneal ulcer[6]. Tacrolimus (FK 506) is a macrolide antibiotic isolated from *Streptomyces tsukubaensis spp* that has an immunomodulatory action[4]. The effects observed with its use are the combination of local immunosuppression, goblet cell proliferation and anti-inflammatory effects[7,8].

Recent studies in humans[9,10,11] and animals[12,13] showed good results in dry eye control with the use of essential fatty acids (EFAs), omega-3 (ω-3) and omega-6 (ω -6) due to their ability to produce anti-inflammatory mediators.

Fish oil (FO) is an important source of ω-3 obtained from cold water fish (ex: salmon, tuna and herring)[9] and results in the formation of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) due to ingestion of marine plants containing synthesized ω-3. In addition, other essential oils, such as flaxseeds, require the conversion of β-linolenic acid (ALA) to EPA and DHA[10,11].

EPA has an important anti-inflammatory role, whereas DHA plays an important role in the functioning and development of the retina[14] and the brain[15]. In addition to EPA and DHA, omega 3 is a precursor of lipid mediators, such as resolvins and protectins, which have anti-inflammatory and immunomodulatory actions[16,17].

Several studies have shown that nutritional supplementation with antioxidants, vitamins (A, C and E) and minerals improves visual function[18]. Antioxidants assist in the anti-inflammatory response, corneal healing and tear film stability[19].

The objective of the study was to compare the efficacy of 0.03% topical tacrolimus associated with oral omega 3 with different ratios of EPA, DHA and antioxidants as an adjuvant in the treatment of KCS in dogs.

MATERIALS AND METHODS

Animals

The study was conducted according to the standards of animal experimentation of the UNOESTE Ethical Committee on Animal Use (CEUA) (protocol n° 2939) and the ARVO (Association for Research in Vision and Ophthalmology - Statement for the use of animals in ophthalmic and visual research).

We evaluated forty-five dogs, attended to at the Veterinary Hospital of UNOESTE, in a six month period with no breed or sexual preference. The dogs were diagnosed with KCS and registered under an authorization term (Informed Consent Term). The animals were included in the experiment by observing clinical ophthalmic signs compatible with KCS (ocular secretion, conjunctivitis, corneal opacity and pigmentation) with a slit lamp (Kowa SL-15, Japan) as well as SST ≤10 mm/min and/or TBUT ≤ 10 sec.

Groups

After the diagnosis of KCS, the dogs were randomly assigned to three treatment groups: T group (n=15): tacrolimus 0.03% eye drops (Eye Pharma Laboratory, São Paulo, Brazil), 1 drop, 2x/day, topical, in both eyes for 6 months; TO group (n=15) tacrolimus eye drops 0.03% ((Eye Pharma Laboratory, São Paulo, Brazil), 1 drop 2x / day, topical in both eyes + oral omega 3 (1.5 EPA: 1 DHA - Ograx®-3, Avert Laboratory, São Paulo, Brazil, 1 capsule of 500 mg /7 kg/day) for 6 months; TOA group (n=15) tacrolimus eye drops 0.03% ((Eye Pharma Laboratory, São Paulo, Brazil), 1 drop 2x / day, topical in both eyes + oral omega 3 + antioxidants (1 EPA: 4,5 DHA + vitamin E + vitamin C + selenium- Seniox®, Avert Laboratory, São Paulo, Brazil, 1 capsule of 500 mg/10 kg/day), for 6 months. In addition to conventional treatment with tacrolimus and propylene glycol

based lubricant (Systane®, Alcon, São Paulo, Brazil), antibiotics and anti-inflammatory eye drops with diclofenac sodium were also prescribed (Still®, Allergan, São Paulo, Brazil) for 15 days.

Ophthalmic Exams

Monthly ophthalmic and cytological exams were performed, considering zero (T0) the first day of treatment with bilateral KCS diagnosis and the other time points (T1 to T6). The cytological examination was performed at moments T0, T3, and T6, and histopathological examination was performed at the time of diagnosis (T0) and at the end of the study (T6).

Clinical ophthalmic signs were identified using the portable slit lamp (Kowa, Japan) with or without conjunctivitis, ocular secretion, opacity and corneal pigmentation according to the scoring described in Table 1.

The Schirmer Tear Test (STT) was performed without anesthetic eye drops to evaluate the quantitative portion of the tear, introducing 0.5 cm of the strip into the conjunctival sac for one minute and considering positive testing if ≤10 mm/min.[20]. Tear Film Break-up Time (TBUT) was used to evaluate the qualitative portion of the tear, being measured twice consecutively, and the mean was calculated. After instilling a drop of 1% fluorescein eye drops (Allergan, São Paulo, Brazil) with a slit lamp (Kowa, Japan), the time between the last blink and the appearance of spots or dark spots on the lacrimal film was measured and considered positive for TBUT values ≤ 10 seconds[21].

After the TBUT examination, the eyes were washed with physiological solution and the presence or absence of ulcers in the cornea was determined using the fluorescein test[20]. The eyes were graded according to the severity and extent of the ulcers (0-negative, 1- small superficial ulcer, 2- medium superficial ulcer, 3- extensive superficial

ulcer, 4- small stromal ulcer, 5- medium stromal ulcer, 6- extensive stromal ulcer, 7- descemetocele and 8- keratomalacia or melt ulcer).

The Green Lissamine Test (GLT) was used to evaluate the presence of devitalized cells using the lissamine strip[22] (Ophthalmos, São Paulo, Brazil). The lissamine strip was placed in contact with the tear meniscus at the bottom of the bag, and analysis was performed 2 minutes after. The classification of van Bijsterveld for graduation was used. The palpebral rhyme was divided into 3 areas: lateral bulbar conjunctiva, cornea and medial bulbar conjunctiva. In each of these areas, the following graduation was used: 0- absence of staining, 1-fine dots, 2-dots coarse, and 3-plate. The sum of each of these areas determined the final score, which ranged from 0 to 9.

Cytological and Histopathological Examination

The cytology was performed after ocular cleaning with physiological solution, and samples were taken from cells of the lower conjunctiva with a sterile swab moistened with physiological solution while a blade was made for each eye and stained by the MGG technique (May-Grunwald-Giemsa). Lymphocytes, neutrophils, metaplastic cells and squamous cells were counted under an optical microscope in 10 fields with a 40x objective lens.

The histopathological examination was performed after instillation of Anestésico® (1% tetracaine hydrochloride + 0.1% phenylephrine hydrochloride, Allergan, São Paulo, Brazil) with a withdrawal of 1-3 mm in the fornix of the medial inferior conjunctiva using tweezers and conjunctive scissors. The histological section was placed in a standardized 1x1 cm paper 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), PAS (Merck, USA) and subsequently evaluated for the following parameters. In HE staining: lymphocyte count, neutrophils, presence of squamous metaplasia and presence of edema. In PAS counting the goblet cell density (cells/mm^2) under the optical microscope at 40x.

STATISTICAL ANALYSIS

For the variables STT; TBUT; goblet cell density; and number of squamous cells, metaplastic cells, neutrophils and lymphocytes, we used two analyses of variance (ANOVAs) for Tukey's sample. For the clinical sign variables FT and GLT, we used Friedman's non-parametric test to compare moments, while the Kruskall-Wallis test with Dunn contrast was used to compare the groups. A $P<0.05$ significance level was adopted. The software used for statistical analysis was R, version 3.2.2. (The R Foundation for Statistical Computing, 2015).

RESULTS

In the clinical signs evaluated (Figure 1), all groups showed significant improvement. In the variables of ocular secretion and conjunctivitis, there was total remission in T1 only in the TO group, whereas in the T and TOA groups, remission was found at T3 and T2, respectively. In the variable of opacity, there was total remission in all groups in T2. Regarding corneal pigmentation, the median showed remission throughout the treatment and from T3 in the T, TO and TOA groups, which showed stability without further clinical signal reduction.

In the STT (Figure 2), the three groups showed a significant increase from T1, which represented a significant difference ($P < 0.05$) of all groups compared to T0. At the time T6, the T group presented a significant difference in relation to the other groups. In the TBUT (Figure 2), all groups presented significant differences in relation to the moment T0. However, the TO group presented the best results at all times compared to the other groups.

In FT, all groups presented animals with ulcers of different severities and extension at T0, and all groups showed excellent healing in T1. In the GLT, the performance of all groups was similar, demonstrating improvement in the marking already found in T2.

In the cytological examination (Figure 3), all groups presented a decrease of all the cells, but there was a significant difference only when comparing T0 with the other times. In the histopathological study (Figure 4), all groups showed a decrease of inflammatory cells. The goblet cells showed a significance increased ($P < 0.05$) in TO group (49.7 ± 29.4), in relation to other groups, T (25.5 ± 15.6) and TOA (26.5 ± 11.7).

The aspects of some eyes and cytological and histopathological exams of the T, TO and TOA groups are shown in Figure 5.

DISCUSSION

The use of omega 3 (EPA and DHA) improved the clinical signs of KCS, increasing the STT and TBUT values and promoting resolution of corneal ulcers, because they presented anti-inflammatory characteristics and corroborated the results of other studies[9,10].

In the present study, resolution of ocular secretion, conjunctivitis and increased TBUT were better in the TO group compared to the T and TOA groups. Both TO and TOA groups received omega supplementation, but with different proportions: the TO group (1.5EPA:1DHA) and the TOA group (1EPA:4.5DHA + antioxidants), in which the addition of antioxidants did not seem to have any treatment benefits, had a portion of EPA. According to another study[23], the efficacy in the treatment for dry eye with the use of ω-3 is linked to the dose of the ingested omega; however, the result of our study demonstrates that the effectiveness is related to the proportion of EPA and DHA. Other studies[19] referred to antioxidants as an adjunct in the inflammatory response, corneal healing, and lacrimal film stability. However, in the present study, this adjunct did not bring additional benefits in the treatment of dry eye in dogs.

According to studies, EPA has a pro-inflammatory effect exerting cellular actions that stimulate the production of collagenase and increase the expression of adhesion molecules necessary for leukocyte extravasation. EPA is the precursor of the E series of resolvins, which includes resolvin E1 (RvE1) and resolvin E2 (RvE2)[24,25,26]. Resolvins were first described in the formation of mediator molecules with anti-inflammatory capacity and immunomodulatory properties, including the reduction of proinflammatory leukocytes and cytokine migration, thus leading to a decrease in the inflammatory response in vivo[27].

DHA is more related to its antioxidant properties and is involved in several cognitive processes, while also linked to correct signaling between neurons, in fetal development and retinal function. DHA can also give rise to resolvins D1 (RvD1) and D2 (RvD2), a protectin (or neuroprotetin, when produced by neural tissues) and a maresine, thus having an important anti-inflammatory function in neuronal systems and the formation, development and functioning of the brain and retina[24,25,27].

In the present study, inflammatory cytology and conjunctival biopsy, observed from the neutrophil counts, were lower in the TO group compared to the other groups at time T6, especially the TO group. This may be due to the anti-inflammatory mediator RvE1 (produced by the EPA), which can increase the phagocytic activity of macrophages in order to help tissue cleansing[28].

There was a significant increase in the number of goblet cells between the beginning and the end of treatment, especially the TO group, which was in agreement with other studies that also demonstrated an increase of goblet cells after the administration of omegas in the treatment of KCS in several species[9,10].

Thus, we can conclude that oral omega 3, which contains a higher proportion of EPA than DHA, brought greater benefit to the treatment of dry eye in dogs associated with topical tacrolimus due to improvements in clinical signs, the inflammatory process and an increase in the number of goblet cells. This may be due the greater amount of EPA, which is a precursor of inflammatory mediators such as RvE1 that have anti-inflammatory and immunomodulatory properties.

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Table 1. Evaluation score of clinical ophthalmic signs

Clinical sign	Score
Conjunctivitis	0 = None 1 = Mild conjunctival hyperemia 2 = Moderate to severe Conjunctival hyperemia 3 = Moderate to severe Conjunctival hyperemia and chemosis
Ocular discharge	0 = None 1 = Minor serous discharge 2 = Moderate mucoid discharge 3 = Marked mucopurulent discharge
Corneal Opacity and Corneal Pigmentation	0 = None 1 = <25% 2 = 25-50% 3 = >50%

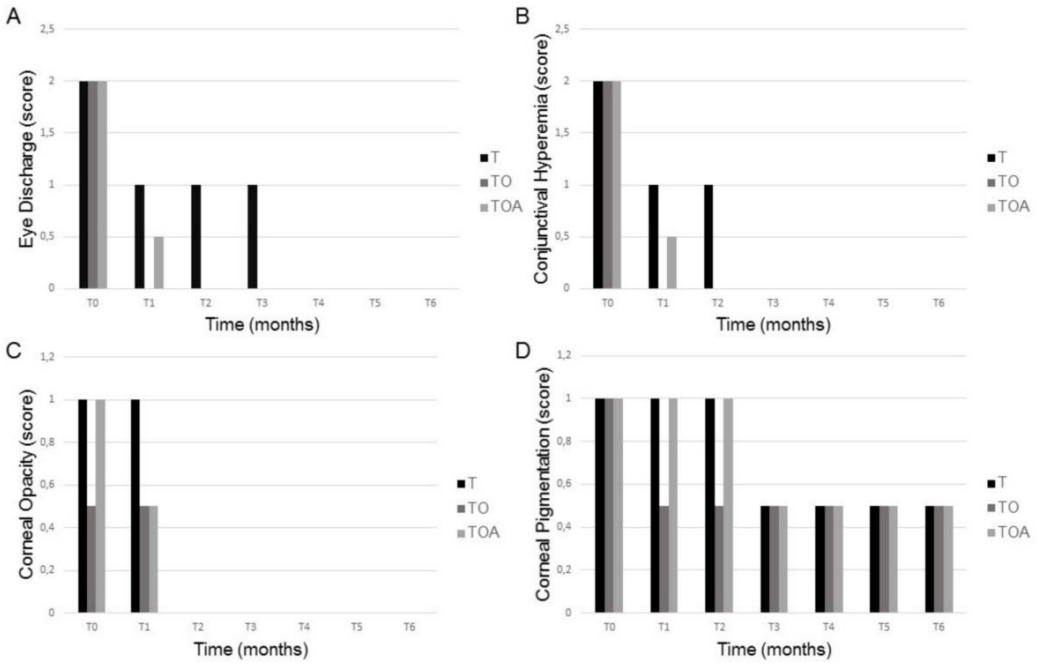

Figure 1. Median score of observed clinical signs from T0 to T6 of the T group (Tacrolimus), the TO group (Tacrolimus + Omega 1.5 EPA: 1 DHA), and the TOA group (Tacrolimus + Omega 1 EPA: 4.5 DHA + Antioxidants): (A) ocular discharge, (B) conjunctival hyperemia (C), corneal opacity and (D) corneal pigmentation.

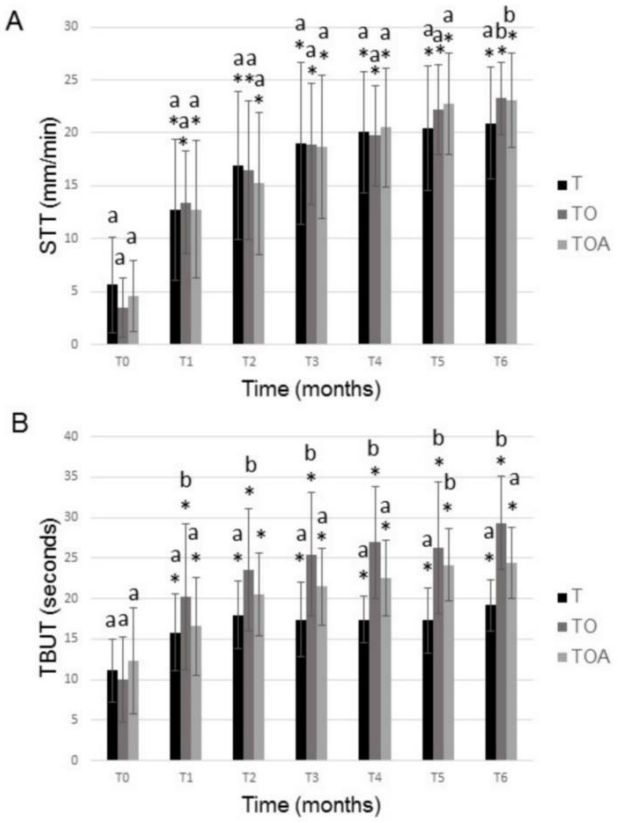

Figure 2: (A) Mean and standard deviation of the Schirmer Tear Test (STT) in mm/min from T0 to T6 of patients in the following groups: T (Tacrolimus), TO (Tacrolimus + Omega 1,5 EPA: 1 DHA), and TOA (Tacrolimus + Omega 1 EPA: 4.5 DHA + Antioxidants) (B) Mean and standard deviation of Tear Film Break-up Time (TBUT) in seconds, T0 to T6, of patients in the T, TO and TOA groups.

*p< 0,05 (Tukey test to compare moments)
[a,b]p< 0,05 (Kruskal-Wallis test to compare groups)

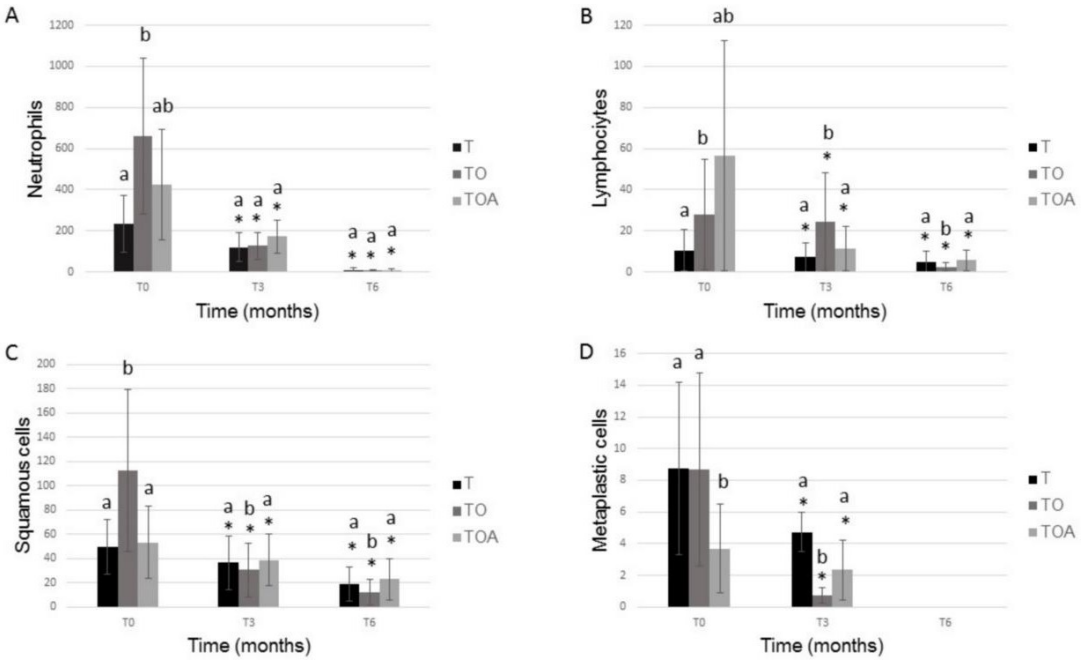

Figure 3. (A) Mean and standard deviation of the neutrophils count in the conjunctival cytology test from T0 to T6 of the T, TO and TOA groups (B) Lymphocytes (C) Squamous cells (D) Metaplastic cells.

*p< 0,05 (Tukey test to compare moments)
[a,b]p< 0,05 (Kruskal-Wallis test to compare groups)

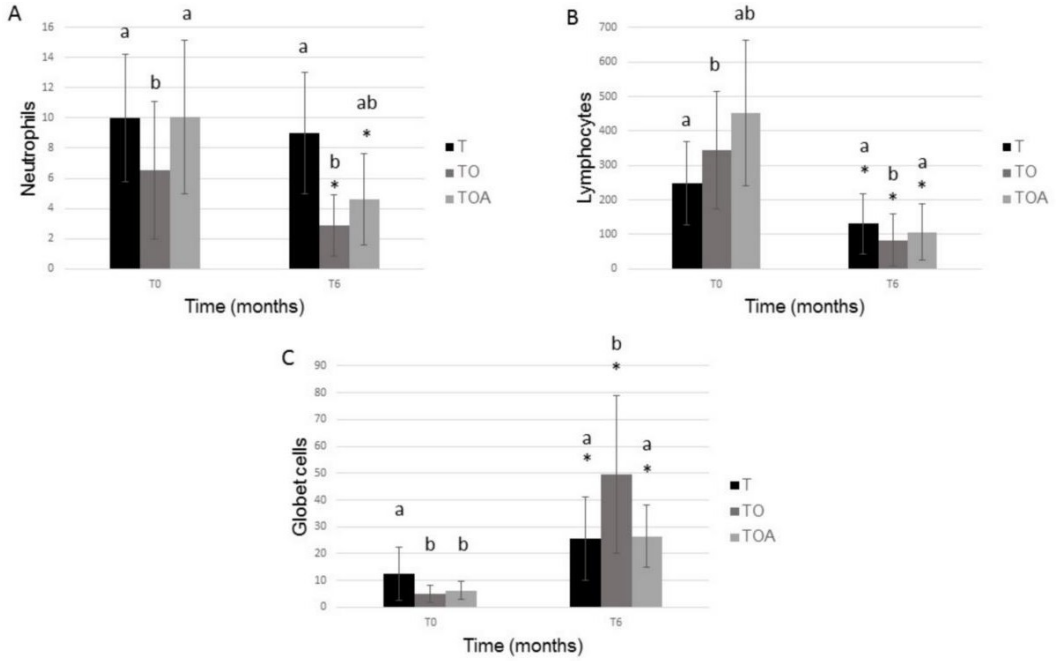

Figure 4. Mean and standard deviation of the neutrophils count in T0 and T6 conjunctival histopathology of the T, TO and TOA groups (B) Mean and standard deviation of the T0 and T6 lymphocyte counts (C) Mean and deviation pattern of goblet cell counts at T0 and T6.

*p< 0,05 (Tukey test to compare moments)
[a,b]p< 0,05 (Kruskal-Wallis test to compare groups)

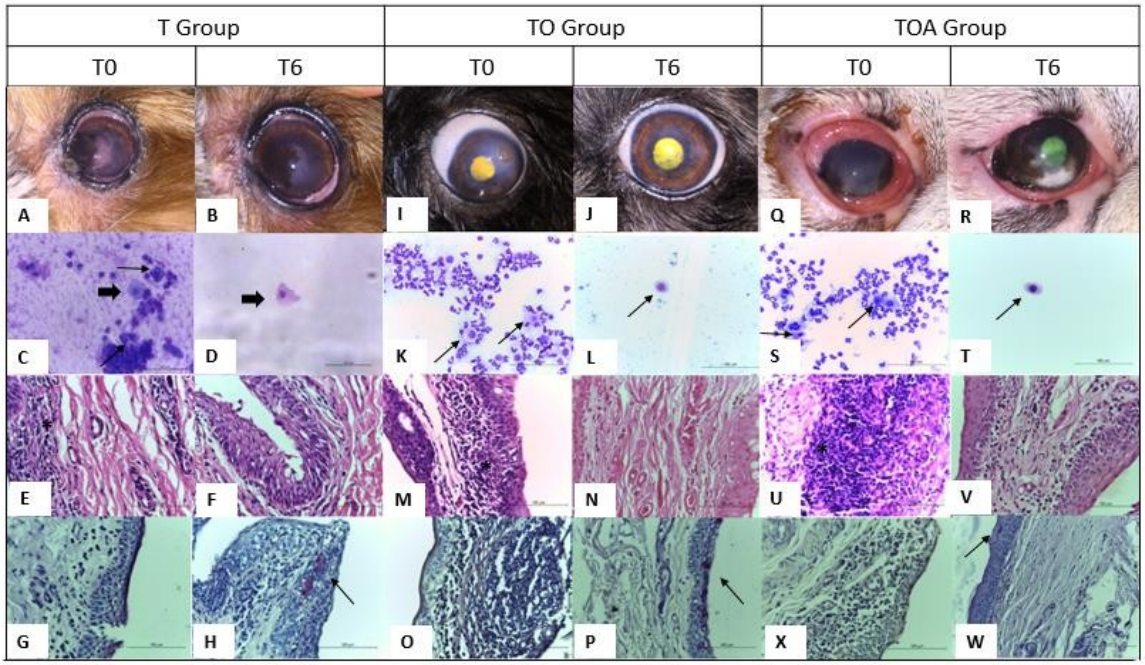

Figure 5. (A) T group, moment T0 left eye, mucoid secretion, opacity and corneal pigmentation (B) Evolution of the patient and persistence of pigmentation (C) Photomicroscopy of T0 conjunctival cytology, presence of neutrophils, squamous cell (thick arrow), metaplastic cell (fine arrow), MGG coloration, 400x magnification (D) T6 moment, presence of squamous cells (thick arrow), MGG coloration, 400x magnification (E) Histopathology at T0, inflammatory infiltrate (*), MGG coloration, 400x magnification (F) T0, reduction of inflammatory process (G) T0, absence of goblet cells, PAS staining, increase of 400x (H) PAS at T6 moment, presence of goblet cells (fine arrow) (I) TO group, T0 moment left eye, mucoid secretion, descemetocele (J) Ulcer healing (K) Photomicroscopy of T0 conjunctival cytology, presence of neutrophils, globet cell (fine arrow), MGG, 400x magnification (L) T6 moment, presence of squamous cell (thick arrow), MGG coloration, 400x magnification (M) Histopathology at T0, inflammatory infiltrate (*), coloration HE, increase of 400x (N) T0, reduction of the inflammatory process (O) T0, absence of goblet cells, PAS staining, increase of 400x (P) PAS, T6, large amount of goblet cells. (Q)TOA group, moment T0 right eye, mucopurulent secretion (R) clinical improvement of patient (S) Photomicroscopy of T0

conjunctival cytology, presence of neutrophils, metaplastic cell (fine arrow), MGG coloration, 400x magnification (T) T6, presence of globet cell (fine arrow), MGG coloration, 400x magnification (U) Histopathology at T0, inflammatory infiltrate (*), HE staining, 400x magnification (V) T0, reduction of inflammatory process (X) T0, absence of goblet cells, PAS staining, increase of 400x (W) PAS at T6 moment, presence of goblet cells.

ANEXO – NORMA DA ARQUIVOS BRASILEIROS DE OFTALMOLOGIA

INSTRUCTIONS TO AUTHORS

ISSN 0004-2749 ***printed version***

Scope and policy

ABO-ARQUIVOS BRASILEIROS DE OFTALMOLOGIA (ABO, ISSN 0004-2749 - printed version and ISSN 1678-2925 - online version) is the official bimonthly publication of the Brazilian Council of Ophthalmology (Conselho Brasileiro de Oftalmologia - CBO). The purpose of the journal is to publish scientific studies in Ophthalmology, Visual Sciences, and Public Health, encouraging research, as well as qualification and updating of the professionals involved in this field.

Methods

Original manuscripts are accepted only in English. Manuscripts are grouped into one of the following categories, based on the methodology used:

Clinical Studies
Descriptive or analytical studies involving humans or evaluating the literature relevant to humans.

Epidemiological Studies
Analytical studies involving results from human populations.

Laboratory Experimental Studies
Descriptive or analytical studies involving animal models or other biological, physical or chemical techniques.

Theoretical Studies
Descriptive studies involving description and theoretical analysis of new hypotheses based on the knowledge available in the literature. Theoretical results must add new information to literature.

Types of Manuscripts

Manuscripts submitted to ABO should fit into one of the following categories according to their format. The maximum number of words, figures, tables and, references for each type of manuscript are in parentheses at the end of the description for each category. The word count of the manuscript includes the text from the beginning of the introduction up to the

end of the discussion; therefore, the following items are not included: title page, abstract, references, acknowledgments, tables and figures, including legends.

Editorials

Editorials are contributed by invitation and should be related to topics of current interest, preferentially related to articles published in the same issue of ABO (title, maximum of 1,000 words, 2 figures or tables, and 10 references).

Original Articles

Original articles present complete experiments with results that have never been published before (title, structured abstract, maximum of 3,000 words, 8 figures or tables, and 30 references). The evaluation of the manuscripts will be based on the following priorities:

1. New and relevant information based on a study that uses appropriate methodology.
2. Repetition of information available in the literature, not previously confirmed locally, based on a study that uses appropriate methodology.
3. Repetition of information available in the literature and previously confirmed locally, based on a study that uses appropriate methodology.

* Manuscripts containing speculative conclusions, unsubstantiated by the results or based on a study with inappropriate methodology will not be accepted.

Case Reports and Case Series

Case reports or case series will be considered for publication when describing rare and original findings that have not been internationally confirmed, or when presenting clinical or surgical responses that can contribute to elucidate the pathophysiology of a disease (title, unstructured abstract, maximum of 1,000 words, 4 figures or tables, and 10 references).

Letters to the Editor

Letters to the editor are considered for publication if they contain comments related to manuscripts previously published in ABO or, exceptionally, the results of original studies with insufficient content to be submitted as Original Article. These letters should present new information or new interpretation of existing information. When the content of the letter refers to an article previously published in ABO, such article should be mentioned in the first paragraph of the letter and included in its reference list. In these cases, the letters will be linked to the article, and the authors of the article will have their right of reply guaranteed in the same issue. Congratulation letters will not be published (title, maximum of 700 words, 2 figures or tables, and 5 references).

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Manuscripts will only be considered for publication if they meet all the journal's requirements. The editorial office will inform the authors if their manuscript fails to meet such requirements. Upon notification, the corresponding author will have 30 days to make the necessary changes in the manuscript. If the deadline is not met, the manuscript will be excluded from the editorial process.

The manuscripts submitted to ABO are initially evaluated by the editors to check for content compliance with the editorial line of the journal. After this assessment, all manuscripts are sent for peer review. The anonymity of reviewers is preserved throughout the whole process. However, the authors of manuscripts do not remain anonymous.

After the initial editorial evaluation, the reviewers' comments can be sent to the authors to guide the changes to be implemented in the text. After implementing the changes suggested by the reviewers, the revised manuscript should be resubmitted along with a letter (which is sent as a supplementary document) with specific indications of all changes made to the manuscript or the reasons why the suggested changes were not made. Manuscripts that are resubmitted without a letter will be withheld until the editorial office receives the letter. The deadline to submit the new version of the manuscript is 30 days after the authors are informed of the need to make changes in their manuscript. Manuscripts will be excluded from the process if authors fail to meet this deadline. The ultimate publication will be based on the final approval of the editors. Manuscripts submitted to ABO should not be simultaneously considered for publication by other journals. In addition, total or partial publication or translation for publication in another language of the manuscripts submitted to ABO should not be considered without the permission of the editors of ABO.

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I. Conception and design, acquisition of data, or analysis and interpretation of data.
II. Draft or critical revision of the article for important intellectual content.
III. Final approval of the version to be published.

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The corresponding author should complete and submit the Author Contribution Statement as a supplementary document.

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- STARD (Diagnostic instruments or techniques)
- PRISMA (Systematic reviews and meta-analyses)
- STROBE (Observational studies)

Manuscript Preparation

Manuscripts should only be submitted online using the appropriate interface of ABO. The following guidelines were based on the format suggested by the International Committee of Medical Journal Editors (ICMJE) and published in the document: Uniform Requirements for Manuscripts Submitted to Biomedical Journals.

Only the manuscripts complying with these guidelines will be considered for analysis.

The text should be sent as a digital file. Only the following formats are accepted: .doc. The text should be typed double-spaced, in 12 point font. The pages should be numbered in Arabic numerals, starting each section on a new page.

The sections should be presented according to the following sequence: Title page (as a separate document); Abstract and Keywords; Introduction; Methods; Results; Discussion; Acknowledgements (if any); References; Tables (optional) and Figures (optional) including legends.

1. Title Page. It should contain: a) title (no more than 135 characters with spaces); b) authors' names as they should appear in print; c) each author's affiliation* (city, state, country and, if applicable, department, school, university); d) corresponding author's name, address, phone number, and email; e) sources of financial support (if any); f) project number and institution responsible for the approval of the Research Ethics Committee; g) statement of conflicts of interests of all authors; h) clinical trial registration number on a public trials registry.

* Professional or academic degrees, as well as job position will not be published.

Approval of the Institutional Review Board (IRB). All retrospective, cross-sectional, or prospective studies involving primary data collection or clinical and surgical reports should include the project number and name of the institution that provided the approval of the IRB on the title page. Studies involving humans should be compliant with the Declaration of Helsinki, whereas studies involving animals should be in accordance with the principles suggested by the Association for Research in Vision and Ophthalmology (ARVO).

As a supplementary document, the corresponding author should send the IRB approval or its report stating that the evaluation of the project by the Committee is not necessary. The author cannot decide on the need for evaluation by the Research Ethics Committee.

Statement of Conflicts of Interest. The title page should contain the statement of conflicts of interest of all authors (even if there is no conflict of interest). For more information about potential conflicts of interest, refer to: World Association of Medical Editors: Conflict of interest in peer-reviewed medical journals.

All authors should send the Form for Disclosure of Potential Conflicts of Interest as supplementary documents.

Clinical Trials. All Clinical Trials shall include on the title page the registration number in an international registry that allows free access to trial information (examples: U.S. National Institutes of Health, Australian and New Zealand Clinical Trials Registry, International Standard Randomised Controlled Trial Number - ISRCTN, University Hospital Medical Information Network Clinical Trials Registry - UMIN CTR, Nederlands Trial Register, Registros Brasileiros de Ensaios Clínicos).

2. Abstract and Keywords. Structured abstract (Objective, Methods, Results, Conclusions) with no more than 300 words. Unstructured abstract with no more than 150 words. Five keywords in English listed by the National Library of Medicine (MeSH - Medical Subject Headings).

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4. Introduction, Methods, Results, and Discussion. Citations in the text should be numbered sequentially in superscript Arabic numerals and in parentheses. The names of the authors should not be cited in the text.

5. Acknowledgements. This section should include the collaboration of people, groups or institutions that deserve to be acknowledged but do not meet the criteria for authorship. Statisticians and medical editors may meet the criteria for authorship and, in this case, should be acknowledged as authors. When they do not meet the criteria for authorship, they should be mentioned in this section. Writers who are not identified in the manuscript cannot be accepted as authors; therefore, professional writers should be acknowledged in this section.

6. References. Citations (references) of authors in the text should be numbered sequentially in the same order as they are cited and identified using superscript Arabic numerals. References should be in accordance with the format suggested by the International Committee of Medical Journal Editors (ICMJE), based on the examples below.

The titles of the journals should be abbreviated according to the style provided by the List of Journal Indexed in Index Medicus of the National Library of Medicine.

The names of all authors should be cited for references with up to six authors. For studies with seven or more authors, cite only the first six authors followed by *et al*.

Examples of references:

Journal Articles
Costa VP, Vasconcellos JP, Comegno PEC, José NK. O uso da mitomicina C em cirurgia combinada. Arq Bras Oftalmol. 1999;62(5):577-80.

Books
Bicas HEA. Oftalmologia: fundamentos. São Paulo: Contexto; 1991.

Book Chapters
Gómez de Liaño F, Gómez de Liaño P, Gómez de Liaño R. Exploración del niño estrábico. In: Horta-Barbosa P, editor. Estrabismo. Rio de Janeiro: Cultura Médica; 1997. p. 47-72.

Annals
Höfling-Lima AL, Belfort R Jr. Infecção herpética do recém-nascido. In: IV Congresso Brasileiro de Prevenção da Cegueira; 1980 Jul 28-30, Belo Horizonte, Brasil. Anais. Belo Horizonte; 1980. v.2. p. 205-12.

Dissertations
Schor P. Idealização, desenho, construção e teste de um ceratômetro cirúrgico quantitativo [dissertation]. São Paulo: Universidade Federal de São Paulo; 1997.

Electronic Documents
Monteiro MLR, Scapolan HB. Constrição campimétrica causada por vigabatrin. Arq Bras Oftalmol. [online journal]. 2000 [cited 2005 Jan 31]; 63(5): [about 4 p.]. Available at: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0004-27492000000500012&lng=pt&nrm=iso

7. Tables. Tables should be numbered sequentially using Arabic numerals in the order they are mentioned in the text. All tables should have a title and a heading for all columns. Their format should be simple, with no vertical lines or color in the background. All abbreviations (even if previously defined in the text) and statistical tests should be explained below the table. The bibliographical source of the table should also be informed when the table is extracted from another study.

Do not include tables in the main document of the manuscript, they should be uploaded as supplementary documents

8. Figures (graphs, photos, illustrations, charts). Figures should be numbered sequentially using Arabic numerals in the order they are mentioned in the text. ABO will publish the figures in black and white at no cost to the authors. Manuscripts with color figures will be published only whether the color figure is considered necessary, otherwise, it will be published in black and white.

Graphs should be in shades of gray, on a white background and without three-dimensional or depth effects. Instead of using pie charts, the data should be included in tables or described in the text.

Photos and illustrations should have a minimum resolution of 300 DPI for the size of the publication (about 2,500 x 3,300 pixels for a full page). The quality of the images is considered in the evaluation of the manuscript.

The main document should contain all figure legends, typed double-spaced and numbered using Arabic numerals.

Do not include figures in the main document of the manuscript; they should be uploaded as supplementary documents.

9. Abbreviations and Acronyms. Abbreviations and acronyms should be preceded by the spelled-out abbreviation on first mention and in the legends of tables and figures (even if they have been previously mentioned in the text). Titles and abstracts should not contain abbreviations and acronyms.

10. Units of Measurement: Values of physical quantities should be used in accordance with the standards of the International System of Units.

11. Language. Texts should be clear to be considered appropriate for publication in a scientific journal. Use short sentences, written in a direct and active voice. Foreign words should be in italics. Therapeutic agents should be mentioned by their generic names with the following information in parentheses: trade name, manufacturer's name, city, state and country of origin. All instruments or apparatus should be mentioned including their trade name, manufacturer's name, city, state and country of origin. The superscript symbol of trademark ® or ™ should be used in all names of instruments or trade names of drugs. Whenever there are doubts about style, terminology, units of measurement and related issues, refer to the AMA Manual of Style 10th edition.

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Checklist
Before submitting their manuscript, authors should make sure that all the following items are available:

- Manuscript prepared in accordance with the instructions to authors.
- Maximum number of words, tables, figures, and references according to the type of manuscript.
- Title page including the clinical trial registration number is not included in the main document
- No figures and tables are included in the main document of the manuscript.
- All figures and tables were uploaded separately as supplementary documents.
- Author Contribution Statement completed and saved as a digital file to be sent as a supplementary document.

- Form for Disclosure of Potential Conflicts of Interest of all authors completed and saved as digital files to be sent as supplementary documents.
- Digital version of the report provided by the Institutional Review Board containing the approval of the project to be sent as a supplementary document.

II SCIENTIFIC ARTICLE

Fractal analysis of palpebral conjunctiva of three treatments of keratoconjunctivitis sicca in dogs

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Running Title: Fractal analysis of three KCS treatments in dogs

ABSTRACT

Objective Using fractal dimension, evaluate the palpebral conjunctiva of dogs with keratoconjunctivitis sicca (KCS) treated with topical tacrolimus associated or not with oral omega in different proportions of EPA, DHA and antioxidants.

Procedure Sixty dogs were evaluated monthly for 6 months by Schirmer Tear Test (STT), tear film break-up time (TBUT) and slit lamp examination. Of these dogs, 45 with KCS were treated as the T group (tacrolimus 0.03% topical), TE group (tacrolimus topical + omegas 1.5EPA: 1DHA oral) and TD group (tacrolimus topical + omegas 1EPA: 4.5DHA + oral antioxidants), and 15 dogs were in the C group (negative control). A total of 180 conjunctival biopsy microscope slides from the beginning and end of the treatment of dogs with KCS, and 30 microscope slides from the dogs of C group were evaluated. For the fractal analysis, the microscope slides in hematoxylin and eosin were photographed (40X), and Image J software was performed. Calculation of the fractal dimension for evaluation and comparison between groups was performed.

Results In the fractal analysis, the C group (1.84) did not significant difference with only the TE group at T6 (1.881), and the values were close to the values found in the fractals parameters of healthy eyes.

Conclusion The fractal analysis quantified the presence of the inflammatory process / edema in the palpebral conjunctiva of dogs with KCS. The oral omega 3 with the highest proportion of EPA associated with topical tacrolimus presented better performance in the reduction of inflammation at the end of the treatment.

Keywords: keratoconjunctivitis sicca, fractal analysis, fish oil, tacrolimus, antioxidants and conjunctiva.

INTRODUCTION

Keratoconjunctivitis sicca (KCS), also known as dry eye, is a chronic inflammatory disease commonly diagnosed in dogs and humans, resulting in deficient production of the aqueous portion of the tear film (quantitative deficiency) and/or excessive tear evaporation (qualitative deficiency) due to inadequate production of the lipid layer, thus decreasing or ceasing the protective function of the tear. [1,2,3,4]

Clinical signs of dry eye include mucoid ocular secretion, conjunctival hyperemia, blepharospasm, corneal vascularization, corneal opacity, and, in advanced cases, ulcer and pigmentation of the cornea. There are several causes of KCS in dogs that can cause decreased lacrimal production: racial predisposition, hypothyroidism, autoimmune diseases, facial nerve palsy, medications (atropine, sulfonamides), surgical excision of the third eyelid gland, distemper and conjunctivitis among others. [5,6,7,8]

The diagnosis is based on the presence of clinical signs and specific tests, such as the Schirmer Tear Test, Tear film break-up time and Fluorescein Test, and additional investigations, such as cytology and conjunctival histopathology, are performed to evaluate the cellular infiltrate and changes in the tissue. Treatment consists of the use of immunosuppressive agents, mainly tacrolimus and cyclosporin, associated with ocular lubricants, while antibiotics and anti-inflammatory drugs are used secondarily. [8,9,10,11,12]

Recent studies in medicine and veterinary medicine have shown good results in dry eye control with the use of essential fatty acids (AGEs), omega-3 (ω-3) and omega-6 (ω-6), due to their ability to produce anti-inflammatory mediators as an alternative therapy for patients with various forms of lacrimal deficiency. [13,14,15]. One of the most important sources of ω-3 is fish oil (FO), which has pre-formed eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). This is due to the ingestion of marine plants containing the synthesized ω-3, while linseed oil requires the conversion of β-linolenic acid (ALA) into EPA

and DHA. [16,17]. EPA and DHA have anti-inflammatory properties because they affect lipoprotein metabolism and decrease the production of other components, including cytokines, interleukin 1-β and tumor necrosis factor α, which have pro-inflammatory effects. [22]. In addition, DHA plays an important role in the functioning and development of the retina [18] and brain [19], predominantly found in most cell membranes. DHA influences the physical properties of brain membranes, the characteristics of their receptors, cellular interactions and enzymatic activities. [20]

Fractal analysis has been used in several areas of medicine, as a quantitative and specific way of measuring changes in images (histological, photographs, X-ray). The term fractal means breaking or producing irregular pieces. This analysis is an important tool to evaluate irregular histological structures and thus quantify the change after some specific intervention. The analysis or fractal dimension has been used to measure geometric and irregular structures. By means of a mathematical formula in a software, one can evaluate a certain complex area. One of the most used methods is box-counting, in which the (r) and number of squares are needed to cover the image (Nr), and the fractal dimension consists of the slope of (r) values and the number of squares Nr.[23,24, 25]. According to some studies, the fractal analysis may be an important means of differentiating normal and pathological states of vascularization of the canine retina. [26]

To calculate the fractal analysis, we must take into account the type of image (radiographic, histological, resonance) and the method of analysis. From the standardization of healthy tissue, it will be possible to make a correlation with tissues modified by some pathology.

However, despite being a technique with good applicability for histological and quantitative analysis, we did not find specific studies that used fractal analysis to evaluate the tissue of the palpebral conjunctiva and alterations to it caused by dry eye. The use of this

technique may pave the way for a standardized measurement of histological findings and the diagnosis of KCS, thus allowing for a more accurate analysis of the results.

The aim of the present study was to analyze the alterations in the palpebral conjunctiva tissue of healthy animals and compare it with the animals affected by KCS while quantitatively comparing the efficacy of three treatments of keratoconjunctivitis sicca in dogs as follows: tacrolimus associated or not with omegas in different proportions of EPA, DHA and antioxidants.

MATERIALS AND METHODS

Animals

The study was approved by the Ethics Committee on Animal Use (CEUA) of UNOESTE (Protocol nº.3901) and was carried out according to the rules of ARVO (Association for Research in Vision and Ophthalmology - Statement for the use of animals in ophthalmic and visual research).

Forty-five dogs, attended to at the Unoeste Veterinary Hospital, were diagnosed with bilateral KCS, with no racial, age and sexual preference, and were randomly allocated to the three dry eye treatment groups. The animals were included in the experiment by observing clinical ophthalmic signs with slit lamp (Kowa SL-15, Japan) (ocular discharge, conjunctival hyperemia, corneal opacity and pigmentation) and STT $\leq$ 10 mm /min and /or TBUT $\leq$ 10 seconds. We also used 15 dogs, with no racial, age or sexual preference, with healthy eyes that were sourced from the Kennel of Unoeste.

Groups

After the diagnosis of KCS, the dogs were randomly assigned to three treatment groups: T group (n = 15): tacrolimus 0.03% eye drops (Eye Pharma Laboratory, São Paulo, Brazil), 1 drop, 2x / day, topical, in both eyes for 6 months; TE group (n = 15) tacrolimus eye drops 0.03% (Eye Pharma Laboratory, São Paulo, Brazil), 1 drop 2x / day, topical in both eyes + oral omega 3 (1.5 EPA: 1 DHA - Ograx Avert, São Paulo, Brazil, 1 capsule of 500 mg / 7 kg / day or Ograx®-3 1000 / Laboratory Avert, 1 capsule of 1000 mg / 14 kg / day), for 6 months; TD group (n = 15) tacrolimus eye drops 0.03% (Eye Pharma Laboratory, São Paulo, Brazil), 1 drop 2x / day, topical in both eyes + oral omega 3 (1 EPA: 4, 5 DHA + Vitamin E + Vitamin C + Selenium - (Seniox® 500 / Avert Laboratory, São Paulo, Brazil, 1 capsule 500 mg / 10 kg / day or Seniox® 1000 / Laboratory Avert, São Paulo, Brazil, 1 capsule 1000 mg / 20 kg / day) for 6 months; and Control Group (n = 15), dogs with healthy eyes without treatment.

Ophthalmic Exams

Clinical ophthalmic signs were identified using a portable slit lamp (SL-15, Kowa, Japan) in the presence or absence of conjunctivitis, ocular secretion, corneal opacity (fibrosis, edema, infiltrate or vascularization) and corneal pigmentation, according to the scores described in Table 1, in which they were evaluated before treatment (T0) and after 6 months of treatment (T6). For the control group, we measured only time zero (T0) to establish the normal parameters of healthy dog ophthalmic exams in the region of Presidente Prudente, SP, Brazil, for comparison with the KCS-positive animals and treated groups (T, TE and TD) in the same regions.

The Schirmer Tear Test (STT) (Schirmer®-Ophthalmos , São Paulo, Brazil) was performed without anesthetic eye drops to evaluate the quantitative portion of the tear. Values <10 mm / min were considered positive for KCS, and tear film break-up time (TBUT) was used to evaluate the qualitative portion of the tear. After instilling 1 drop of the fluorescein eye drops (Fluoresceína®, Allergan, São Paulo, Brazil), with the aid of the slit lamp time (seconds) of tear film rupture, values <10 seconds were considered as KCS.

Histopathological Examination

The conjunctival biopsy was performed after three instillations of anesthetic eye drops (1% tetracaine hydrochloride + 0.1% phenylephrine hydrochloride, Allergan, São Paulo, Brazil). With absence of local sensitivity, a fragment of a size 1-3 mm of the medial inferior fornix of the conjunctiva was removed with the aid of ophthalmic instruments (clamp and conjunctive scissors (HR, São Paulo, Brazil). The fragment was placed on a 1x1 cm piece of paper and fixed in formalin, and then processed and stained by the hematoxylin and eosin (HE) technique (Dolles, São Paulo, Brazil).

Fractal Analysis

Sixty slides from each treatment group (T, TE, TD) were analyzed, with 30 microscope slides before treatment and 30 slides after treatment. There were 30 microscope slides for the control group. The LEICA ICC 50 HD microscope was used to visualize the images of the HE microscope slides in the 40x objective and was coupled to a video camera.

For fractal analysis, the images went through the process of binarization, making them black and white. Later, fractal dimension analysis was performed using the ImageJ software (Fig. 1), which is available free of charge on the Internet (http://rsbweb.nih.gov/ij/), and the Box-Counting method.

STATISTICAL ANALYSIS

The Kolmogorov-Smirnov test was used to test the normal distribution of the fractal dimensions. For the non-parametric analysis, the Kruskal-Wallis method was used with Dunn`s test. A significance level of $P<0.05$ was adopted. The software used was the GraphPad Prism® statistic (GraphPad software, La Jolla, CA, USA).

RESULTS

The clinical signs observed before and after treatment are described in Fig. 2. There was improvement in the signs of conjunctivitis, hyperemia, ocular secretion, pigmentation and opacity of the cornea in the T, TE and TD groups from T0 to T6. There was a significant ($p <0.05$) increase in STT (Fig. 3A) and TBUT (Fig. 3B) in the treatment groups.

In the fractal analysis, the control group, when compared to the other groups, presented a significant difference with only the TE-T6 group ($p <0.0001$). After the treatment, the values approached the parameters found in healthy eyes: TE -T6 1.81 (1.75-1.85) and Control 1.84 (1.82-1.85) (Fig. 4). In the comparison between moments of the same group, there was a significant difference ($p <0.0001$) between the TE-T0 and TE-T6 groups, which had values of 1.70 (1.65-1.78) and 1.81 (1.75 -1.85), respectively (Figure 4). When comparing the groups at the same time, the T-T6 group 1.67 (1.64-1.70) presented significant difference with the other TE-T6 groups, with a value of 1.81 (1.75-1.85), and TD-T6, which had a value of 1.79 (1.70-1.83), ($p <0.0001$) (Figure 4).

Some illustrative cases of different histological aspects and respective Fractal Dimension values within the treatment groups T, TE and TD are shown in Figure 5.

DISCUSSION

In the present study, the fractal analysis technique was applied with the objective of evaluating the inflammatory process and edema through the measurement of the inflammatory infiltrate in the tissue of the conjunctiva. When submitted to the binarization process, HE-stained microscope slides demonstrated that the nucleus and tissue fibers were detached from the rest of the cells; thus, it was possible to measure the size of the infiltrate and edema by fractal dimension analysis.

Currently, the histological analysis of the muscle tissue and the conjunctiva is performed in a qualitative way, through tissue characterization from cell morphology, size, nucleus position, presence of inflammatory infiltrate, and count of inflammatory cells, etc.[27,15]. However, this type of analysis is dependent on the analyzer and may vary from one observer to another. [28]. The analysis through the fractal dimension, besides quantifying the histological changes, has the advantage of being independent of the evaluator since it is an automatic analysis performed by the software.

The present study demonstrated that through fractal analysis, patients with dry eye present with a smaller fractal dimension when compared to healthy patients due to greater conjunctive edema and, consequently, greater laxity of the fibers of the conjunctiva. This result is not in agreement with other fractal analysis studies when other tissues, such as cardiomyocytes [25] and stretch muscle injury [29], have been studied. These studies noted an increase in the fractal dimension for both cardiac morphological changes and the formation of collagen in stretched muscle.

The TE group, which received treatment with omega-3 (1.5 EPA: 1 DHA) associated with 0.03% tacrolimus eye drops, showed a greater improvement in both clinical signs and fractal dimension values when compared to the T group, which received only 0.03% tacrolimus eye drops, and the TD group, which received 0.03% tacrolimus eye drops

associated with omega 3 + antioxidants (1 EPA: 4.5 DHA + vitamin E + vitamin C + selenium).

The difference between the groups that used oral omega 3, TE and TD, resides mainly in the different proportions of EPA and DHA. In the TE group (1.5 EPA: 1 DHA) and the TD group (1 EPA: 4.5 DHA), the addition of antioxidants did not bring any apparent benefits in the treatment of KCS, but rather a greater proportion of EPA. According to studies, EPA has a pro-inflammatory effect that exerts cellular actions to stimulate the production of collagenase and increase the expression of adhesion molecules necessary for extravasation of leukocytes, thus justifying the result obtained from the TE group in T6 that presented a fractal value similar to that of the control group. DHA is more related to its antioxidant properties and is involved in several cognitive processes, in addition to correct signaling between the neurons, fetal development and retinal function [19-22].

The fractal dimension has already been used in several areas of medicine, such as oncology, neurology, ophthalmology, radiology and cardiology, [31,32] and is useful in the characterization and identification of irregular and complex structures. Although studies of fractal dimension in veterinary medicine are few, only the area of ophthalmology has described one study of the retina in dogs [26]; thus, new research with a standardized measurement of histological evaluation may contribute to a more reliable diagnosis to evaluate the effectiveness of treatments.

The present study demonstrated that fractal analysis was able to quantify the presence of the inflammatory process / edema in conjunctival tissue in dogs with KCS and that oral omega 3 is efficient in improving clinical signs and inflammatory process in the treatment for KCS in dogs, especially those containing a higher concentration of EPA than DHA.

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Table 1. Evaluation score of clinical ophthalmic signs

Clinical sign	Score
Conjunctivitis	0 = None 1 = Mild conjunctival hyperemia 2 = Moderate to severe Conjunctival hyperemia 3 = Moderate to severe Conjunctival hyperemia and chemosis
Ocular discharge	0 = None 1 = Minor serous discharge 2 = Moderate mucoid discharge 3 = Marked mucopurulent discharge
Corneal Opacity and Corneal Pigmentation	0 = None 1 = <25% 2 = 25-50% 3 = >50%

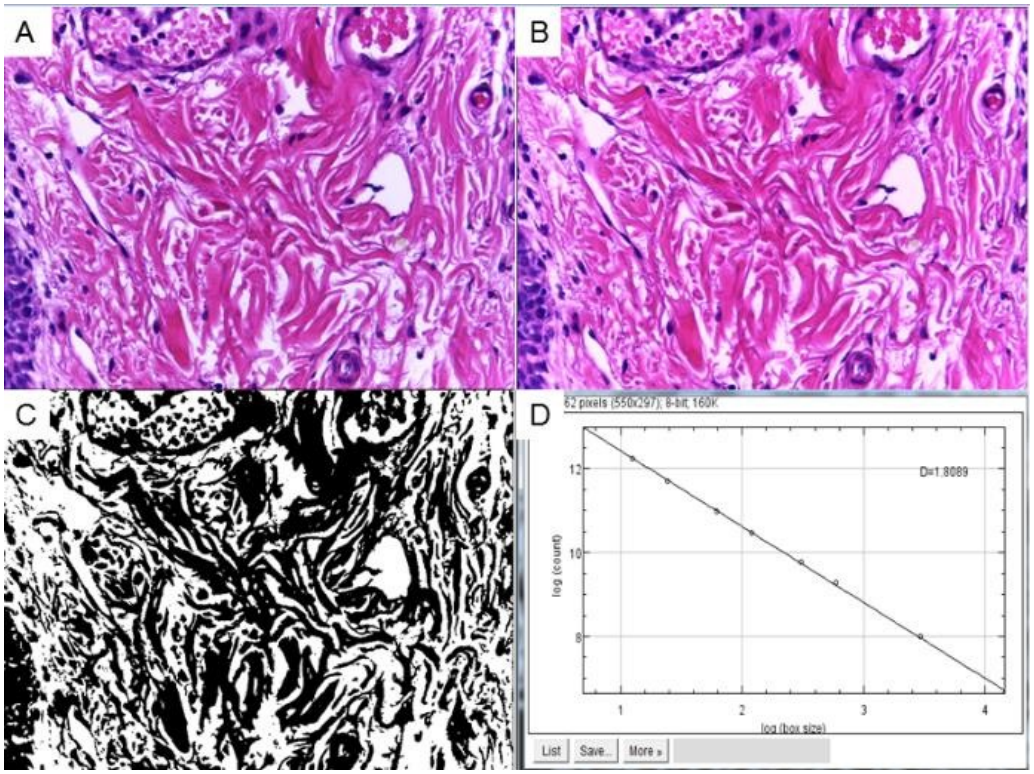

Figure 1. A) Histological section of the palpebral conjunctiva stained in Hematoxylin and Eosin (HE), 40x magnification. B) Binarization process. C) Image of HE after the binarization process. The connective tissue in black and the rest (space between the fibers) in white are observed. D) Linear regression by the overlapping of squares (N) of progressively smaller sides (r), where Nr is the number of r-side squares required to cover the image with each chosen size. The fractal dimension is the slope of the regression line of the two log values.

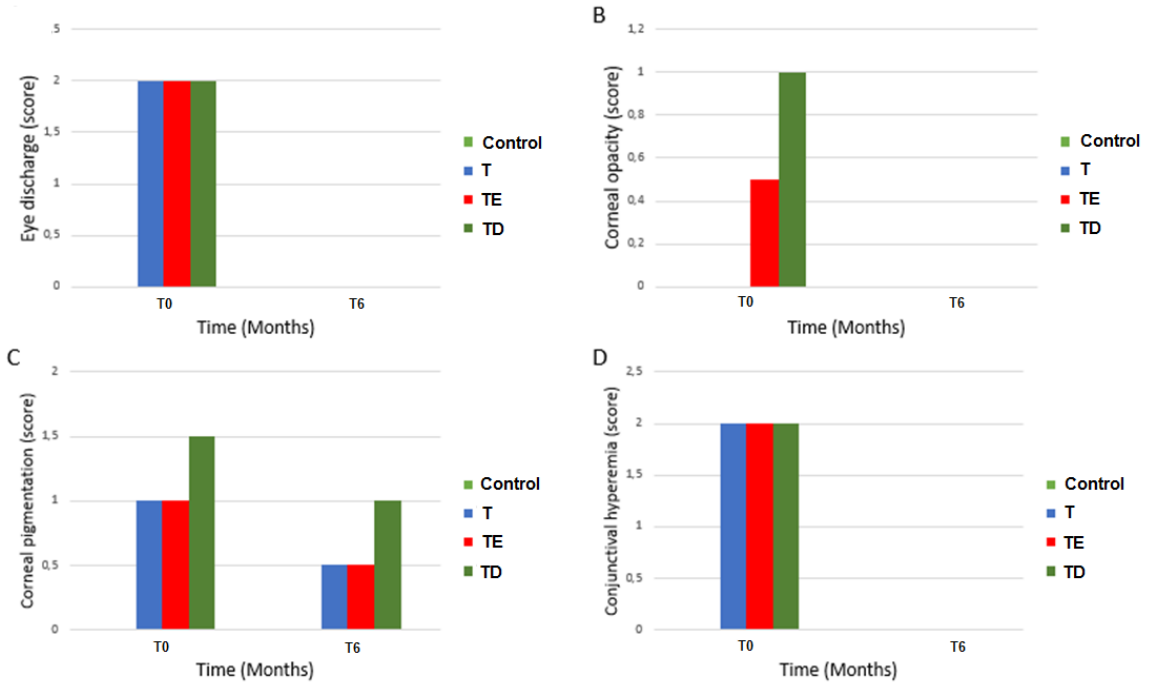

Figure 2. Median of clinical signs observed at T0 and T6 (Table 1). (A) Eye discharge, (B) Corneal opacity, (C) Corneal pigmentation and (D) Conjunctival hyperemia.

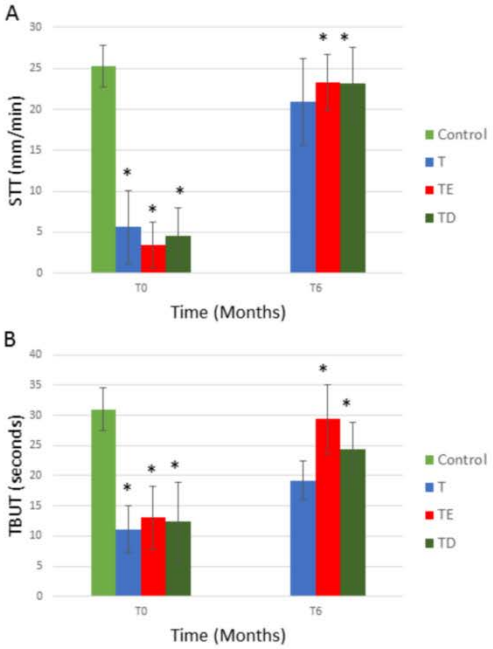

Figure 3. Mean and Standard Deviation of the values obtained from the (A) Schirmer Tear Test (SST) [values ≤10 mm / min (positive for KCS)] in mm/min and (B) Tear Film Break-up time (TRFL) test [TRFL ≤10 seconds (positive for KCS)] in seconds in dogs.

* p <0.05 (Kruskal-Wallis test for comparison between groups).

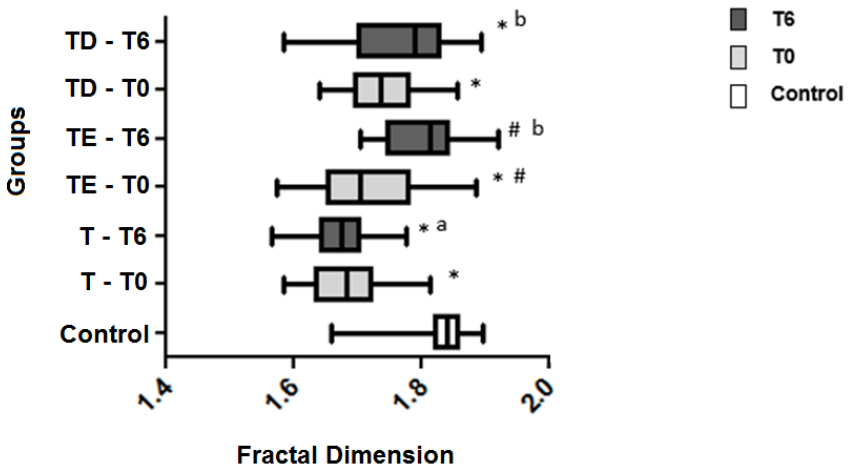

Figure 4. Fractal dimension analysis microscope slides stained in HE. * p <0.0001 vs control group; # p <0.0001 intragroup; ab p <0.0001 intergroup (Kruskal-Wallis and Dunn).

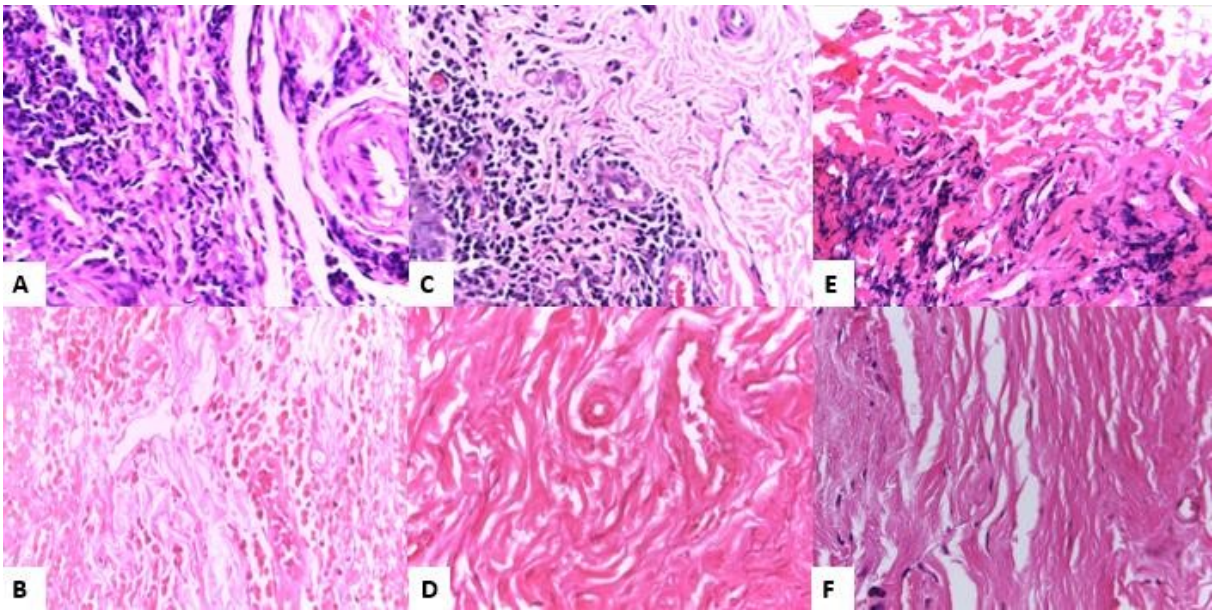

Figure 5. Histological sections of the palpebral conjunctiva stained in Hematoxylin and Eosin (HE), increased by 40x. (A) Moment T0, T group, Fractal Dimension = 1,655. (B) Moment T6, T group, Fractal Dimension = 1.687. (C) Moment T0, TE group, Fractal Dimension = 1.63. (D) Moment T6, TE group, Fractal Dimension = 1,881. (E) Moment T0, TD group, Fractal Dimension = 1,66. (B) Moment T6, TD group, Fractal Dimension = 1,795.

ANEXO – NORMA DA VETERINARY OPHTHALMOLOGY

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2. Barnett KC. *Color Atlas of Veterinary Ophthalmology.* Williams and Wilkins, Baltimore, 1990.

3. Davidson MG. Equine ophthalmology. In: *Veterinary Ophthalmology* 2nd edition (ed. Gelatt KN). Lea and Febiger: Philadelphia, 1991; 576-610
4. Maggs DJ, Nasisse MP. Effects of oral L-lysine supplementation on the ocular shedding rate of feline herpesvirus (FHV-1) in cats (abstract). *28th Annual Meeting of the American College of Veterinary Ophthalmologists* 1997; 101: 67-78.

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