PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
MESTRADO EM CIÊNCIA ANIMAL

USO DO TACROLIMUS 0,03% COLÍRIO DILUÍDO EM ÓLEO DE OLIVA OU DE SEMENTE DE LINHAÇA NO TRATAMENTO DE CERATOCONJUNTIVITE SECA EM CÃES

LUÍS FELIPE DA COSTA ZULIM

Presidente Prudente - SP
2016

PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
MESTRADO EM CIÊNCIA ANIMAL

USO DO TACROLIMUS 0,03% COLÍRIO DILUÍDO EM ÓLEO DE OLIVA OU DE SEMENTE DE LINHAÇA NO TRATAMENTO DE CERATOCONJUNTIVITE SECA EM CÃES

LUÍS FELIPE DA COSTA ZULIM

Dissertação apresentada Pró-Reitoria de Pesquisa e Pós-Graduação, Universidade do Oeste Paulista, como parte dos requisitos para obtenção do título de Mestre em Ciência Animal.

Orientadora: Profª Drª Sílvia M.C.F. Andrade

Presidente Prudente - SP
2016

636.708977
Z94u

Zulim, Luís Felipe da Costa
O uso do tacrolimus 0,03% colírio diluído em óleo de oliva ou de semente de linhaça no tratamento de ceratoconjuntivite seca em cães / Luís Felipe da Costa Zulim. – Presidente Prudente, 2016.
67 f.: il.

Dissertação (Mestrado em Ciência Animal) - Universidade do Oeste Paulista – Unoeste, Presidente Prudente, SP, 2016.
Bibliografia.
Orientador: Sílvia M. C.Franco Andrade.

1. Ceratoconjuntivite Seca. 2. Cães. 3. Tacrolimo. I. Título.

LUÍS FELIPE DA COSTA ZULIM

USO DO TACROLIMUS 0,03% COLÍRIO DILUÍDO EM ÓLEO DE OLIVA OU DE SEMENTE DE LINHAÇA NO TRATAMENTO DE CERATOCONJUNTIVITE SECA EM CÃES

Dissertação apresentada a Pró-Reitoria de Pesquisa e Pós-Graduação, Universidade do Oeste Paulista, como parte dos requisitos para obtenção do título de Mestre em Ciência Animal.

Presidente Prudente, 05 de maio de 2016.

BANCA EXAMINADORA

_____
Profa. Dra Sílvia M. C. F. Andrade
Universidade do Oeste Paulista – Unoeste
Presidente Prudente – SP

_____
Profa. Dra Cecília Braga Laposy
Universidade do Oeste Paulista – Unoeste
Presidente Prudente – SP

_____
Profa. Dra Cláudia Valéria Seullner Brandão
Universidade Estadual Paulista – UNESP
Botucatu – SP

DEDICATÓRIA

Este trabalho é dedicado aos meus pais, amigos, professores e animais.

AGRADECIMENTOS

A conclusão deste trabalho só foi possível graças à orientação e auxílio dos professores e alunos:

Dra Sílvia M.C. Franco Andrade
Carolina S. G. Pereira
Aline Gutierez
Hugo Benguela
Bruna Foglia
Aline Batista
Gabriel Molinari
Dra Rosa B. Nogueira
Dra Cecília Laposy
Dra Gisele Nai
Dra Cláudia Valeria Brandão
Dr Rogério Giufridda

“[...] Fale, e eu esquecerei; Ensina-me, e eu poderei lembrar; Envolva-me, e eu aprenderei. [...]”

Benjamin Franklin

RESUMO

O uso do tacrolimus 0,03% colírio diluído em óleo de oliva ou de semente de linhaça no tratamento de ceratoconjuntivite seca em cães.

O presente estudo teve como objetivo comparar a eficácia do colírio de tacrolimus 0,03% diluído em dois tipos de veículo, óleo de semente de linhaça e óleo de oliva, no tratamento de ceratoconjuntivite seca (CCS) em cães. Foram utilizados 60 cães, sendo vinte cães saudáveis como grupo controle negativo e 40 cães diagnosticados com CCS bilateral que foram alocados aleatoriamente em dois grupos, tacrolimus em óleo de oliva (TO) e tacrolimus em óleo de semente de linhaça (TL). Os cães foram avaliados mensalmente com exames oftálmicos, Teste Lacrimal de Schirmer (TLS), Teste de Ruptura do Filme Lacrimal (TRFL), Teste de Fluoresceína (TF), Teste de Rosa Bengala (TRB) e citologia conjuntival e no início e final do estudo com exame histopatológico de conjuntiva .Em ambos os grupos, os sinais clínicos, o TLS, TRFL, TF e TRB apresentaram melhora significativa com um mês de tratamento. Ao término do estudo na avaliação citológica e histopatológica, ambos os grupos apresentaram diminuição de neutrófilos, sendo ela mais significativa no grupo TL, e ambos grupos apresentaram aumento de células caliciformes. Desta forma podemos concluir que o colírio de tacrolimus 0,03% diluído em óleo de oliva e em óleo de semente de linhaça foi eficiente no tratamento de CCS e o grupo TL apresentou melhor desempenho quanto a diminuição de neutrófilos. Assim, o óleo de semente de linhaça pode ser uma nova alternativa como diluente do tacrolimus.

Palavras-chave: olho seco, tacrolimus, óleo de oliva, óleo de linhaça, ômegas e cães

ABSTRACT

Use of 0.03% tacrolimus eye drops in olive oil or linseed oil for the treatment of keratoconjunctivitis sicca in dogs

This study aimed to compare the efficacy of tacrolimus 0.03% eye drops diluted in two types of vehicle, flaxseed oil and olive oil in the treatment of keratoconjunctivitis sicca (CCS) in dogs. 60 dogs were used, twenty healthy dogs as a negative control group and 40 dogs diagnosed with bilateral CCS that were randomly divided into two groups, tacrolimus in olive oil (TO) and tacrolimus in flaxseed oil (TL). The dogs were evaluated monthly with ophthalmologic exams, Tear Test Schirmer (TLS), Break Test Tear Film (TRFL), Fluorescein test (TF), Test of Rose Bengal (TRB) and conjunctival cytology and at the beginning and end of the study with histopathological examination of the conjunctiva .In both groups, clinical signs, TLS, TRFL, TF and TRB showed significant improvement with one month of treatment. At study end in cytological and histopathologic evaluation, both groups showed a decrease of neutrophils, it is more significant in the TL group, and both groups showed an increase of goblet cells. Thus we can conclude that the drops of tacrolimus 0.03% diluted in olive oil and flaxseed oil was effective in the treatment of CCS and the TL group performed better as the decrease in neutrophils. Thus, the linseed oil may be a new alternative as the tacrolimus diluent.

Key-words: dry eye, olive oil, linseed oil, dogs, tacrolimus, omegas.

LISTA DE SIGLAS

TO – Tacrolimus diluído em óleo de oliva
TL – Tacrolimus diluído em óleo de linhaça
TLS – Teste lacrimal de Schirmer
TRFL – Tempo de ruptura do filme lacrimal
TF- Teste de fluoresceína
TRB – Teste de rosa bengala
CCS – Ceratoconjuntivite seca

SUMÁRIO

1 ARTIGO CIENTÍFICO

Uso do tacrolimus 0,03% colírio diluído em óleo de oliva ou de semente de linhaça no tratamento de ceratoconjuntivite seca em cães.

Luís Felipe da Costa Zulim[1], Gisele Alborgetti Nai[2], Rogério Giuffrida[1], Carolina Silva Guimarães Pereira[1], Hugo Benguella[3], Aline Gutierrez Cruz[3], Bruna Toledo Duran Foglia[3], Aline da Silveira Batista[4] e Silvia Franco Andrade[1].

1. Post Graduate Program in Animal Science, Oeste Paulista University (UNOESTE), Presidente Prudente, SP, Brazil

2. Department of Anatomy Pathology, Faculty of Medicine (UNOESTE)

3. Faculty of Veterinary Medicine (UNOESTE)

4. Resident of Clinical Laboratory of the Veterinary Hospital (UNOESTE)

Contact address: S. F. Andrade Tel.: +55 18 3229 2067; Fax +55 18 3229 2036

e-mail: silviafranco@unoeste.br

Running Title: Uso de óleo de oliva ou linhaça tópico no olho seco cães

RESUMO

Objetivo Comparar a eficácia do colírio de tacrolimus 0,03% diluído em dois tipos de veículo, óleo de semente de linhaça e óleo de oliva, no tratamento de ceratoconjuntivite seca (CCS) em cães.

Procedimento Foram utilizados 60 cães. Vinte cães saudáveis foram alocados no grupo controle e 40 cães diagnosticados com CCS bilateral foram alocados aleatoriamente em dois grupos, tacrolimus em óleo de oliva (TO) e tacrolimus em óleo de semente de linhaça (TL). Foram avaliados mensalmente com exames oftálmicos, Teste Lacrimal de Schirmer (TLS), Teste de Ruptura do Filme Lacrimal (TRFL), Teste de Fluoresceína (TF) e Teste de Rosa Bengala (TRB), mensalmente com citologia e no início e fim do estudo com exame histopatológico.

Resultados Em ambos os grupos, os sinais clínicos, a cicatrização das úlceras de córneas, os exames oftálmicos TLS, TRFL e TRB, apresentaram melhora significativa com um mês após o tratamento. Ao final do experimento, ambos os grupos 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, sendo que no grupo TL em ambas as análises a diminuição de neutrófilos foi mais significativa.

Conclusão O colírio de tacrolimus 0,03% diluído em óleo de oliva e em óleo de semente de linhaça foi eficiente no tratamento de CCS. Não houve diferença significativa nos parâmetros avaliados entre os grupos, somente na diminuição de neutrófilos que foi mais significativa no TL. Assim, o óleo de semente de linhaça pode ser uma nova alternativa como diluente do tacrolimus.

Palavra-chave: olho seco, tacrolimus, óleo de oliva, óleo de linhaça, ômegas e cães

INTRODUÇÃO

A ceratoconjuntivite seca (CCS) ou olho seco é uma oftalmopatia inflamatória crônica comum em cães e humanos caracterizada por uma diminuição da camada aquosa do filme lacrimal (quantitativa) e/ou por uma deficiência na porção lipídica ou de mucina (qualitativa), causando um processo inflamatório progressivo principalmente na córnea, conjuntiva e glândulas lacrimais, na maioria das vezes de origem imunomediada.[1-3] Os sinais clínicos da CCS incluem hiperemia conjuntival, irritação ocular, quemose, blefaroespasmo, fotofobia, secreção mucoide e mucopurulenta, vascularização e pigmentação da córnea e até perda da visão. As principais causas da CCS em cães são principalmente imunomediadas e secundariamente por deficiência lacrimal congênita, predisposição racial, induzida por drogas, neurogênica, iatrogênica, infecciosa, senilidade e retirada da glândula da terceira pálpebra.[3-7]

A terapia tópica consiste principalmente no uso de lubrificantes associados ao uso de imunossupressores, tais como ciclosporina, tacrolimus e pimecrolimus, além de secundariamente, se necessário, no uso de anti-inflamatórios, antibióticos e mucolíticos.[8-12] O tacrolimus (FK506) é um antibiótico macrolídeo isolado da *Streptomyces tsukubaensis*, que tem um efeito similar a ciclosporina. O efeito do tacrolimus é uma combinação de imunossupressão local, proliferação de células caliciformes, supressão da apoptose de células lacrimais e efeito anti-inflamatório.[11-13]

Os óleos vegetais são ricos em ácidos graxos essenciais (AGE) como os ômegas 3 e 6, considerados anti-inflamatórios naturais, além de vitaminas, minerais entre outras substâncias e têm grande aplicação em preparações farmacêuticas tópicas, inclusive como veículos para colírios. Os principais óleos vegetais usados topicamente são os de oliva, linhaça, amêndoas, semente de uva e macadâmia.[14-16]

O óleo de oliva, *Olea europeae*, contém grande quantidade de ácido graxo monoinsaturado (MUFA), cerca de 70-80% de ácido oleico, 10-15% de ácido graxo saturado (ácido palmítico), e uma pequena quantidade de ácido graxo poli-insaturado (PUFA) em torno de 5 a 10% de ômega 3, 6 e 9. Possui propriedades anti-inflamatórias, antinoceptivas, imunoestimulantes e antimicrobianas.[15-17] Há um estudo em coelhos induzidos com KCS e tratados com o óleo de oliva puro ou associado à ciclosporina demonstrando excelentes resultados do óleo de oliva no controle dos sintomas da KCS.[18]

O óleo de semente de linhaça é considerado atualmente uma das maiores fontes de AGE do tipo ômega 3 e ômega 6, sendo recomendado, por via oral, como uma terapia adjuvante na síndrome de Sjögren e KCS em pacientes humanos.[19-22] Estudos em coelhos, relataram melhora nos sintomas da KCS induzida experimentalmente, com o uso do óleo de semente linhaça em várias preparações (orais, tópicas e associação das duas), além do uso do óleo em associação com tacrolimus e ciclosporina.[18,23,24] O óleo de linhaça é considerado um agente anti-inflamatório natural devido o seu potencial para a síntese de mediadores anti-inflamatórios, tais como a prostaglandina E1 (PGE1) e tromboxano A1 (TXA 1).[14,21,22]

O objetivo deste estudo foi avaliar a eficácia do tacrolimus 0,03% colírio diluído em dois veículos diferentes, o óleo de oliva que já é comumente utilizado para esta finalidade, e óleo de semente de linhaça que ainda não foi testado como diluente do tacrolimus no tratamento de ceratoconjuntivite seca em cães.

MATERIAIS E MÉTODOS

Animais

O estudo foi aprovado pela Comissão de Ética no Uso de Animais (CEUA) da UNOESTE, protocolo nº1794, e está de acordo com as normas da ARVO (Association for Research in Vision and Ophthalmology –Statement for the use of animals in ophthalmic and

visual research). Foram selecionados 40 cães com CCS bilateral, dentre eles as raças mais prevalentes foram: Lhasa apso (30%), Poodle (17,5%), Sem raça definida (15%), Pinscher (7,5%), Shit-zu (5%) e demais raças completando 25% com um animal de cada (Border Collie, Cocker Spaniel, PitBull, Beagle, Yorkshire Terrier, Bull Terrier, Rotweiller, Pug, Maltês), 25 fêmeas (62,5%) e 15 (37,5%), com idade média de 6,7±3,9 e peso médio de 10,3 ±7,7.Os animais foram inclusos no experimento a partir da observação dos sinais clínicos oftálmicos (secreção ocular, conjuntivite, opacidade e pigmentação de córnea) e TLS≤ 10 mm/min e/ou TRFL≤10 seg. Foram utilizados 20 cães saudáveis, sem raça definida, com idade média de 3,5±2,4 e peso médio 10,3±7,7 sendo 9 machos (45%) e 11 fêmeas (55%), como grupo controle negativo para parâmetro da normalidade dos exames TLS, TRFL, citologia e histopatologia conjuntival.

Grupos

A partir do diagnóstico de CCS os animais foram alocados aleatoriamente em dois grupos de tratamento: grupo TO (n=20) colírio de tacrolimus 0,03% diluído em óleo de oliva (Ophthalmos, São Paulo, Brasil) e o grupo TL (n=20) colírio de tacrolimus diluído em óleo de semente de linhaça (Ophthalmos, São Paulo, Brasil), 1 gota duas vezes ao dia, durante seis meses, além do tratamento complementar com lubrificante a base de propilenoglicol (Systane®, Alcon, São Paulo, Brasil), 1 gota duas vezes ao dia, durante seis meses, e se necessário, colírio antibiótico com base no exame de cultura e antibiograma (1 gota, 4 vezes ao dia, durante 15 dias) e colírio anti-inflamatório a base diclofenaco sódico (1 gota 2, vezes ao dia, durante 15 dias) (Still®, Allergan, São Paulo, Brasil).

Momentos e exames oftálmicos

Foram realizados exames oftálmicos e citológicos mensais, sendo considerado o momento zero (M0) o primeiro dia de atendimento com diagnóstico de CCS bilateral e os

demais momentos (M1 a M6) de tratamento. Foi realizado o exame histopatológico no momento do diagnóstico (M0) e ao de término do estudo (M6).

Os sinais clínicos oftálmicos foram identificados pelo mesmo clínico (LFCZ) utilizando a lâmpada de fenda portátil (Kowa, Japan) com a presença ou não de conjuntivite, secreção ocular, opacidade e pigmentação de córnea conforme escore descrito na Tabela 1.

O Teste Lacrimal de Schirmer (TLS) (Teste de Schirmer ® - Ophthalmos, São Paulo, Brasil) foi realizado sem colírio anestésico para avaliar a porção quantitativa da lágrima, introduzido 0,5 cm da tira no saco conjuntival durante um minuto e considerado positivo ≤10 mm/min.[25] O Tempo de Ruptura do Filme Lacrimal (TRFL) foi utilizado para avaliar a porção qualitativa da lágrima, sendo mensuradas duas vezes consecutivas e a média calculada. Após instilar uma gota de colírio de fluoresceína® 1 % (Allergan, São Paulo, Brasil), com uma lâmpada de fenda (Kowa, Japan), foi observado o tempo entre o último piscar e o aparecimento de manchas ou pontos escuros no filme lacrimal, sendo considerados positivos para TRFL valores ≤ 10 segundos.[26]

Após o exame de TRFL, os olhos foram lavados com solução fisiológica e então foi avaliada a presença ou não de úlceras na córnea,[25] e graduado em um escore de acordo com sua gravidade e extensão (0 - negativo, 1 - úlcera superficial pequena, 2 - úlcera superficial média, 3 - úlcera superficial extensa, 4 - úlcera estromal pequena, 5 - úlcera estromal média, 6 - úlcera estromal extensa, 7 - descemetocele e 8 – ceratomalácia ou úlcera em "*melt*").

O Teste de Rosa Bengala (TRB) para avaliar a presença de células desvitalizadas sendo utilizado o colírio de Rosa Bengala a 1% (Ophthalmos, São Paulo, Brasil), com instilação prévia de colírio anestésico. Foi considerado positivo para CCS quando alguma área da córnea ou conjuntiva corou em rosa.[25] O escore do TRB foi graduado em 0 a 3: 0- negativo; 1-somente a conjuntiva corada; 2- somente a córnea corada e 3- conjuntiva e córnea corada.

Exame citológico e histopatológico

A citologia foi realizada após a limpeza ocular com solução fisiológica e instilação de colírio anestésico (Allergan®, São Paulo, Brasil), foram colhidas amostras de células da conjuntiva inferior com swab estéril umedecido com solução fisiológica e confeccionado uma lâmina para cada olho, em seguida fixadas em metanol, e corada pela técnica de MGG (May-Grunwald-Giemsa). Foi realizada a contagem de linfócitos, neutrófilos, células metaplásicas e células escamosas, em microscópio óptico, 10 campos, na objetiva de 40x.

O exame histopatológico foi realizado após instilação de colírio anestésico (Allergan®, São Paulo, Brasil) com a retirada de 1-3 mm no fórnix da conjuntiva inferior medial, com o auxílio de uma pinça e tesoura de conjuntiva. O corte histológico foi alocado em um fragmento de papel em tamanho padronizado de 1x1 cm fixados em formol, e inclusão em parafina (Dinâmica Reagentes Analíticos, São Paulo, Brasil). Com auxílio de micrótomo rotativo foram obtidos cortes de 5μm de espessura da conjuntiva, os quais foram corados pelas técnicas de hematoxilina e eosina (HE) (Dolles, São Paulo, Brasil), PAS (Merck, EUA) e posteriormente avaliados os seguintes parâmetros; No HE: a contagem de linfócitos e neutrófilos, e PAS: para contagem da densidade de células caliciformes (células/mm^2), em objetiva de 40x, no microscópio óptico.

Análise Estatística

Para as variáveis TLS e TRFL, densidade de células caliciformes e número de células escamosas, células metaplásicas, neutrófilos e linfócitos, utilizamos duas análises de variância (ANOVA) para amostra pareada com contraste pelo método de Tukey. Para variáveis TF e TRB, utilizamos teste não paramétrico de Friedman para comparar momentos e Kruskall-Wallis com contraste Dunn para comparer os grupos. Foi adotado nível de $P>0,05$ de signifcância O software utilizado para análise estatística foi o Bioest 5.3

RESULTADOS

Os resultados dos exames realizados no grupo controle negativo para obtenção dos parâmetros de normalidade estão descritos na Tabela 2.

Nos sinais clínicos avaliados (Figura 1) ambos os grupos apresentaram melhora e não houve diferença estatística significativa ($p > 0,05$) entre os grupos TO e TL, apenas diferença estatística significativa ($p>0,05$) entre o M0 e demais momentos. Nas variáveis secreção ocular, conjuntivite e opacidade corneal houve remissão total já em M1.Pigmentação corneal, a mediana mostrou remissão total em M5.

No TLS (Figura 2) o grupo TO e TL apresentaram aumento significativo já no M1, houve diferença estatística ($p < 0,05$) no M2, M3, M5 e M6 entre grupos, onde o grupo TO apresentou valores maiores do que o grupo TL, e ambos os grupos apresentaram diferença estatística ($p<0,05$) quando comparado o M0 aos demais momentos, aproximando seus valores ao grupo controle negativo (Tabela 2). No TRFL (Figura 2) os dois grupos apresentaram aumento significativo já no M1 e se mantiveram até M6 com valores próximos ao grupo controle negativo (Tabela 2), não houve diferença estatística significativa ($p>0,05$) entre os grupos, mas houve diferença estatística quando comparado o M0 com demais momentos.

No TF (Figura 3) os dois grupos apresentaram diversos tipos de úlceras em sua gravidade e extensão em M0, e ambos os grupos mostraram excelente cicatrização já em M1, não houve diferença significativa ($p>0,05$) entre os grupos, apenas entre M0 e demais momentos ($p<0,05$). No TRB (Figura 4), em M0 houve prevalência de córnea e conjuntiva corada, de M1 a M4 no grupo TO houve prevalência de somente córnea corada, enquanto no grupo TL M1 e M2, somente conjuntiva, no grupo TO houve resolução total em M5,

enquanto no grupo TL, em M4, apresentando diferença estatística significativa entre M0 e demais momentos em ambos os grupos.

No exame citológico (Figura 5) ambos os grupos apresentaram diminuição de todas as células, porém houve diferença estatística entre grupos apenas no parâmetro da contagem de neutrófilos, sendo significativamente menor a contagem ($P<0,05$) do grupo TL em relação ao grupo TO no momento M6 e houve diferença significativa entre os grupos e quando comparado o M0 com os demais momentos. No histopatológico (Figura 6) ambos os grupos apresentaram diminuição de células inflamatórias, sendo significativamente menor a contagem de neutrófilos ($P<0,05$) do grupo TL em relação ao TO no M6. Houve aumento do número de células caliciformes e não houve diferença estatística entre os grupos. Ambos os grupos apresentaram melhora nos exames, chegando mais próximo dos parâmetros da normalidade (grupo controle negativo) (Figura 7). O exame citológico e histopatológico no M0 e M6 do grupo TO do animal N10 está descrito na Figura 8 e do grupo TL do animal N3 está descrito na Figura 9.

DISCUSSÃO

Alguns estudos em KCS experimentalmente induzida em coelhos e tratada com imunossupressores tópicos mais utilizados atualmente, ciclosporina e tacrolimus, diluídos em óleos vegetais (amêndoa, oliva e óleo de semente de linhaça), e também o uso isolado desses óleos, demonstraram que não só os imunossupressores, mas também os óleos apresentaram eficácia no controle dos sintomas da KCS, principalmente pelo efeito anti-inflamatório que esses óleos possuem devido principalmente à presença de ômegas 3 e 6 em sua composição, que induzem a formação de mediadores anti-inflamatórios, tais como a prostaglandina E1 (PGE1) e tromboxano A1 (TXA 1).[14,18,21-23] Nesses estudos, o óleo de semente de linhaça sempre apresentou um desempenho melhor que os demais óleos, amêndoa[23] e oliva[18], isto

talvez ocorra devido o óleo de semente de linhaça possui uma concentração maior ômegas 3 e 6 que os demais óleos estudados.[20-22] Um outro estudo em KCS experimentalmente induzida em coelhos com o uso do óleo de semente de linhaça em várias vias de administração (oral, tópica e oral e tópica associada) também demonstrou eficácia no controle dos sintomas e aumento das células caliciformes.[24]

Cães da raça Beagle tratados com tacrolimus diluído em óleo de oliva apresentaram melhora dos sinais clínicos (secreção, pigmentação e hiperemia) e sem diferença estatística quando comparado a outro imunossupressor, ciclosporina, e também apresentaram aumento significativo do TLS já no primeiro momento, assim como em nosso estudo.[12]

Nos resultados de TLS e TRFL houve aumento significativos nos valores obtidos e ao final do experimento não houve diferença estatística significativa entre os grupos TO e TL. Isto está de acordo com o descrito na pesquisa com ciclosporina 0,02% diluída em óleo de oliva e óleo de linhaça,[18] em CCS experimentalmente induzida em coelhos, porém no estudo do uso do tacrolimus 0,03% diluído em óleo de amêndoas e linhaça,[23]o grupo somente com linhaça tópico apresentou um aumento mais significativo. Valores de TLS também foram significativamente maiores no estudo do uso da linhaça por via oral e tópica em CCS experimentalmente induzida em coelhos.[24]

No presente estudo houve uma excelente resolução das úlceras de córnea em ambos os grupos, TO e TL, sem diferença estatística significativa entre eles. Isto também está de acordo com as outras pesquisas,[18,23] inclusive na resolução da ceratomalácia (úlcera em "*melting*") descrita no estudo de óleo de linhaça por via tópica, oral e associada em coelhos com CCS induzida. [24]

Também no teste TRB, ao final do experimento, não houve diferenças significativas entre os grupos, demonstrando que houve diminuição na presença de células desvitalizadas na

córnea e conjuntiva induzida pela CCS, que está de acordo com outros trabalhos que usaram esses óleos associados à imunossupressores tópicos. [18,23]

No presente estudo, a única diferença significativa estatisticamente no final do tratamento foi no parâmetro contagem de neutrófilos, que foi estatisticamente menor no grupo TL. A diminuição do número de células inflamatórias também foi descrito em um estudo com cães com CCS e tratados com ciclosporina tópica a 2%.[27] A literatura sugere que a patogênese da CCS baseia-se no infiltrada inflamatório, intensa presença de linfócitos e moderada presença de neutrófilos, e o infiltrado inflamatório é a causa principal da destruição do tecido glandular.[1,2] Estudo realizado com cães infectados com o vírus da cinomose e com CCS mostrou no exame histopatológico severo infiltrado inflamatório, com predominância de linfócitos e neutrófilos e um pequeno número de células caliciformes.[6]

No presente estudo houve aumento significativo do número de células caliciformes entre o início e fim do tratamento, não havendo diferença estatística entre os grupos. Outros estudos também demonstraram aumento das células caliciformes com tratamento com ômegas [14,24] ou imunossupressores[9-13,18,23,24] no tratamento da CCS em diversas espécies.

Desta maneira, podemos concluir que o colírio de tacrolimus 0,03% diluído em óleo de oliva (TO) e em óleo de linhaça (TL) foram eficientes no tratamento de CCS, e o TL mostrou melhor desempenho na diminuição de neutrófilos. Assim, o óleo de linhaça pode ser uma nova alternativa como diluente do tacrolimus.

AGRADECIMENTOS

Os autores agradecem a Pós-Graduação da UNOESTE pelo apoio financeiro e ao Laboratório Ophthalmos pela doação dos materiais para a realização da pesquisa.

BILIOGRAFIA

1. Stevenson W, Chauhan SK, Dana R. Dry eye disease: an immune-mediated ocular surface disorder. *Archives of Ophthalmology* 2012; 130:90–100.

2. Williams DL. Immunopathogenesis of keratoconjunctivitis sicca in the dog. *Veterinary clinics of North America. Small Animal Practice* 2008;38:251-68.

3. Miller PE. Lacrimal system. In: Slatter's Fundamentals of Veterinary Ophthalmology, 4thedn. (edsMaggs DJ, Miller PE, Ofri R) Saunders Elsevier, St. Louis, 2008; 157–174.

4. Matheis FL, Reinhardt LW, Spiess BM. Canine neurogenic keratoconjunctivitis sicca:11 cases. *Veterinary Ophthalmology* 2012; 15:288–290.

5. Westermeyer HD, Ward DA, Abrams K. Breed predisposition to congenital alacrima in dogs. *Veterinary Ophthalmology* 2009;12:1–5.

6. Almeida D.E, Roveratti C, Brito FL, et al. Conjunctival effects of canine distemper virus-induced keratoconjunctivitis sicca. *Veterinary Ophthalmology* 2009,12:211–215.

7. Naranjo C, Fondevila D, Altet L et al. Evaluation of the presence of Leishmania spp. by real-time PCR in the lacrimal glands of dogs with leishmaniosis. *Veterinary Journal* 2012;193:168–73.

8. Grahn BH; Storey ES. Lacrimomimetics and lacrimostimulants. *Veterinary Clinics of North America: Small Animal Practice* 2004; 34:739–753.

9. Izci C, Celik I, Alkan F, Ogurtan Z, et al. Histologic characteristics and local cellular immunity of the gland of the third eyelid after topical ophthalmic administration of 2% cyclosporine for treatment of dogs with keratoconjunctivitis sicca. *American Journal of Veterinary Research* 2002;63: 688–694.

10. Ofri R, Lambrou GN, Allgoewer I, Graenitz U, Pena TM et al. Clinical evaluation of pimecrolimus eye drops for treatment of canine keratoconjunctivitis sicca: a comparison with cyclosporine A. *Veterinary Journal* 2009;179: 70–77.

11. Berdoulay YA, English RV, Naldelstein B. Effect of topical 0.02% tacrolimus aqueous suspension on tear production in dog with keratoconjunctivitis sicca. *Veterinary Ophthalmology* 2005; 8:225–232.

12. Hendrix VDH, Adkins EA, Ward, DA et al. An investigation comparing the efficacy of topical ocular application of tacrolimus and cyclosporine in dogs. *Veterinary Medicine International* 2011; 2011:487592

13. Moskovici BK, Holzchuh R, Naves FES et al. Treatment of Sjögren's syndrome dry eye using 0.03% tacrolimus eyedrop: Prospective double-blind randomized study. *Contact Lens & Anterior Eye* 2015;38:373–8.

14. Barabino S, Rolando M, Camicione P et al. Systemic linoleic and γ-linolenic acid therapy in dry eye syndrome with an inflammatory component. *Cornea* 2003;22:97–101.

15. Waterman E, Lockwood B. B. Active components and clinical applications of olive oil. *Alternative Medicine Review* 2007;12:331–342.

16. Wardhana EE, Surachmanto EAD. The role of omega-3 fatty acids contained in olive oil on chronic inflammation. *Acta Medica Indonesiana* 2011;43:138-42.

17. Eidi A, Moghadam-Kia S, Moghadam JZ et al. Antinociceptive and anti-inflammatory effects of olive oil (*Olea europeae L.*) in mice. *Pharmaceutical Biology* 2012; 50:332-37.

18. Parrilha LR, Nai GA, Giuffrida R et al. Comparison of 1% cyclosporine eye drops in olive oil and in linseed oil to treat experimentally-induced keratoconjunctivitis sicca in rabbits. *Arquivos Brasileiros de Oftalmologia* 2015, 78(5):295-9.

19. Hassan-Zadeh A, Sahari MA, Barzegar M, Optimization of the -3 extraction as a functional food from flaxseed. *International Journal of Food Sciences and Nutrition* 2008; 59: 526-534.

20. Roncone M, Bartlett H, Eperjesi, F. Essential fatty acids for dry eye: A review. Contact Lens & Anterior Eye 2010; 33:49-54.

21. Wojtowicz, JC, Butovich I, Uchiyama E et al. Pilot, prospective, randomized, double-masked, placebo-controlled clinical trial of an omega-3 supplement for dry eye. *Cornea* 2011; 30:308-314.

22. Aragona P, Bucolo C, Spinella R et al. Systemic omega-6 essential fatty acid treatment and PGE1 tear content in Sjögren's syndrome patients. *Investigative Ophthalmology Visual Science* 2005; 46:4474–4479.

23. Sgrignoli MR, Yamasaki L, Sanches OC *et al*. Comparison of topical 0.03% tacrolimus in almond and linseed oil to treat experimentally induced keratoconjunctivitis sicca in rabbits. *International Journal of Ophthalmic Pathology* 2013; **2**:3.

24. Neves ML, Yamasaki L, Sanches OC et al. Use of linseed oil to treat experimentally induced keratoconjunctivitis sicca in rabbits. *Journal of Ophthalmic Inflammation and Infection* 2013;3:4:1–5.

25. Maggs DJ. Basic diagnostic techniques. In: Slatter's Fundamentals of Veterinary Ophthalmology, 4th edn. (eds Maggs DJ, Miller PE, Ofri R) Saunders Elsevier, St. Louis, 2008; 81–106.

26. Saito A1, Kotani T. Estimation of lacrimal level and testing methods on normal beagles. *Veterinary Ophthalmology* 2001;4:7–11.

27. Izci C, Celik I, Alkan F, et al. Clinical and light microscopic studies of the conjunctival tissues of dogs with bilateral keratoconjunctivitis sicca before and after treatment with topical 2% cyclosporine. *Biotechnic & Histochemistry* 2015, 90(3): 223–230

Tabela 1. Escore dos sinais clínicos avaliados

Sinais clínicos	Escore
Conjuntivite	0 = ausente 1 = leve hiperemia conjuntival 2 = moderada a severa hiperemia conjuntival 3 = moderada a severa hiperemia conjuntival e quemose
Secreção ocular	0 = ausente 1 = leve secreção serosa 2 = moderada secreção mucóide 3 = severa secreção mucóide
Opacidade corneal	0 = ausente 1 = < 25% de extensão da córnea 2 = 25-50% 3 = > 50%
Pigmentação corneal	0 = ausente 1 = < 25% de extensão da cornea 2 = 25-50% 3 = > 50%

Tabela 2. Média e desvio padrão do Teste Lacrimal de Schirmer (TLS), Tempo de ruptura do filme lacrimal (TRFL), citologia conjuntival e histopatologia conjuntival, T0, grupo controle negativo (n=20).

Exames Oftálmicos	
TLS (mm/min)	27,3±4,1
TRFL (seg)	23,3±5,1
Citologia Conjuntival	
Linfócitos (células/10CGA)	0,1±0,4
Neutrófilos (células/10CGA)	0±0
Células Metaplásicas (células/10CGA)	0,5±2,1
Células Escamosas (células/10CGA)	0,1±0,3
Histopatologia Conjuntival	
Linfócitos (células/5CGA)	26,7±24,2
Neutrófilos (células/5CGA)	2,6±4,4
Células Caliciformes (células/mm^2)	28,3±16,0

*Contagem de células em10 campos na citologia e 5 campos na histopatologia, objetiva de 40x.

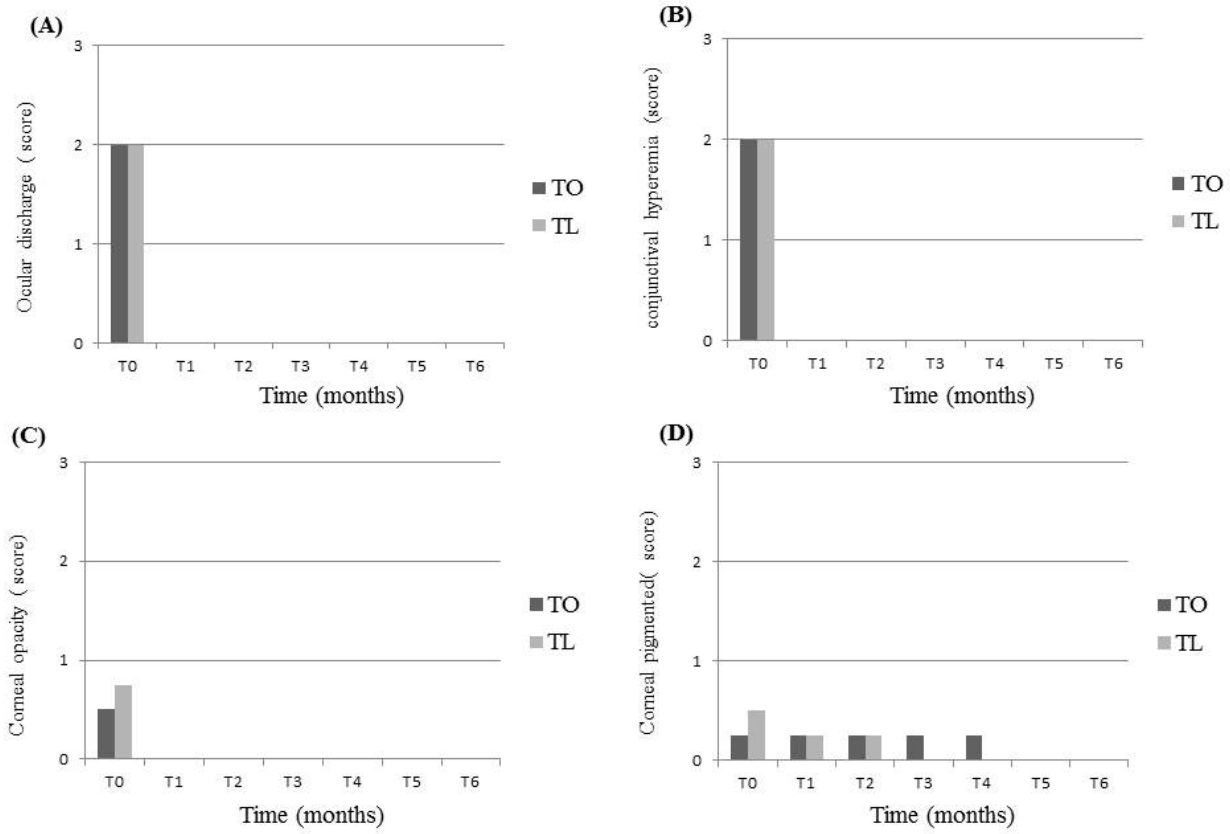

Figura 1. Mediana do escore dos sinais clínicos observados de M0 a M6, dos grupos TO (óleo de oliva) e TL (linhaça) (A) Secreção ocular (B) Hiperemia conjuntival (C) Opacidade corneal e (D) Pigmentação corneal.

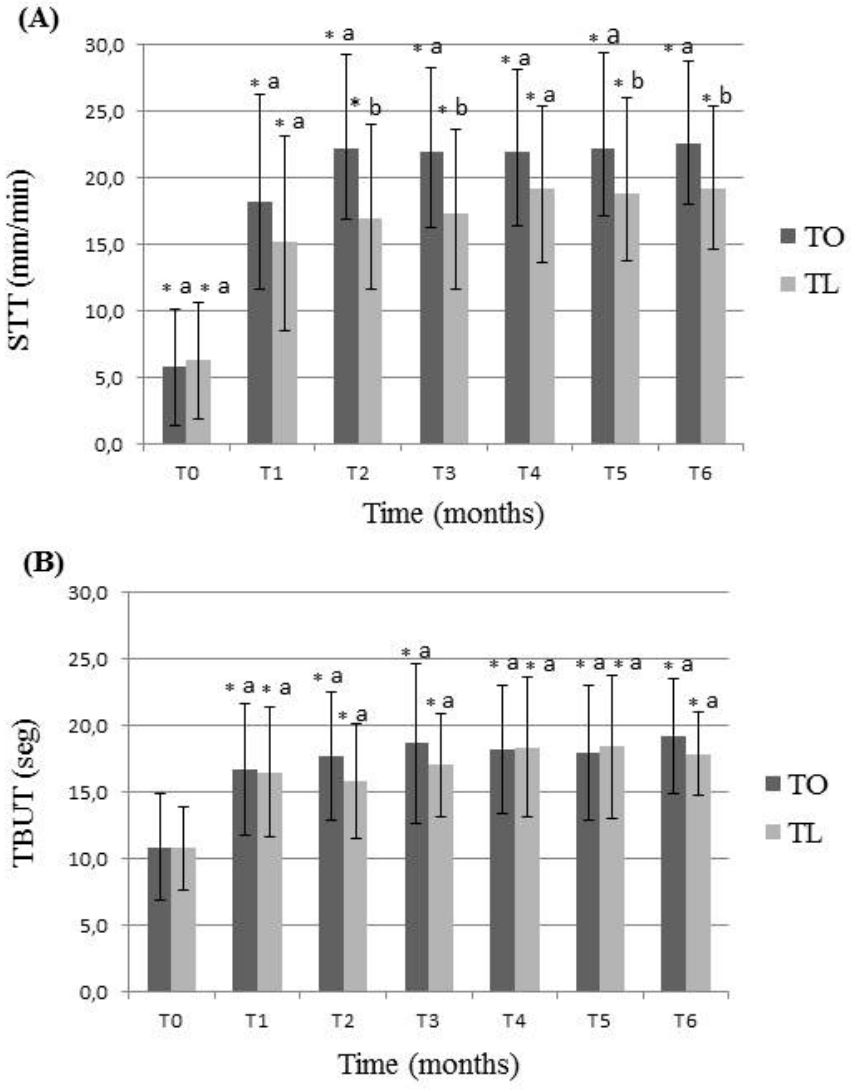

Figura 2. (A) Média e desvio padrão do Teste lacrimal de Schirmer (TLS) [a] em mm/min de M0 a M6, dos pacientes do grupo TO (tacrolimus diluído em óleo de oliva) e TL (tacrolimus diluído em óleo de linhaça) (B) Média e desvio padrão do Tempo de ruptura do filme lacrimal (TRFL)[b] em segundos, M0 a M6, dos pacientes do grupo TO e TL.

[a] Valores ≤ 10 mm/min positivo para CCS
[b] Valores ≤ 10 segundos positivo para CCS
*p< 0,05 (Teste de Tukey para comparar momentos)
[a,b]p< 0,05 (Teste de Kruskal Wallis para comparar grupos)

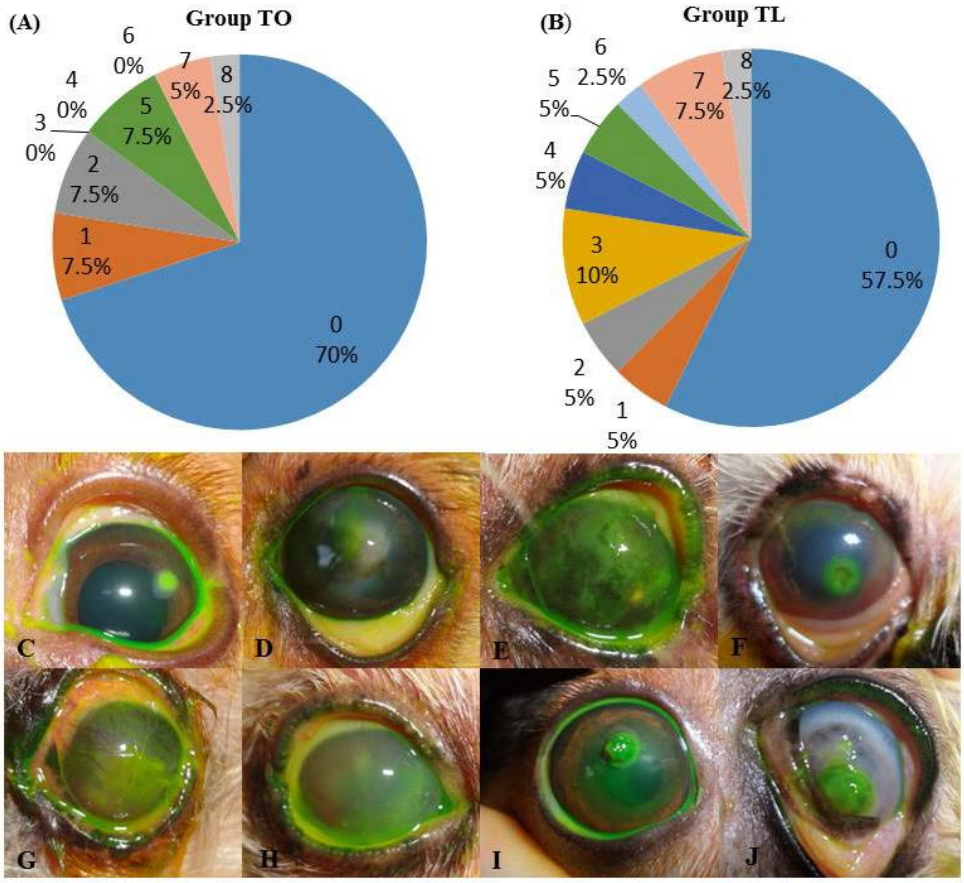

Figura 3. Teste de fluoresceína. Gráficos das porcentagens dos tipos de úlcera* em M0, (A) grupo TO e (B) grupo TL. Fotos dos animais do experimento demonstrando a classificação dos tipos de úlceras: (C) (1) Úlcera superficial pequena em olho esquerdo (OE), animal N2 do grupo TO; (D) Úlcera superficial média, OE, animal N5 do grupo TO; (E) Úlcera superficial extensa, OE, animal N10 do grupo TL; (F) Úlcera estromal pequena, olho direito (OD), animal N24 do grupo TL; (G) Úlcera estromal média, OE, animal N14 do grupo TL; (H) Úlcera estromal extensa, OD, animal N 17 do grupo TO (I) Descemetocele, OD, animal N32 do grupo TO. (J) úlcera em melting, OE, do animal N31 do grupo TL.

*0-negativo, 1- úlcera superficial pequena, 2- úlcera superficial média, 3- úlcera superficial extensa, 4- úlcera estromal pequena, 5- úlcera estromal média, 6- úlcera estromal extensa, 7- descemetocele e 8- melting.

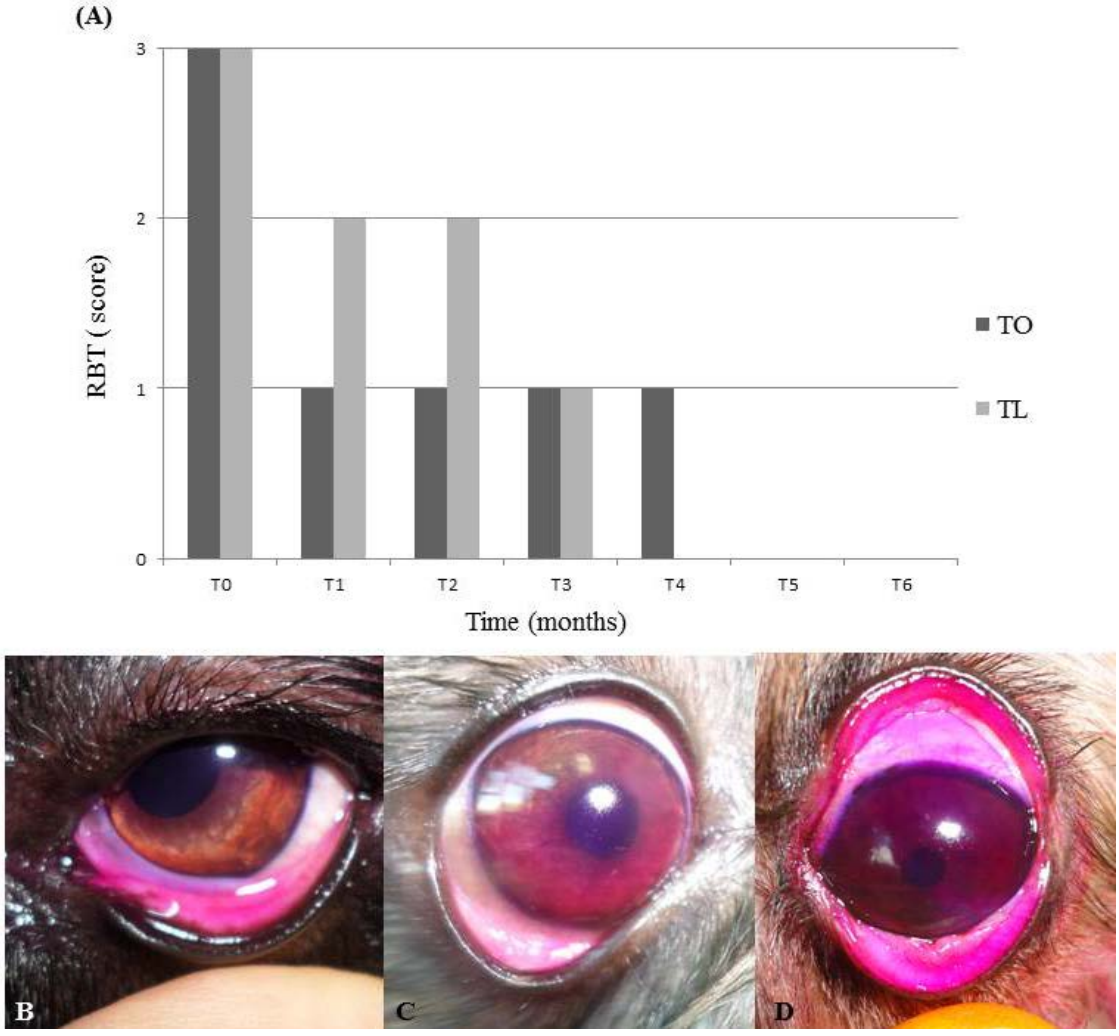

Figura 4. Teste de Rosa Bengala (TRB). (A) Mediana do TRB dos grupos TO e TL. Fotos dos animais do experimento demonstrando a classificação por escore do TRB: (B) Somente conjuntiva corada (OE), paciente N12, grupo TL; (C) somente córnea corada (OE), paciente N5, TO; (D) córnea e conjuntiva corada (OD), paciente N13, TL.

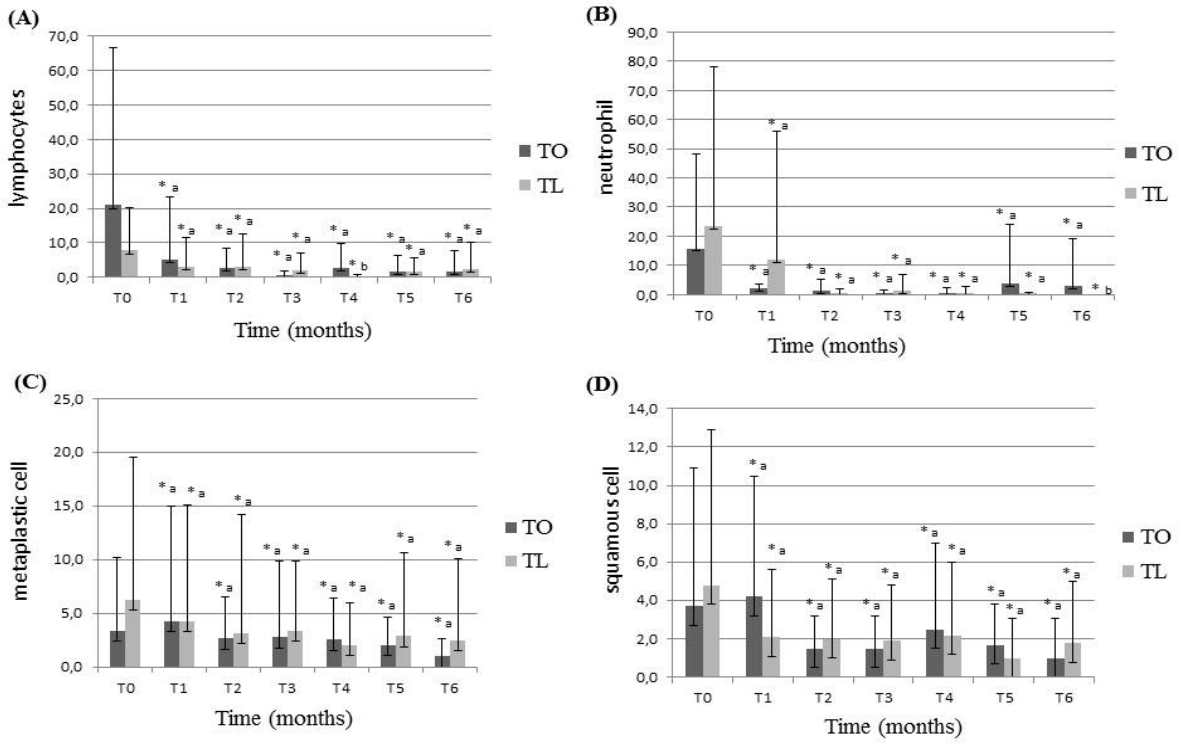

Figura 5. (A) Média e desvio padrão da contagem de linfócitos no exame de citologia conjuntival do M0 ao M6, grupo TO e TL (B) Neutrófilos (C) Células metaplásicas (D)Células escamosa.

*p< 0,05 (Teste de Tukey para comparar momentos)
[a,b]p< 0,05 (Teste de Kruskal Wallis para comparar grupos)

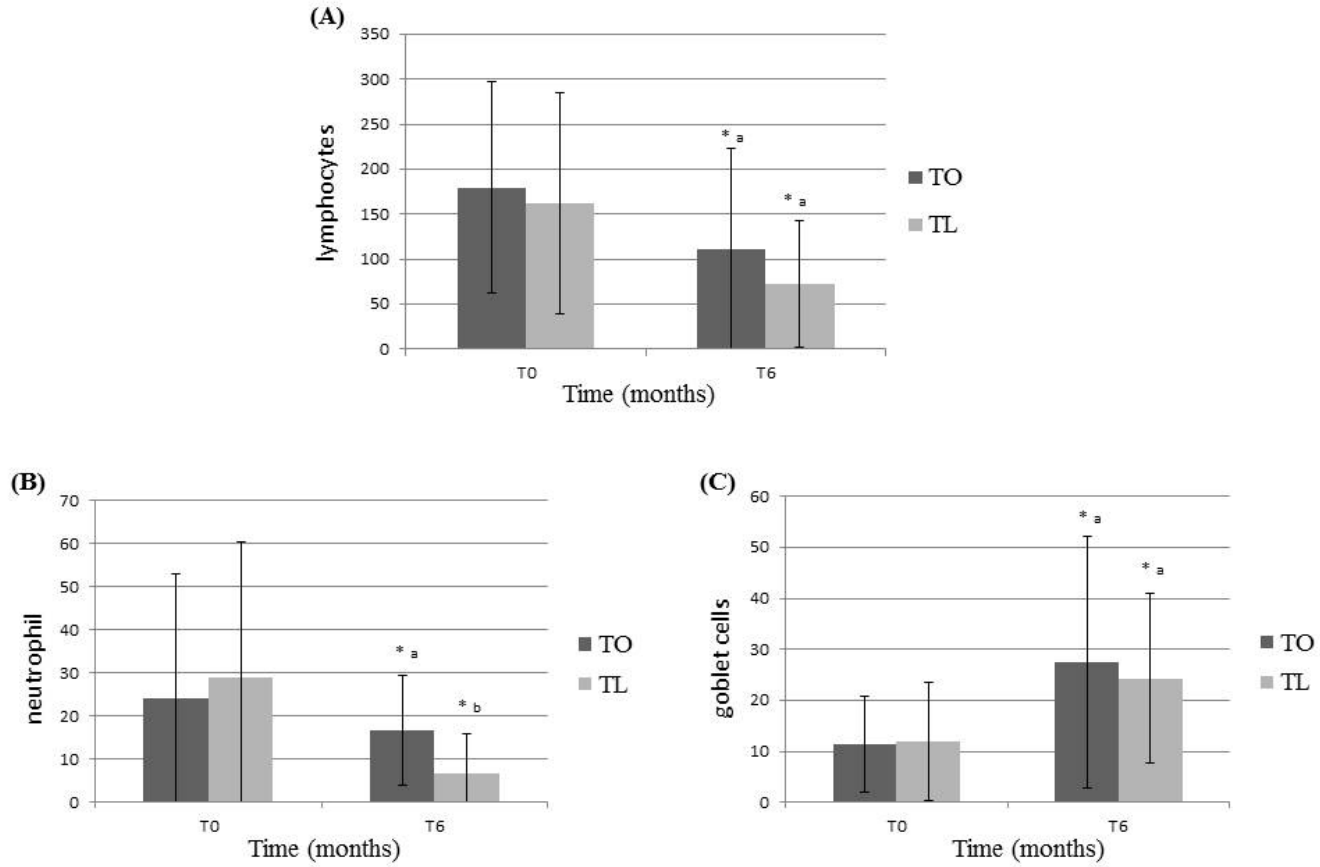

Figura 6. (A) Média e desvio padrão da contagem de linfócitos na histopatologia conjuntival no M0 e M6, do grupo TO e TL (B) Média e desvio padrão da contagem de neutrófilos no T0 e T6 (C) Média e desvio padrão da contagem de células caliciformes no T0 e T6.

*p< 0,05 (Teste de Tukey para comparar momentos)
[a,b]p< 0,05 (Teste de Kruskal Wallis para comparar grupos)

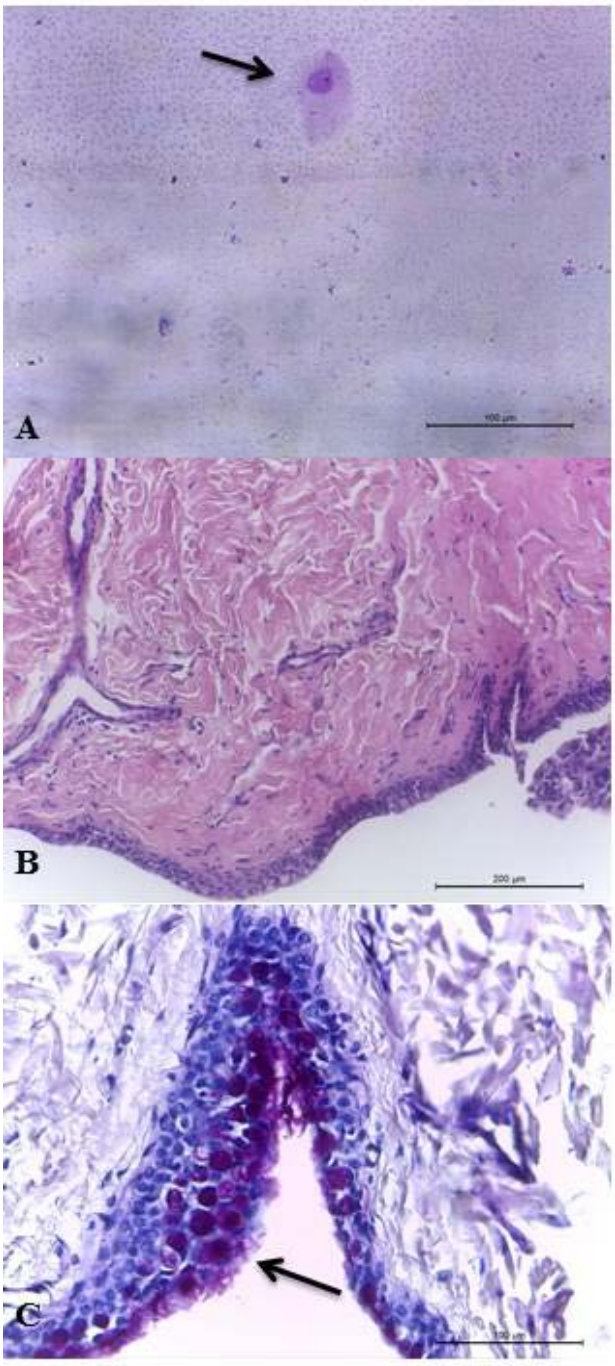

Figura 7. Fotomicroscopia do grupo controle (A) Citologia conjuntival com presença de célula escamosa (seta) coloração de MGG, aumento de 400x (B) Histopatologia, sem alteração tecidual coloração de Hematoxilina-eosina, aumento de 200x (C) Células caliciformes (seta) coloração PAS, aumento de 400x.

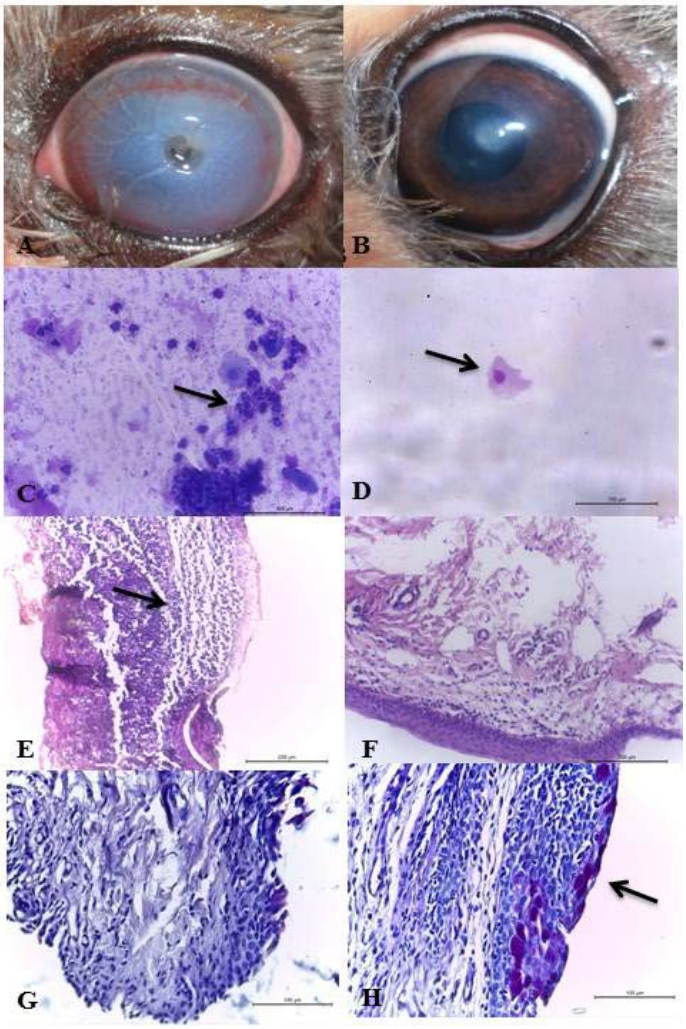

Figura 8. Grupo TO (A) M0 olho esquerdo, paciente N10 ceratomalácia e vascularização corneal (B) M6 cicatrização da úlcera (C) Fotomicroscopia da Citologia conjuntival do M0, presença de neutrófilos (seta), coloração MGG, aumento de 400x (D) T6, presença célula escamosa (seta), MGG, aumento de 400x (E) Histopatologia no M0, infiltrado inflamatório (seta). Coloração HE, aumento de 200x (F) M6, diminuição de infiltrado inflamatório (G) M0, ausência de células caliciformes. Coloração PAS, aumento de 400x (H) PAS M6 grande quantidade de células caliciformes (seta).

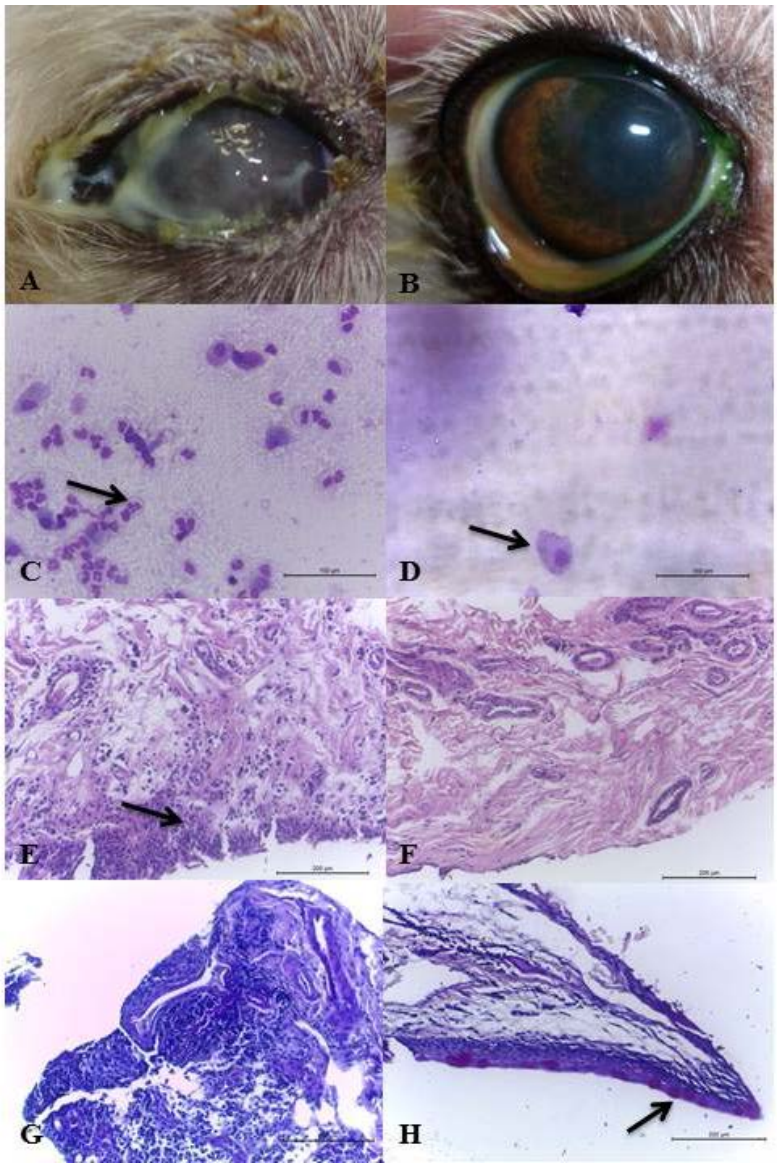

Figura 9. grupo TL(A) M0 olho direito, paciente N3, Secreção mucoide e pigmentação corneal (B) M6 Evolução clínica (C) Fotomicroscopia da citologia conjuntival do M0, presença de células neutrófilos (seta), coloração MGG, aumento de 400x (D) M6, presença de célula escamosa (seta) (E) Histopatologia no M0, infiltrado inflamatório (seta) Coloração HE, aumento de 200x (F) M6, diminuição de infiltrado inflamatório (G) M0, ausência de células caliciformes. Coloração PAS, aumento de 400x (H) PAS M6 grande quantidade de células caliciformes (seta).

Use of 0.03% tacrolimus eye drops in olive oil or linseed oil for the treatment of keratoconjunctivitis sicca in dogs

Luís Felipe da Costa Zulim[1], Gisele Alborgetti Nai[2], Rogério Giuffrida[1], Carolina Silva Guimarães Pereira[1], Hugo Benguella[3], Aline Gutierrez Cruz[3], Bruna Toledo Duran Foglia[3], Aline da Silveira Batista[4] and Silvia Franco Andrade[1]

1. Post Graduate Program in Animal Science, Oeste Paulista University (UNOESTE), Presidente Prudente, SP, Brazil

2. Department of Anatomy Pathology, Faculty of Medicine (UNOESTE)

3. Faculty of Veterinary Medicine (UNOESTE)

4. Resident of Clinical Laboratory of the Veterinary Hospital (UNOESTE)

Contact address: S. F. Andrade Tel.: +55 18 3229 2067; Fax +55 18 3229 2036

e-mail: silviafranco@unoeste.br

Running Title: Tacrolimus eye drops in olive oil or linseed oil in KCS dogs

ABSTRACT

Objective To compare the efficacy of tacrolimus 0.03% eye drops diluted in two vehicle types, linseed oil and olive oil, for the treatment of keratoconjunctivitis sicca (KCS) in dogs.

Procedure Sixty dogs were used; 20 healthy dogs were allocated to the control group, and 40 dogs diagnosed with bilateral KCS were randomly allocated to two groups: tacrolimus in olive oil (TO) and tacrolimus in linseed oil (TL). The animals were evaluated monthly using ophthalmic examinations, Schirmer Tear Test (STT), Tear Film Break-up Time (TBUT), Fluorescein Test (FT) and Rose Bengal Test (RBT), as well as monthly cytological examinations and histopathological examinations at the beginning and end of the study.

Results In both groups, the clinical signs, healing of corneal ulcers and STT, TBUT and RBT ophthalmic examinations improved significantly after one month of treatment. At the end of the experiment, in the cytological analysis, both groups presented decreases in lymphocytes, neutrophils and metaplastic and squamous cells, whereas in the histopathological analysis, decreases in lymphocytes and neutrophils and an increase in goblet cells were observed; in the TL group, the decrease in neutrophils was more significant in both analyses.

Conclusion Tacrolimus 0.03% eye drops diluted in olive oil and linseed oil were efficient in the treatment of KCS. There were no significant differences between the groups in the evaluated parameters, except for the reduction in neutrophils, which was more significant in the TL group. Thus, linseed oil might present a new alternative as a tacrolimus eye drop diluent.

Key-words: dry eye, olive oil, linseed oil, dogs, tacrolimus, omegas.

INTRODUCTION

Keratoconjunctivitis sicca (KCS), or dry eye, is a common chronic inflammatory ophthalmopathy in dogs and humans that is characterized by a decrease in the aqueous layer of the tear film (quantitative) and/or a deficiency in the lipid or mucin layer (qualitative), causing a progressive inflammatory process, primarily in the cornea, conjunctiva and lacrimal glands, usually of an immunomediated origin.[1-3] The clinical signs of KCS include conjunctival hyperemia, eye irritation, chemosis, blepharospasm, photophobia, mucoid and mucopurulent secretion, vascularization and pigmentation of the cornea, and even a loss of vision. The causes of KCS in dogs are primarily immunomediated and secondarily through congenital lacrimal deficiency, breed predisposition, drug-induced, neurogenic, iatrogenic, or infectious causes (distemper and leishmaniosis), senility and removal of the gland of the third eyelid.[3-7]

Topical therapy consists predominantly of the use of lubricants associated with the use of immunosuppressants, such as cyclosporine, tacrolimus and pimecrolimus, and secondarily, if necessary, the use of anti-inflammatories, antibiotics and mucolytics.[8-12] Tacrolimus (FK506) is a macrolide antibiotic isolated from *Streptomyces tsukubaensis*, and it has an effect similar to cyclosporine. The effects of tacrolimus are a combination of local immunosuppression, goblet cell proliferation, suppression of lacrimal cell apoptosis and anti-inflammatory action.[11-13]

Vegetable oils are rich in essential fatty acids (EFAs), such as omegas 3 and 6, which are considered natural anti-inflammatories, as well as vitamins, minerals and other substances. These oils have wide application in topical pharmaceutical preparations, including use as a vehicle for eye drops. The principal vegetable oils used topically are olive, linseed, almond, grape seed and macadamia.[14-16]

Olive oil, from *Olea europeae*, contains large quantities of monounsaturated fatty acids (MUFAs), 70-80% oleic acids, 10-15% saturated fatty acids (palmitic acid), and a small quantity of polyunsaturated fatty acids (PUFAs), including approximately 5 to 10% of omegas 3, 6 and 9. It has anti-inflammatory, antinociceptive, immunomodulatory and antimicrobial properties.[15-17] A study in rabbits with induced KCS that were treated with pure olive oil or in combination with cyclosporine demonstrated excellent results for olive oil in controlling the symptoms of KCS.[18]

Linseed oil is currently considered one of the largest sources of the EFAs omega 3 and omega 6, and it is recommended orally as an adjuvant therapy in Sjögren KCS syndrome in human patients.[19-22] Studies in rabbits have reported an improvement in symptoms of experimentally induced KCS with the use of linseed oil both in various preparations (oral, topical and a combination of the two) and in combination with tacrolimus and cyclosporine.[18,23,24] Linseed oil is considered a natural anti-inflammatory agent due to its potential for the synthesis of anti-inflammatory mediators, such as prostaglandin E1 (PGE1) and thromboxane A1 (TXA 1).[14,21,22]

The objective of this study was to evaluate the efficacy of tacrolimus 0.03% eye drops diluted in two different vehicles, olive oil, which is already commonly used for this purpose, and linseed oil, which has not been tested as a tacrolimus diluent in the treatment of KCS in dogs.

MATERIALS AND METHODS

Animals

The study was approved by the Ethics Committee on Animal Use (CEUA) of UNOESTE (protocol no.1794), and it was undertaken in accordance with the rules of ARVO (Association for Research in Vision and Ophthalmology – Statement for the use of animals in

ophthalmic and visual research). Forty dogs with bilateral KCS were selected, of which the most prevalent breeds were Lhasa apso (30%), poodle (17.5%), mongrel dog (15%), pinscher (7.5%), shih-tzu (5%) and other breeds representing 25%, with one breed each (border collie, cocker spaniel, pit bull, beagle, Yorkshire terrier, bull terrier, Rottweiler, pug, Maltese), 25 females (62.5%) and 15 males (37.5%), with a mean age of 6.7 ± 3.9 and a mean weight of 10.3 ± 7.7 kg. The animals were included in the experiment through the observation of ophthalmic clinical signs (ocular discharge, conjunctivitis, corneal opacity pigmentation), as well as STT ≤ 10 mm/min and/or TBUT ≤10 sec. Twenty healthy mongrel dogs from the kennel of the university were used, with a mean age of 3.5 ± 2.4 years and a mean weight of 10.3 ± 7.7 kg, 9 males (45%) and 11 females (55%), as a negative control group for the normal parameters of the STT, TBUT and cytological and conjunctival histopathological examinations.

Groups

After the diagnosis of KCS, the animals were randomly divided into two treatment groups: TO group (n = 20), tacrolimus 0.03% eye drops diluted in olive oil (Ophthalmos Laboratory, São Paulo, Brazil); and the TL group (n = 20), tacrolimus 0.03% eye drops diluted in linseed oil (Ophthalmos Laboratory, Sao Paulo, Brazil), 1 drop twice per day for six months, in addition to complementary treatment with propylene glycol-based lubricant (Systane®, Alcon, Sao Paulo, Brazil), 1 drop, twice per day for six months, and if necessary, antibiotic eye drops based on the examination of culture and sensitivity (1 drop, 4 times per day for 15 days) and diclofenac sodium-based anti-inflammatory eye drops (1 drop, twice per day for 15 days) (Still®, Allergan, São Paulo, Brazil).

Moments and ophthalmic examinations

Ophthalmic and cytological examinations were performed monthly, with the moment zero (M0) considered the first day of treatment after the diagnosis of bilateral KCS and the other moments (M1 to M6) determined monthly during treatment. Histopathological examinations were performed at the time of diagnosis (M0) and at study completion (M6). The ophthalmologic clinical signs were identified by the same examiner (LFCZ) using a portable slit lamp (Kowa, Japan) in the presence or absence of conjunctivitis, ocular secretions, corneal opacity and pigmentation, in accordance with the scores described in Table 1.

The Schirmer Tear Test (STT) (Teste de Schirmer® - Ophthalmos Laboratory, São Paulo, Brazil) was performed without anesthetic drops to evaluate the quantitative portion of tears; a 0.5-cm strip was inserted into the conjunctival sac for one minute, and ≤10 mm/min was considered positive.[25] The Tear Film Break-up Time (TBUT) was used to evaluate the qualitative portion of the tear; two consecutive measures were obtained, and the average as calculated. After the application of one drop of Fluorescein 1% eye drops (Allergan, São Paulo, Brazil), the time was observed between the final blink and the appearance of stains or dark spots in the tear film using a slit lamp (Kowa, Japan); TBUT values ≤ 10 seconds were considered positive.[26]

The fluorescein test (FT) was performed with a fluorescein 1% eye drop(Allergan, São Paulo, Brazil. The eye was washed with saline solution, and the presence or absence of corneal ulcers was evaluated[25] and scored according to its severity and extension (0-negative, 1-small superficial ulcer, 2-medium superficial ulcer, 3-extensive surface ulcer, 4-small stromal ulcer, 5-medium stromal ulcer, 6-extensive stromal ulcer, 7-descemetocele and 8-keratomalacia).

The Rose Bengal Test (RBT) was performed to evaluate the presence of devitalized cells using Rose Bengal 1% eye drops (Ophthalmos Laboratory, São Paulo, Brazil) after a prior application of anesthetic eye drops. The result was considered positive for KCS when any area of the cornea or conjunctiva flushed pink.[25] The RBT score was graduated from 0-3: 0-negative; 1-only the conjunctiva stained; 2-only the cornea stained; and 3-conjunctiva and cornea stained.

Cytological and histological examinations

The cytological examination was performed after eye cleansing with saline and application of anesthetic eye drops (Allergan®, São Paulo, Brazil). Samples of lower conjunctival cells were harvested with a sterile swab moistened with saline and microscope slide glass for each eye; the samples were then fixed in methanol and stained using the MGG technique (May-Grunwald-Giemsa). Lymphocyte, neutrophil, metaplastic cell and squamous cell counts were performed under an optical microscope in 10 fields using a 40x objective.

The histopathological examination was performed after the application of anesthetic eye drops (Allergan®, São Paulo, Brazil), with the removal of 1-3 mm from the medial portion inferior of the fornix of the conjunctiva, with the aid of forceps and conjunctiva scissors. The histological section was placed on a fragment of paper of standard size, 1x1 cm, and was fixed in formalin and embedded in paraffin (Dinâmica Reagentes Analíticos, São Paulo, Brazil). With the aid of a rotary microtome, 5-μm-thick cuts were obtained from the conjunctiva, which was stained using hematoxylin and eosin (HE) (Dolles, São Paulo, Brazil) and PAS techniques (Merck, USA), and the following parameters subsequently evaluated: for HE, the count of lymphocytes and neutrophils; and for PAS, goblet cell density count (cells/mm^2) with an optical microscope using a 40x objective.

Statistical analysis

For the STT I and TBUT variables, the goblet cell density, and the numbers of squamous cells, metaplastic cells, PMNs and MNs, we used two-way analysis of variance (ANOVA) for paired samples with contrasts by Tukey's method. For the FT and RBT variables, we used Friedman's nonparametric test for the comparison of time points and the Kruskal-Wallis test with contrasts by Dunn's method for the comparison of groups. A significance level of $p<0.05$ was adopted. The software used for statistical analysis was BioStat, version 5.3.

RESULTS

The results of tests performed in the negative control group to obtain the normality parameters are described in Table 2. Regarding the clinical signs evaluated (Figure 1), both groups presented improvements, and there were no statistically significant differences ($p<0.05$) between the TO and TL groups; there were only statistically significant differences ($p>0.05$) between M0 and other moments. For the variables of ocular discharge, conjunctivitis and corneal opacity, there was complete remission of signs at M1. For corneal pigmentation, the median showed complete remission at M5.

On the STT (Figure 2), the TO and TL groups presented significant increases at M1, and there was a significant difference ($p <0.05$) at M2, M3, M5 and M6 between the groups, with the TO group presenting higher values than the TL group and both groups demonstrating statistically significant differences ($p<0.05$) when comparing M0 with the other moments, with values close to the negative control group (Table 2). On the TBUT (Figure 2), the two groups presented significant increases at M1, which continued until M6, with values close to the negative control group (Table 2). There was no statistically significant difference ($p>0.05$)

between the groups; however, there was a significant difference when comparing M0 with the other moments.

On the FT (Figure 3), at T0, the two groups presented various types of ulcers in terms of severity and extension, and both groups presented excellent healing at M1; there were no significant differences ($p>0.05$) between the groups except for between M0 and the other moments ($p<0.05$). On the RBT (Figure 4), at M0, there was a prevalence of stained cornea and conjunctiva from M1 to T4 in the TO group, and there was a prevalence of only stained cornea; in the TL group, at M1 and M2, only conjunctiva was stained. In the TO group, there was full resolution at M5, whereas in the TL group, there was full resolution at M4, with statistically significant differences between M0 and other moments in both groups.

In the cytological examination (Figure 5), both groups presented decreases in all cells, and there were no significant differences between the groups, except in the parameter of neutrophils, which presented a significantly lower count ($p <0.05$) in the TL group than in the TO group at M6, with a significant difference between the groups and when comparing M0 with other moments. In the histopathological examination (Figure 6), both groups presented decreases in inflammatory cells, with a significantly lower neutrophil count ($P <0.05$) in the TL group than in the TO group at M6. There was an increase in the number of goblet cells with no significant difference between the groups. Both groups presented an improvement in the examinations, with values close to the normal parameters (negative control group) (Figure 7). The cytological and histopathological examinations at M0 and M6 for animal N10 from the TO group are shown in Figure 8 and for animal N3 from the TL group in Figure 9.

DISCUSSION

Some studies of KC that was experimentally induced in rabbits and treated with the most commonly used topical immunosuppressants, cyclosporine and tacrolimus, diluted in vegetable oils (almond, olive oil and linseed oil) and studies of the isolated use of these oils have demonstrated that not only the immunosuppressants but also the oils showed efficacy in controlling symptoms of KCS, especially having anti-inflammatory effects mainly due to the presence of omegas 3 and 6 in their composition, which induce the formation of anti-inflammatory mediators, such as prostaglandin E1 (PGE1) A1 and thromboxane (TXA 1).[14,18,21-23] In these studies, the linseed oil always performed better than the other oils, almond[23] and olive[18], which perhaps occurred because linseed oil has a greater concentration of omegas 3 and 6 than do the other oils studied.[20-22] Another study on experimentally induced KCS in rabbits, using linseed oil through various routes of administration (oral, topical and oral and topical in combination), also demonstrated efficacy in the control of symptoms and an increase in goblet cells.[24]

Beagle dogs treated with tacrolimus diluted in olive oil presented an improvement in clinical signs (secretion, pigmentation and hyperemia) and no significant difference compared to another immunosuppressant: cyclosporine. A significant increase in the STT was also found at the first time point, as in the present study.[12]

In the results from the STT and TBUT, there was a significant increase in the values obtained, while there was no statistically significant difference between the TO and TL groups at the end of the study, which was in agreement with the research on cyclosporine 0.02% diluted in olive oil and linseed oil[18] in experimentally induced KCS in rabbits. However, in a study using tacrolimus diluted in 0.03% almond oil and linseed oil[23], the group with only topical application of linseed oil presented a more significant increase. The STT

values were also significantly higher in the study of the use of linseed oil applied orally and topically to treat experimentally induced KCS in rabbits.[24]

In the present study, there was excellent resolution of corneal ulcers in both the TO and TL groups, with no statistically significant difference between them, which was also in agreement with other studies[18,23], including the resolution of keratomalacia (*"melting"* corneal ulcer) described in a study with linseed oil applied topically and orally in association with KCS induced in rabbits.[24]

Additionally, on the RBT test, at the end of the experiment, there were no significant differences between the groups, demonstrating that there was a decrease in the presence of devitalized cells in the cornea and conjunctiva induced by KCS, in agreement with other studies that used these oils in combination with topical immunosuppressants.[18,23]

In the present study, the only statistically significant difference at the end of treatment was in the neutrophil count parameter, which was statistically lower in the TL group. A decrease in the number of inflammatory cells was also described in a study of dogs with KCS treated with 2% topical cyclosporine.[27] The literature has suggested that the pathogenesis of KCS is based on inflammatory infiltrate, the intense presence of lymphocytes and the moderate presence of neutrophils and that the inflammatory infiltrate is the main cause of the destruction of the glandular tissue.[1,2] A study performed in dogs infected with distemper virus and KCS showed severe inflammatory infiltrate on histopathological examination, with a predominance of lymphocytes and neutrophils and a small number of goblet cells.[6]

In the present study, there was a significant increase in the number of goblet cells between the beginning and end of treatment, with no significant difference between the groups. Other studies have also demonstrated an increase in goblet cells from treatment with omegas[14,24] or immunosuppressants [9-13,18,23,24] in the treatment of KCS in several species.

Thus, we conclude that the tacrolimus 0.03% eye drops diluted in olive oil (TO) and linseed oil (TL) were effective for the treatment of KCS. There were no significant differences between the groups except for in the reduction of neutrophils, which was more significant in the TL group. Thus, linseed oil could present a new alternative as a tacrolimus eye drop diluent.

ACKNOWLEDGEMENTS

We would like to thank the Post-graduate Program in Animal Science of Universidade do Oeste Paulista, Presidente Prudente (SP), Brazil, for financial support and the Laboratory Ophthalmos® for the donation of some of the materials necessary for the present research.

REFERENCES

1. Stevenson W, Chauhan SK, Dana R. Dry eye disease: an immune-mediated ocular surface disorder. *Archives of Ophthalmology* 2012; 130:90–100.

2. Williams DL. Immunopathogenesis of keratoconjunctivitis sicca in the dog.*Veterinary clinics of North America. Small Animal Practice* 2008;38:251-68.

3. Miller PE. Lacrimal system. In: Slatter's Fundamentals of Veterinary Ophthalmology, 4thedn. (edsMaggs DJ, Miller PE, Ofri R) Saunders Elsevier, St. Louis, 2008; 157–174.

4. Matheis FL, Reinhardt LW, Spiess BM. Canine neurogenic keratoconjunctivitis sicca:11cases.*Veterinary Ophthalmology* 2012; 15:288–290.

5. Westermeyer HD, Ward DA, Abrams K.Breed predisposition to congenital alacrima in dogs. *Veterinary Ophthalmology* 2009;12:1–5.

6. Almeida D.E, Roveratti C, Brito FL, et al. Conjunctival effects of canine distemper virus-induced keratoconjunctivitis sicca. *VeterinaryOphthalmology*2009,12:211–215.

7. Naranjo C, Fondevila D, Altet L et al. Evaluation of the presence of Leishmania spp. by real-time PCR in the lacrimal glands of dogs with leishmaniosis. *Veterinary Journal* 2012;193:168–73.

8.Grahn BH; Storey ES. Lacrimomimetics and lacrimostimulants. *Veterinary Clinics of North America: Small Animal Practice* 2004; 34:739–753.

9. Izci C, Celik I, Alkan F, Ogurtan Z, et al. Histologic characteristics and local cellular immunity of the gland of the third eyelid after topical ophthalmic administration of 2% cyclosporine for treatment of dogs with keratoconjunctivitis sicca. *American Journal of Veterinary Research* 2002;63: 688–694.

10. Ofri R, Lambrou GN, Allgoewer I, Graenitz U, Pena TM et al. Clinical evaluation of pimecrolimus eye drops for treatment of canine keratoconjunctivitis sicca: a comparison with cyclosporine A. *Veterinary Journal* 2009;179: 70–77.

11. Berdoulay YA, English RV, Naldelstein B. Effect of topical 0.02% tacrolimus aqueous suspension on tear production in dog with keratoconjunctivitis sicca. *Veterinary Ophthalmology* 2005; 8:225–232.

12. Hendrix VDH, Adkins EA, Ward, DA et al. An investigation comparing the efficacy of topical ocular application of tacrolimus and cyclosporine in dogs.*Veterinary Medicine International* 2011; 2011:487592

13. Moskovici BK, Holzchuh R, Naves FES et al. Treatment of Sjögren's syndrome dry eye using 0.03% tacrolimus eyedrop: Prospective double-blind randomized study. *ContactLens& Anterior Eye*2015;38:373–8.

14. Barabino S, Rolando M, Camicione P et al. Systemic linoleic and γ-linolenic acid therapy in dry eye syndrome with an inflammatory component. *Cornea* 2003;22:97–101.

15. Waterman E, Lockwood B. B. Active components and clinical applications of olive oil. *Alternative Medicine Review* 2007;12:331–342.

16. Wardhana EE, Surachmanto EAD. The role of omega-3 fatty acids contained in olive oil on chronic inflammation. *Acta Medica Indonesiana*2011;43:138-42.

17. Eidi A, Moghadam-Kia S, MoghadamJZet al. Antinociceptive and anti-inflammatory effects of olive oil (*Olea europeae L*.) in mice. *PharmaceuticalBiology* 2012; 50:332-37.

18. Parrilha LR, Nai GA, Giuffrida R etal.Comparison of 1% cyclosporine eye drops in olive oil and in linseed oil to treat experimentally-induced keratoconjunctivitis sicca in rabbits. *ArquivosBrasileiros de Oftalmologia* 2015, 78(5):295-9.

19. Hassan-Zadeh A, Sahari MA, Barzegar M, Optimization of the -3 extraction as a functional food from flaxseed. *International Journal of Food Sciences and Nutrition* 2008; 59: 526-534.

20. Roncone M, Bartlett H, Eperjesi, F. Essential fatty acids for dry eye: A review. Contact Lens & Anterior Eye 2010; 33:49-54.

21. Wojtowicz, JC, Butovich I, Uchiyama E et al. Pilot, prospective, randomized, double-masked, placebo-controlled clinical trial of an omega-3 supplement for dry eye. *Cornea* 2011; 30:308-314.

22. Aragona P, Bucolo C, Spinella R et al. Systemic omega-6 essential fatty acid treatment and PGE1 tear content in Sjögren'ssyndrome patients. *Investigative Ophthalmology Visual Science* 2005; 46:4474–4479.

23. Sgrignoli MR, Yamasaki L, Sanches OC *et al*. Comparison of topical 0.03% tacrolimus in almond and linseed oil to treat experimentally induced keratoconjunctivitis sicca in rabbits. *International Journal of Ophthalmic Pathology* 2013; **2**:3.

24. Neves ML, Yamasaki L, Sanches OC et al. Use of linseed oil to treat experimentally induced keratoconjunctivitis sicca in rabbits. *Journal of Ophthalmic Inflammation and*

Infection 2013;3:4:1–5.

25.Maggs DJ. Basic diagnostic techniques. In: Slatter's Fundamentals of Veterinary Ophthalmology, 4thedn. (edsMaggs DJ, Miller PE, Ofri R) Saunders Elsevier, St. Louis, 2008; 81–106.

26. Saito A1, KotaniT.Estimation of lacrimal level and testing methods on normal beagles.*Veterinary Ophthalmology* 2001;4:7–11.

27. Izci C, Celik I, Alkan F, et al. Clinical and light microscopic studies of the conjunctival tissues of dogs with bilateral keratoconjunctivitis sicca before and after treatment with topical 2% cyclosporine. *Biotechnic&Histochemistry* 2015, 90(3): 223–230

Table 1. Scores of the evaluated clinical 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	0 = None
	1 = <25%
	2 = 25-50%
	3 = >50%
Corneal pigmentation	0 = None
	1 = <25%
	2 = 25-50%
	3 = >50%

Table 2. Mean and standard deviation of the Schirmer Tear Test (STT), Tear Film Break-up Time (TBUT), conjunctival cytology and conjunctival histopathology parameters, from healthy dogs in the negative control group (n=20)

Ophthalmic examinations	
STT (mm/min)	27.3±4.1
TBUT (seconds)	23.3±5.1
Conjunctival cytology	
Lymphocytes (cells/10 HPF)	0.1±0.4
Neutrophils (cells/10 HPF)	0±0
Metaplastic cells (cells/10 HPF)	0.5±2.1
Squamous cells (cells/10 HPF)	0.1±0.3
Conjunctival histopathology	
Lymphocytes (cells/5 HPF)	26.7±24.2
Neutrophils (cells/5 HPF)	2.6±4.4
Goblet cells (cells/mm^2)	28.3±16.0

*Cell count in 10 fields and 5 fields, 40x objective.

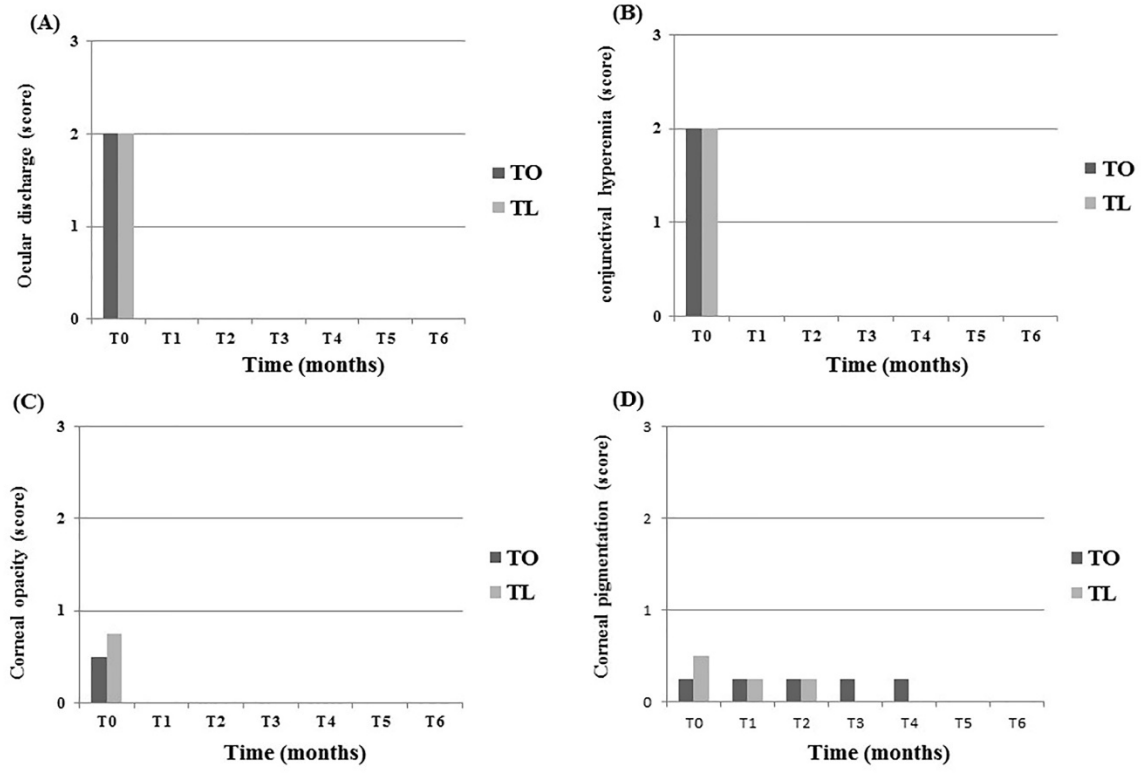

Figure 1. Median score of clinical signs observed from M0 to M6, in the TO (olive oil) and TL groups (linseed): (A) ocular discharge, (B) conjunctival hyperemia, (C) corneal opacity and (D) corneal pigmentation.

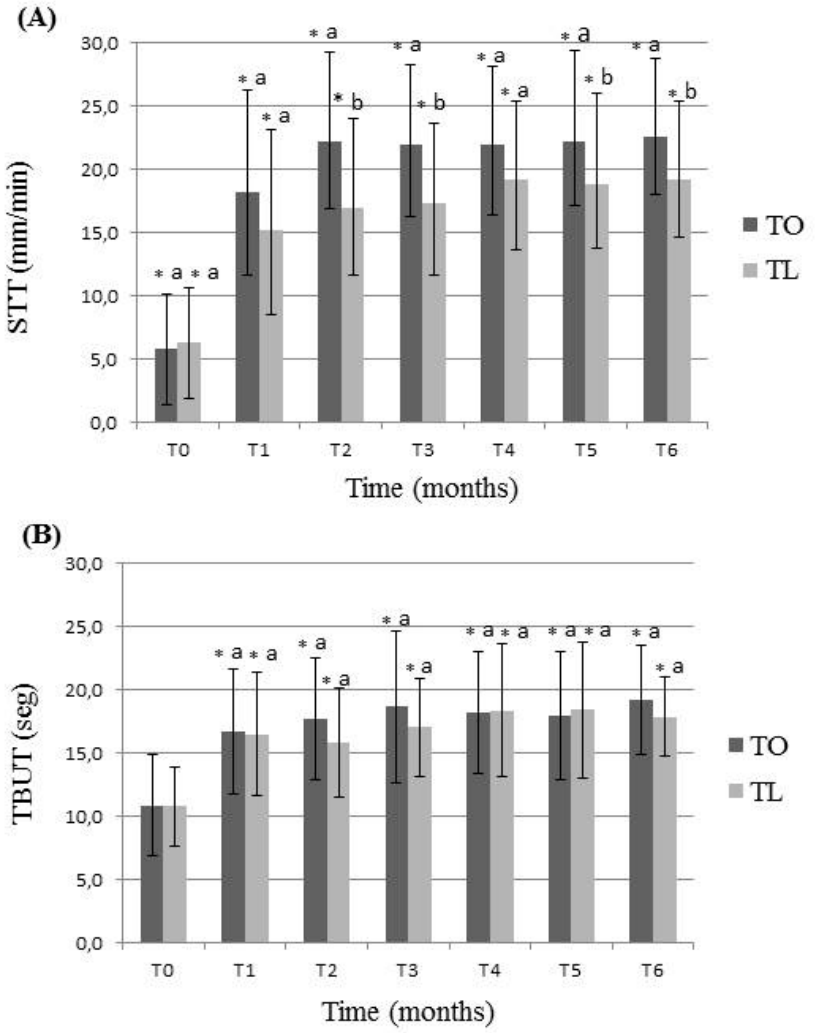

Figure 2. (A) Mean and standard deviation of results of the Schirmer tear test (STT)[a] in mm/min from M0 to M6, in the animals from the TO (tacrolimus diluted in olive oil) and TL groups (tacrolimus diluted in linseed oil); (B) Mean and standard deviation of the Tear Film Break-up Time (TBUT)[b] in seconds, M0 to M6, in the animals from the TO and TL groups.

[a]Values ≤10 mm/min positive for KCS
[b]Values ≤10 seconds positive for KCS
*p<0.05 (Tukey's test to compare time points)
[a,b]p<0.05 (Kruskal-Wallis test to compare groups)

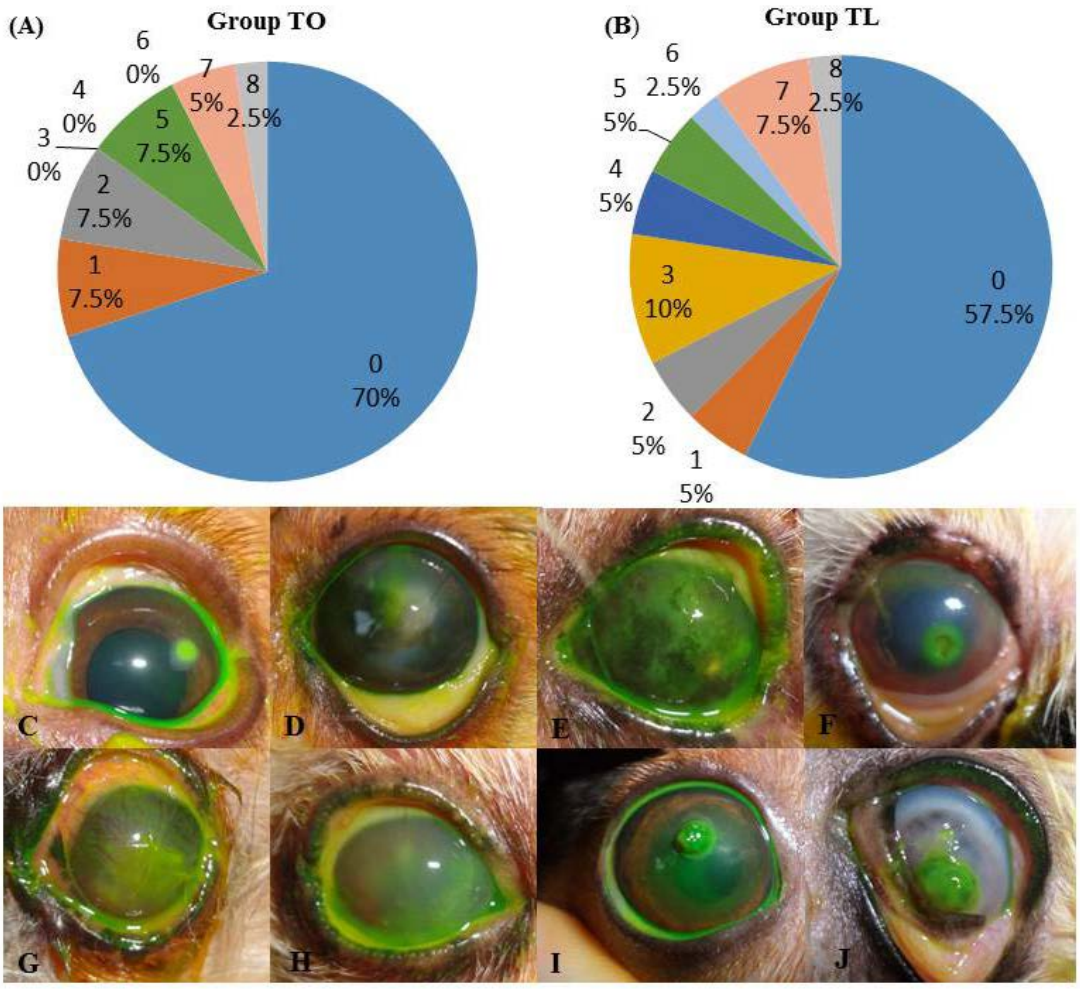

Figure 3. Fluorescein test (FT). Graphs of the percentages of ulcer types * at M0 (A) for the TO group and (B) the TL group. Photos of animals' eyes in the present study demonstrating the classification of types of ulcers: (C) small surface ulcer in the left eye (OS), animal N2 from the TO group; (D) medium surface ulcer, OS, animal N5 from the TO group; (E) extensive surface ulcer, OS, animal N10 from the TL group; (F) small stromal ulcer, right eye (OD), animal N24 from the TL group; (G) medium stromal ulcer, OS, animal N14 from the TL group; (H) extensive stromal ulcer, OD, animal N17 from the TO group; (I) Descemetocele, OD, animal N32 from the TO group; (J) keratomalacia, OS, animal N31 from the TL group.

*0-negative, 1- small superficial ulcer, 2- medium superficial ulcer, 3- extensive surface ulcer, 4-small stromal ulcer, 5-medium stromal ulcer, 6-extensive stromal ulcer, 7-descemetocele, and 8-keratomalacia.

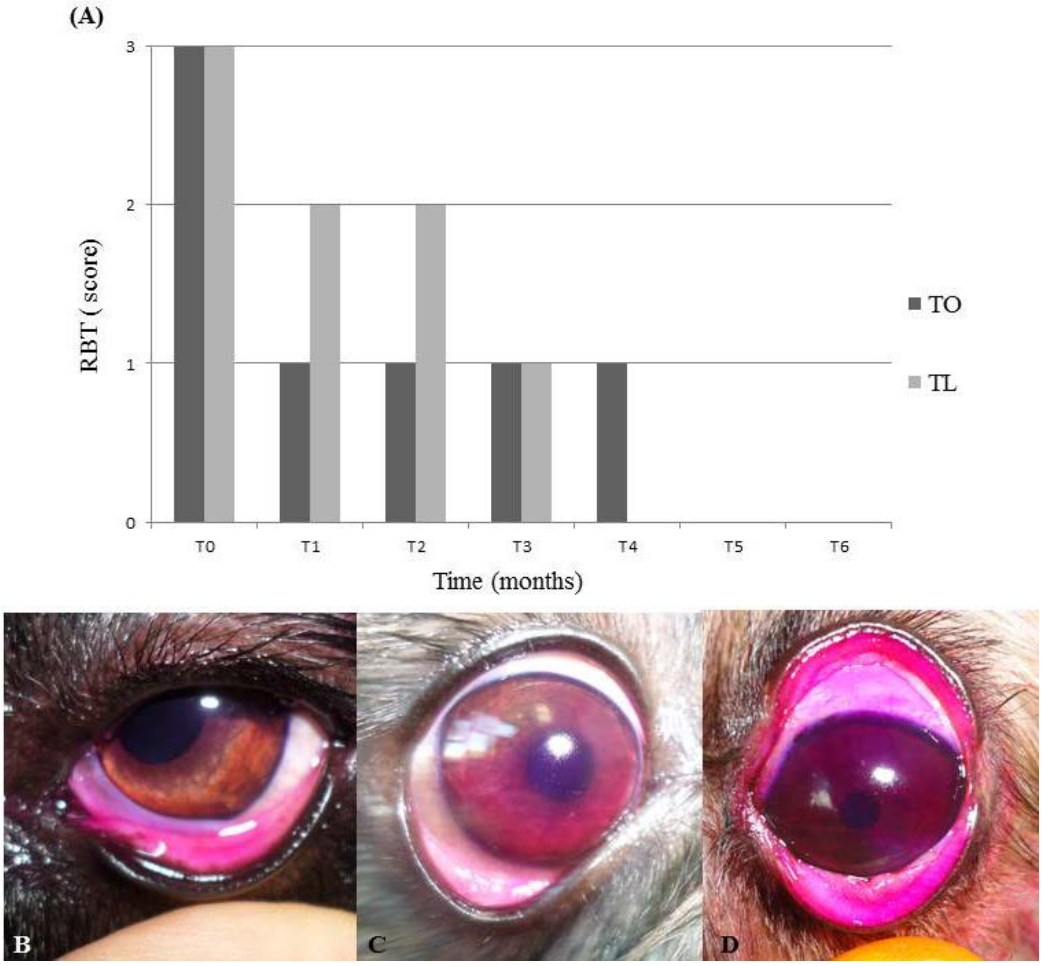

Figure 4. Rose Bengal Test (RBT). (A) Median of the RBT score in the TO and TL groups. Photos of experimental animals demonstrating the RBT classification scores: (B) only conjunctiva stained (OS), animal N12, from the TL group; (C) only cornea stained (OS), animal N5, from the TO group; (D) cornea and conjunctiva stained (OD), animal N13, from the TL group.

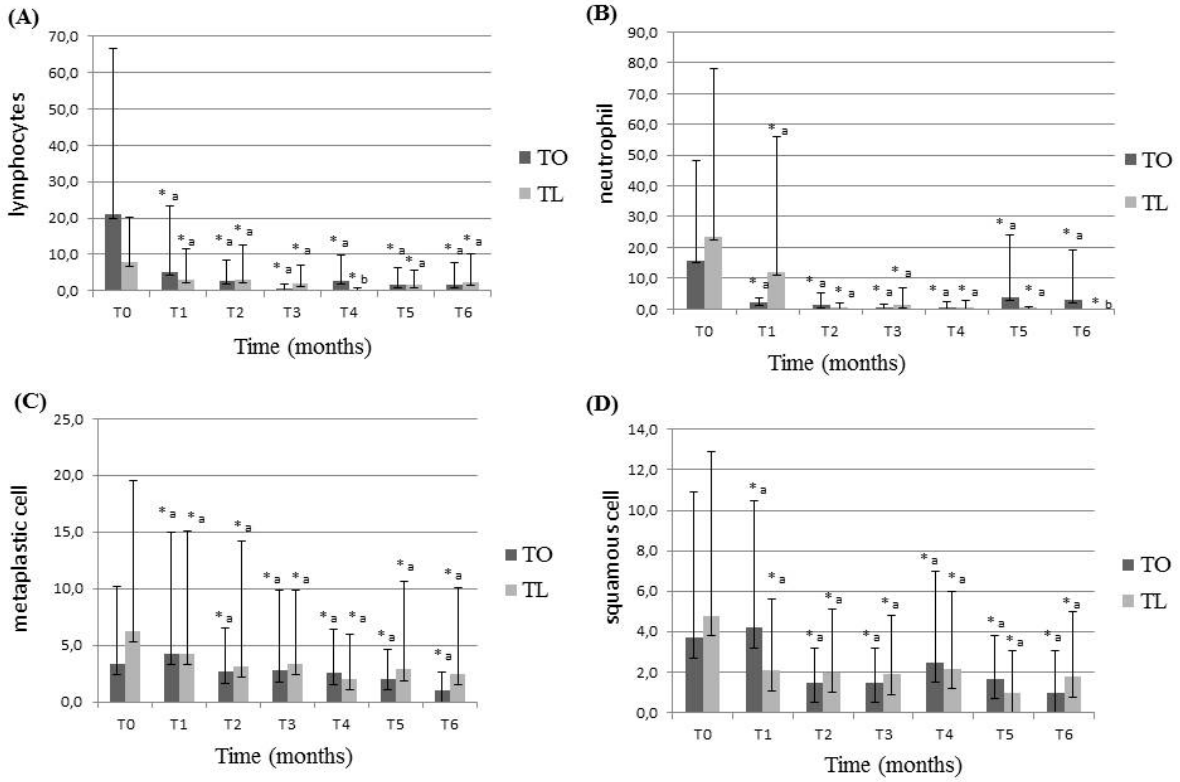

Figure 5. (A) Mean and standard deviation of the lymphocyte counts in the conjunctival cytology examination from M0 to M6, in the TO and TL groups (B) Mean and standard deviation of the neutrophil counts from M0 to M6 (C) Mean and standard deviation of the metaplastic cell counts from M0 to M6 (D) Mean and standard deviation of the squamous cell counts from M0 to M6.

*$p<0.05$ (Tukey's test to compare time points)
[a,b]$p< 0.05$ (Kruskal-Wallis test to compare groups)

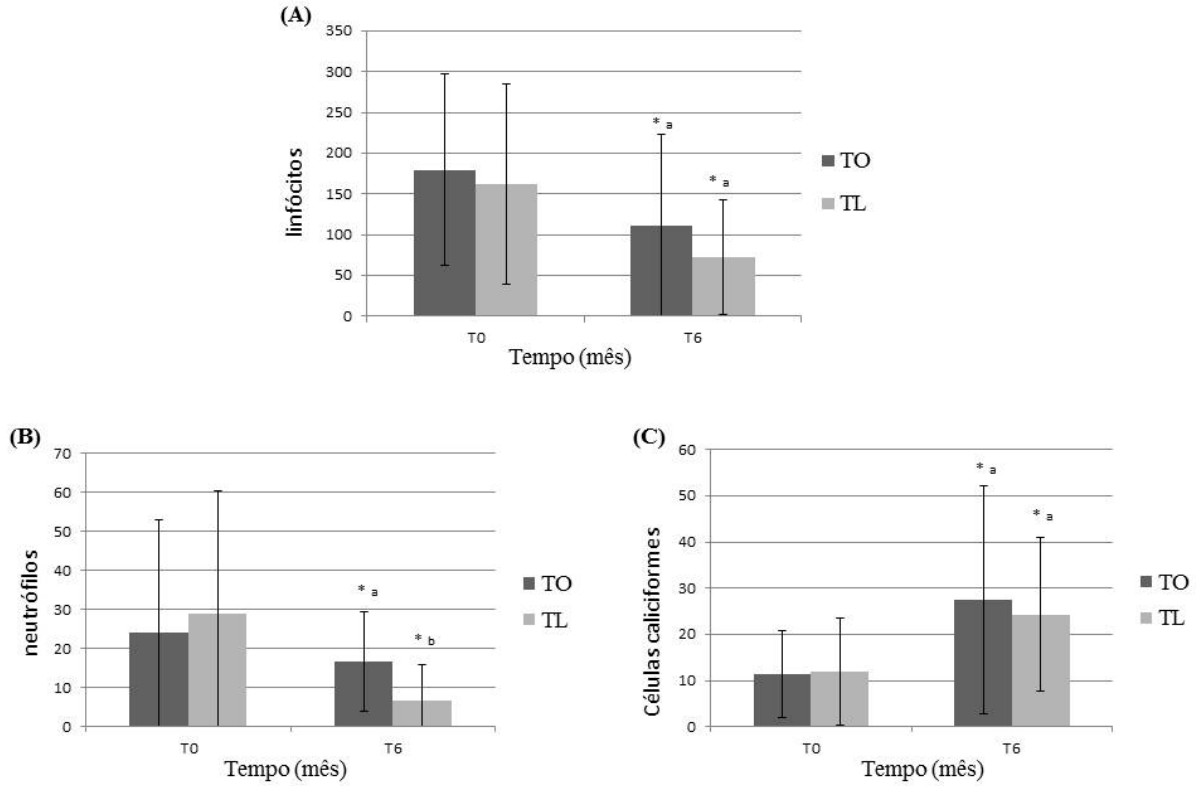

Figure 6. (A) Mean and standard deviation of the lymphocyte counts in conjunctival histopathology at M0 and M6, in the TO and TL groups (B) Mean and standard deviation of the neutrophil counts at M0 and M6 (C) Mean and standard deviation of the goblet cell counts at M0 and M6.

*$p<0.05$ (Tukey's test to compare time points)
[a,b]$p<0.05$ (Kruskal-Wallis test to compare groups)

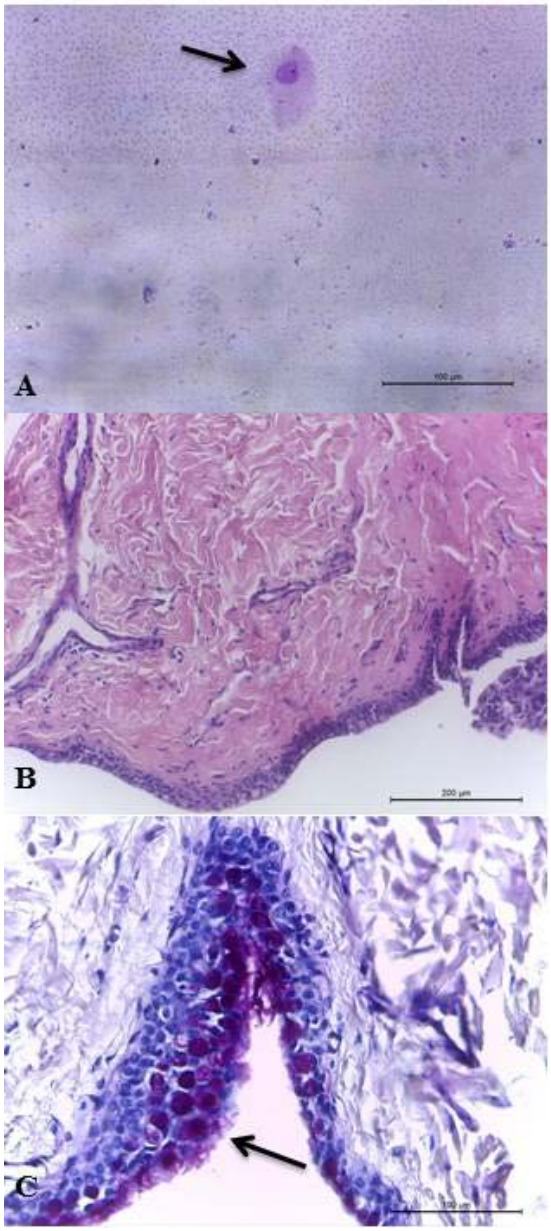

Figure 7. Light microscopy of the control group: (A) Conjunctival cytology with the presence of a squamous cell (arrow) MGG staining, 400x magnification; (B) Histopathology, without alteration in tissue staining with hematoxylin-eosin, 200x magnification; (C) Histopathology, with a large number of goblet cells (arrow), PAS staining, 400x magnification.

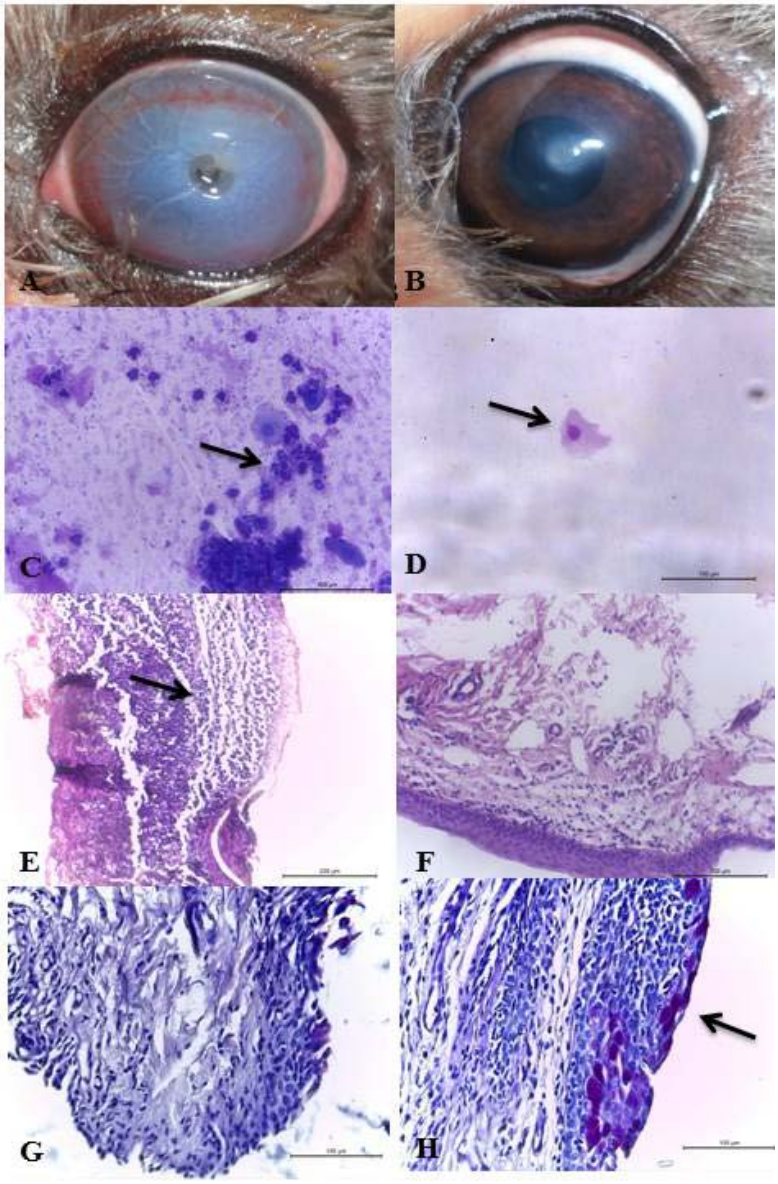

Figure 8. (A) M0, left eye, animal N10 from the TO group, keratomalacia and corneal vascularization; (B) M6 ulcer healing; (C) Light microscopy of the conjunctival cytology at M0, presence of neutrophils (arrow), MGG staining, 400x magnification; (D) Cytology, M6, presence of squamous cell (arrow), MGG, 400x magnification; (E) Histopathology at M0, inflammatory infiltrate (arrow). HE staining, 200x magnification; (F) Histopathology at M6, decrease in inflammatory infiltrate; (G) Histopathology at M0, absence of goblet cells, PAS staining, 400x magnification; (H) PAS histopathology at M6, large quantity of goblet cells (arrow).

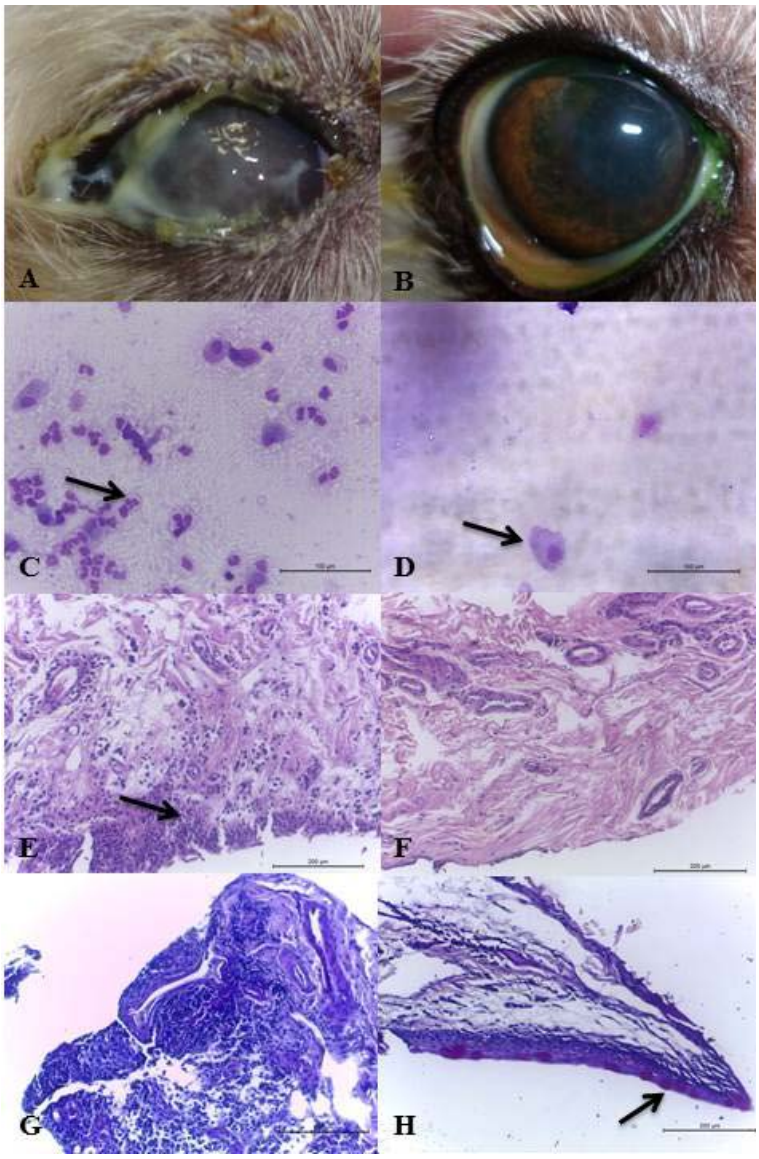

Figure 9. (A) M0, right eye, animal N3 from the TL group, mucoid secretion and corneal pigmentation; (B) M6 clinical evolution; (C) Light microscopy of the conjunctival cytology at M0, presence of neutrophils (arrow), MGG staining, 400x magnification; (D) Cytology, M6, presence of squamous cells (arrow); (E) Histopathology at M0, inflammatory infiltrate (arrow), HE staining, 200x magnification; (F) Histopathology at M6, decrease in inflammatory infiltrate; (G) Histopathology at M0, absence of goblet cells, PAS staining, 400x magnification; (H) PAS histopathology at M6 large quantity of goblet cells (arrow).

ANEXO – NORMAS PARA PUBLICAÇÃO NA REVISTA VETERINARY OPHTHALMOLOGY

Veterinary Ophthalmology publishes original material relating to all aspects of clinical and investigational veterinary and comparative ophthalmology. The following types of material will be published:

- Original articles including clinical (prospective and retrospective clinical studies) and investigational studies. Research studies involving animals must have the approval of the institution's animal care and use committee and be acceptable to the Editor.
- Review articles (including papers which clarify, summarize and critically evaluate the current literature). These will be invited by the Editor or a member of the editorial board.
- Case reports (patient-based studies; either single or multiple animals). Authors of case reports rejected by our journal will be offered the option of having their manuscript, along with any related peer review comments, automatically transferred for consideration by the editorial team of *Clinical Case Reports*, an open access journal.
- Viewpoint articles (papers which challenge existing concepts or present an alternative interpretation of available information) are usually invited by the Editor or a member of the editorial board.
- Short communications: Brief research and clinical communications (limited to 6 pages and 12 references).
- Letters to the editor.

All original research and review articles will be peer reviewed by at least two independent referees. Submission

EarlyView

We are happy to announce that *Veterinary Ophthalmology* is now part of the Wiley-Blackwell Early View service. All articles will now be published online in advance of their appearance in a print issue. These articles are fully peer reviewed, edited and complete – they only lack page numbers and volume/issue details – and are considered fully published from the date they first appear online. This date is shown with the article in the online table of contents. Because Early View articles are considered fully complete, please bear in mind that changes cannot be made to an article after the online publication date even if it has not yet appeared in print.

Beginning January 1, 2007, Veterinary Ophthalmology accepts manuscripts only through our submission website. To submit a manuscript, please follow the instructions below:

Getting Started

1. Launch your web browser (supported browsers include Internet Explorer 6 or higher, Netscape 7.0, 7.1, or 7.2, Safari 1.2.4, or Firefox 1.0.4) and go to the *Veterinary Ophthalmology* ScholarOne Manuscripts homepage http://mc.manuscriptcentral.com/vop
2. Log-in or click the 'Create Account' option if you are a first-time user of ScholarOne Manuscripts.
3. If you are creating a new account:
   - After clicking on 'Create Account' enter your name and e-mail information and click 'Next'. Your e-mail information is very important.
   - Enter your institution and address information as prompted then click 'Next.'
   - Enter a user ID and password of your choice (we recommend using your e-mail address as your user ID) and then select your area of expertise.
   - Click 'Finish' when done.
4. Log-in and select 'Author Center.'

Submitting Your Manuscript

1. After you have logged in, click the 'Submit a Manuscript' link on the Author Center screen.
2. Enter data and answer questions as prompted.
3. Click on the 'Next' button on each screen to save your work and advance to the next screen.
4. You will be prompted to upload your files:
   - Click on the 'Browse' button and locate the file on your computer.
   - Select the description of the file in the drop down next to the Browse button.
   - When you have selected all files you wish to upload, click the 'Upload' button.
5. Review your submission (in both PDF and HTML formats) before sending to the Editors. Click the 'Submit' button when you are done reviewing.

You may stop a submission at any phase and save it to submit later. After submission, you will receive a confirmation via e-mail. You can also log-on to ScholarOne Manuscripts at any time to check the status of your manuscript. The Editors will send you information via e-mail once a decision has been made.A covering letter, signed by all authors, must be included. This should state that the work has not been published and is not being considered for publication elsewhere, and that all authors meet the journal's criteria for authorship (see below). Information on any financial or other conflict of interest which may have biased the work should be provided (even if precautions were taken and authors are satisfied that bias was avoided).

Copyright Transfer Agreement

If your paper is accepted, the author identified as the formal corresponding author for the paper will receive an email prompting them to login into Author Services; where via the Wiley Author Licensing Service (WALS) they will be able to complete the license agreement on behalf of all authors on the paper.

For authors signing the copyright transfer agreement

If the OnlineOpen option is not selected the corresponding author will be presented with the copyright transfer agreement (CTA) to sign. The terms and conditions of the CTA can be previewed in the samples associated with the Copyright FAQs below:

CTA Terms and Conditions http://authorservices.wiley.com/bauthor/faqs_copyright.asp

For authors choosing OnlineOpen

If the OnlineOpen option is selected the corresponding author will have a choice of the following Creative Commons License Open Access Agreements (OAA):

- Creative Commons Attribution License OAA
- Creative Commons Attribution Non-Commercial License OAA
- Creative Commons Attribution Non-Commercial -NoDerivs License OAA

To preview the terms and conditions of these open access agreements please visit the Copyright FAQs hosted on Wiley Author Services http://authorservices.wiley.com/bauthor/faqs_copyright.aspand visit http://www.wileyopenaccess.com/details/content/12f25db4c87/Copyright--License.html.

If you select the OnlineOpen option and your research is funded by The Wellcome Trust and members of the Research Councils UK (RCUK) or the Austrian Science Fund (FWF) you will be given the opportunity to publish your article under a CC-BY license supporting you in complying with your Funder requirements. For more information on this policy and the

Journal's compliant self-archiving policy please visit: http://www.wiley.com/go/funderstatement.

Manuscript Style

The manuscripts must be in Microsoft Word format (.doc or .docx). The manuscript (including footnotes, references, figure legends, and tables) must be double-space typed, using 12-point Times New Roman font, 1-inch margins, and left justification. Original Research papers and Review Articles should usually not be longer than 5000 words. Viewpoint articles will not normally exceed 2000 words, and reviews of books and information materials should be less than 1000 words long.

Title Page

The title page should include a descriptive title for the article, the names [first name, initials of middle name(s), surnames], qualifications and affiliations of all authors, and the full postal address, fax, e-mail (if available), and telephone number of the author to whom correspondence should be addressed. A suggested running title of not more than 50 characters including spaces should be included.

Abstract and Keywords

The abstract should be on a separate page and should not exceed 250 words. Where possible, the abstract should be structured. Suggested headings for abstracts of primary research are: Objective; Animal studied, Procedure(s), Results, and Conclusions.

Key words are used by indexes and electronic search engines, and should appear after the abstract. Use the heading 'Key words', typed in bold and followed by a colon, and then the key words separated by commas. Include up to six key words. Also enter the key words where prompted during the submission process.

Main Text

This should begin on a separate page. Sections within the main text should be appropriately sub-headed: Introduction; Materials and methods, Results, and Discussion. Abbreviations and footnotes should be avoided where possible.

References

These should be in the Vancouver style. References should be numbered sequentially as they occur in the text and identified in the main text by arabic numbers in brackets after the punctuation. The reference list should be typed on a separate sheet from the main text, and references should be listed numerically. The following are examples of the style. All authors should be listed and journal titles and page ranges should not be abbreviated. If there are more than 3 more, please list the first 3 authors, followed by *et al*.

1. Bagley LH, Lavach JD. Comparison of postoperative phacoemulsification results in dogs with and without diabetes mellitus: 153 cases (1991-1992). *Journal of the American Veterinary Medical Association* 1994; 205: 1165-1169.
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.

Please note that work that has not been accepted for publication and personal communications should not appear in the reference list, but may be referred to in the text (e.g. M. van der Burgh, personal communication). Also, it is the authors' responsibility to obtain permission from colleagues to include their work as a personal communication.

Electronic Artwork

Figures must be uploaded as separate files and not be embedded in the main text file. Please save vector graphics (e.g. line artwork) in Encapsulated Postscript Format (EPS), and bitmap files (e.g. half-tones) in Tagged Image File format (TIFF). Detailed information on our digital illustration standards is available on the Wiley Homepage at:http://authorservices.wiley.com/bauthor/illustration.asp.

The figures should be referred to as 'Fig.' and numbered consecutively in the order in which they are referred to in the text. Captions to figures, giving the appropriate figure number, should be typed on a separate page at the end of the manuscript; captions should not be written on the original drawing or photograph. In the fulltext online edition of the journal, figure legends may be truncated in abbreviated links to the full screen version. Therefore, the first 100 characters of any legend should inform the reader of key aspects of the figure. Further guidelines regarding the submission of artwork can be found at http://authorservices.wiley.com/bauthor/illustration.asp

Video Files

The journal will consider up to 2 video files to accompany articles. For video files to be accepted, they must clearly show a dynamic condition that can not be adequately captured in still images. The Editor and/or Associate Editors will scrutinize all video submissions very carefully to assure they meet the intent of providing unique information. Video files of routine imaging findings will not be accepted. Up to 2 video files will be considered for each paper. Video files must be submitted in Quicktime format and each file must be less than 5MB in size. The video files will accompany the online version of the manuscript only; reference to the video file should be made in the print version of the paper.

Tables

Clear tables which contain essential data are welcome. Format tables with the table function in a word processor, such as MS Word, on a separate page with the legend typed above. Column headings should be brief, with units of measurement in parentheses. All abbreviations must be defined in footnotes to the table. Number tables consecutively in the order they occur in the text, with Arabic numerals.

Acknowledgements

Acknowledgements should be brief and must include reference to sources of financial and logistical support. Author(s) should clear the copyright of material they wish to reproduce from other sources and this should be acknowledged.

Author Editing Services

Authors for whom English is a second language may choose to have their manuscript

professionally edited before submission or during the review process. Authors wishing to pursue a professional English-language editing service should make contact and arrange payment with the editing service of their choice. For more details regarding the recommended services, please refer to http://authorservices.wiley.com/bauthor/english_language.asp . Japanese authors can also find a list of local English improvement services at http://www.wiley.co.jp/journals/editcontribute.html. All services are paid for and arranged by the author, and use of one of these services does not guarantee acceptance or preference for publication.

Peer Review

All articles submitted for consideration as original clinical and investigational papers or review articles will be peer reviewed by at least two independent referees, one of which is an editorial board member, and a statistician, if appropriate. We aim to give authors a decision (rejection, rejection with encouragement to rework and resubmit, or acceptance subject to revision/copy editing) within three months of manuscript submission.

Page Proofs and Offprints

Proofs will be sent via e-mail as an Acrobat PDF (portable document format) file. The e-mail server must be able to accept attachments up to 4 MB in size. Acrobat Reader will be required in order to read this file. This software can be downloaded (free of charge) from the following Web site:

http://www.adobe.com/products/acrobat/readstep2.html

This will enable the file to be opened, read on screen, and printed out in order for any corrections to be added. Further instructions will be sent with the proof. Proofs will be posted if no e-mail address is available; in your absence, please arrange for a colleague to access your e-mail to retrieve the proofs.

Page proofs must be returned to Wiley Periodicals within 3 days of receipt, by fax if international or convenient, and by express mail: only typographical errors can be corrected at this stage.

Authors will be provided with electronic offprints of their paper. Electronic offprints are sent to the first author at his or her first email address on the title page of the paper, unless advised otherwise. Consequently, please ensure that the name, address and email of the receiving author are clearly indicated on the manuscript title page if he or she is not the first author of the paper. Paper offprints may be purchased using the order form supplied with proofs.

Further Information

If you wish to discuss prospective submissions or to clarify the guidance outlined above, please contact Dr. David A Wilkie at the editorial office (Tel: 1-614-292-8664; Fax 1-614-292-7667; email:wilkie.1@osu.edu).

Further details about the peer review process and arrangements for the final submission of accepted articles and proofs will be sent to authors of accepted manuscripts and are available from the editorial office.

Author Services

Veterinary Ophthalmology currently offers article tracking for authors. This is a reminder to our authors to enroll in Wiley-Blackwell's Author Services production tracking service
You need to register in order to add your article to the article tracking system and be able to track your article online. As well as tracking the production of your article online, as a registered author you can also:

- choose to receive e-mail alerts on article status
- get free access to your article when it is published online (both HTML and PDF versions)
- Authors, Editors and Contributors can receive a 25% discount on all Wiley books (including Wiley-Blackwell titles previously published by Blackwell Publishing)
- nominate up to 10 colleagues to be notified upon publication and also receive free access
- invite your co-authors to also track the article production
- keep a list of favorite journals with quick links to Author Guidelines and submission information

You can also register and choose not to receive e-mails, but simply check progress online at your own convenience.

NIH Policy
Wiley-Blackwell supports authors by posting the accepted version of articles by NIH grant-holders to PubMed Central. The accepted version is the version that incorporates all amendments made during peer review, but prior to the publisher's copyediting and typesetting. This accepted version will be made publicly available 12 months after publication in the journal. The NIH mandate applies to all articles based on research that has been wholly or partially funded by the NIH and that are accepted for publication on or after April 7, 2008. For more information about the NIH's Public Access Policy, visit http://publicaccess.nih.gov

OnlineOpen
Available to authors of primary research articles who wish to make their article available to non-subscribers on publication, or whose funding agency requires grantees to archive the final version of their article. With OnlineOpen, the author, the author's funding agency, or the author's institution pays a fee to ensure that the article is made available to non-subscribers upon publication via Wiley Online Open, as well as deposited in the funding agency's preferred archive.

Prior to acceptance there is no requirement to inform an Editorial Office that you intend to publish your paper OnlineOpen if you do not wish to. All OnlineOpen articles are treated in the same way as any other article. They go through the journal's standard peer-review process and will be accepted or rejected based on their own merit.

For the full list of terms and conditions, seehttp://wileyonlinelibrary.com/onlineopen#OnlineOpen_Terms
Any authors wishing to send their paper OnlineOpen will be required to complete the payment form available from our website at: https://authorservices.wiley.com/bauthor/onlineopen_order.asp