UNIVERSIDADE ESTADUAL PAULISTA - UNESP

CÂMPUS DE JABOTICABAL

ORGANIZAÇÃO ESTRUTURAL DA CROMATINA EM CÉLULAS EPITELIAIS DA CONJUNTIVA PALPEBRAL DE CÃES COM CERATOCONJUNTIVITE SECA, ANTES E APÓS TRATAMENTO LOCAL COM CICLOSPORINA A

Daniela Moura dos Santos

Médica Veterinária

2018

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Dissertação apresentada à Faculdade de Ciências Agrárias e Veterinárias – Unesp, Câmpus de Jaboticabal, como parte das exigências para a obtenção do título de Mestre em Cirurgia Veterinária.

2018

S237o

Santos, Daniela Moura dos
Organização estrutural da cromatina em células epiteliais da conjuntiva palpebral de cães com ceratoconjuntivite seca, antes e após tratamento local com ciclosporina A / Daniela Moura dos Santos. – – Jaboticabal, 2018
x, 84 p. : il. ; 29 cm

Dissertação (mestrado) - Universidade Estadual Paulista, Faculdade de Ciências Agrárias e Veterinárias, 2018
Orientador: José Luiz Laus
Banca examinadora: Cristiane dos Santos Honsho, Ivan Ricardo Martinez Pádua
Bibliografia

1. Citogenética. 2. Epigenética. 3. Imunomodulador. 4. Oftalmologia. 5. Olho seco. 6. Superfície ocular. I. Título. II. Jaboticabal-Faculdade de Ciências Agrárias e Veterinárias.

CDU 619:617:636.7

UNIVERSIDADE ESTADUAL PAULISTA

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CERTIFICADO DE APROVAÇÃO

TÍTULO DA DISSERTAÇÃO: ORGANIZAÇÃO ESTRUTURAL DA CROMATINA EM CÉLULAS EPITELIAIS DA CONJUNTIVA PALPEBRAL DE CÃES COM CERATOCONJUNTIVITE SECA, ANTES E APÓS TRATAMENTO LOCAL COM CICLOSPORINA A

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Jaboticabal, 28 de fevereiro de 2018

DADOS CURRICULARES DA AUTORA

Daniela Moura dos Santos - nascida em 02 de novembro de 1988, na cidade de Porto Alegre, Rio Grande do Sul. Graduou-se em Medicina Veterinária, pela Universidade Federal do Rio Grande do Sul, em dezembro de 2013. Atuou como residente MEC em Clínica Cirúrgica de Pequenos Animais na mesma instituição. Em 2016 ingressou como aluna regular do Programa de Pós-graduação em Cirurgia Veterinária, nível de mestrado, na Faculdade de Ciências Agrárias e Veterinárias (FCAV) - UNESP - Campus de Jaboticabal, sob orientação do Prof. Dr. José Luiz Laus.

AGRADECIMENTOS

Agradeço a Deus que possibilitou que todos os eventos, bons ou ruins, que ocorreram na minha vida, culminassem na realização deste sonho.

Ao professor José Luiz Laus, a quem devo a oportunidade de fazer parte de um grupo de atendimento de excelência e pelo qual tenho enorme admiração e respeito.

A Marcela Aldrovani, meus sinceros agradecimentos por todo apoio durante esses dois anos, pelas explicações pacienciosas, pela dedicação, pelo incentivo nas horas difíceis, por me fazer capaz.

Agradeço a Karina Kobashigawa, Alexandre Sobrinho, Ivan Martinez, Tiago Barbalho, Roberta Crivelaro, Roberta Renzo e Camila Balthazar (minha dupla), por terem me recebido tão bem em 2013 e, por conseguinte, me incitarem o sonho de retornar e poder fazer parte dessa equipe tão competente! As colegas que conheci depois, Thais Morato, Gabriela Madruga, Karina Herencia e Ana Pascoli, obrigada pela amizade e por também me receberem tão bem. Vocês todos me ensinam e me alegram os dias, nutrindo em mim esse sentimento de estar no lugar certo e fazendo a coisa certa para que, um dia, seja uma profissional tão boa quanto vocês.

A todos os colegas da oftalmologia da UFRGS, Flor Chacaltana, Fabiana Quartiero, Paula Hunning e ao professor João Antonio Tadeu Pigatto, que me guiaram nos primeiros passos dessa especialidade.

Aos colegas de residência, os quais ainda me ajudam, cada um na sua área, nos casos que atendo e que me alegram com sua amizade e parceria.

A Marcella Filezio, com quem pude contar para realização deste projeto, com quem passei horas tentando fazer o que parecia impossível, e que me conquistou durante esse tempo, com seu jeito especial de ser, sua competência e carisma.

As minhas “roommates”, Camila Baptista, Daniella Armani e Letícia Luis (agregada da casa e da vida), com quem aprendi que república é muito mais do que uma casa cheia, é dividir espaço, memórias, responsabilidades, alegrias e tristezas. É ter com quem contar no final de um dia difícil. Obrigada pelos momentos maravilhosos que tivemos, pela compreensão nas fases dificeis dos prazos apertados e por todas as coisas que perdi ou esqueci. Obrigada por terem

aprendido, assim como eu aprendi, a respeitar as diferenças, julgar menos, conviver e amar.

A todos os meus amigos de Jaboticabal, em especial a Stephanie Theodoro e o Gilmar Sousa pois tiveram papel fundamental no início dessa história, me hospedando para prestar a prova e também me apoiando em cada etapa, cada dificuldade, cada um do seu jeito e no seu tempo, mas a minha gratidão é constante. As “repúblicas-segundas-casas” e amigos que fiz durante esses anos e que tanto me ajudaram a me sentir em casa mesmo tão longe, que me mostraram que para ser família não é preciso ter o mesmo sangue.

A Maíra Pacheco, por me acompanhar desde a graduação e dividir comigo a paixão pela oftalmologia, histórias, aulas, planos, casos clínicos, enfim, por ser a amiga-irmã que independente da distância se faz sempre presente e com quem eu sei que posso sempre contar.

Aos meus familiares e amigos de Porto Alegre dos quais eu sempre sinto saudades imensas.

Aos professores, funcionários, residentes, pós-graduandos e estagiários do Hospital Veterinário da FCAV, pela ajuda, aprendizado e companhia no dia-a-dia.

Aos tutores e aos cães que participaram da pesquisa, bem como ao canil da Nutrição da Unesp e ao canil Big Bones de Jaboticabal.

A CAPES pela bolsa concedida e a MSD por doar as pomadas utilizadas no estudo.

Por fim e sempre mais importante, não só agradeço mas dedico este trabalho aos meus pais, cujo amor e apoio sempre foram incondicionais. É por eles que busco sempre ser alguém melhor, que possa ser motivo de orgulho e que mereça tanta dedicação e carinho investidos.

Este trabalho foi realizado no Laboratório de Eletrofisiologia Ocular do Serviço de Oftalmologia, Hospital Governador Laudo Natel e Departamento de Clínica e Cirurgia Veterinária, Faculdade de Ciências Agrárias e Veterinárias (FCAV/Unesp), Câmpus de Jaboticabal, com auxílio financeiro da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), do Conselho Nacional de Pesquisa e Desenvolvimento Científico e Tecnológico (CNPq, processo de número 467289/2014-0), da Fundação de Amparo à Pesquisa do Estado de São Paulo (Fapesp, processos de número 2012/17308-5 e 2013/01494-7), e da Pró-Reitoria de Pesquisa da Unesp.

SUMÁRIO

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CERTIFICADO DE APROVAÇÃO DO PROTOCOLO DA PESQUISA PELA COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA)

unesp UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de Jaboticabal

CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

C E R T I F I C A D O

Certificamos que o Projeto intitulado **"Avaliação epigenética da conjuntiva de cães braquicefálicos com ceratoconjuntivite seca (CCS)",** protocolo nº 6.329/16**,** sob a responsabilidade do Prof. Dr. José Luiz Laus, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de junho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 04 de maio de 2016.

Vigência do Projeto	16/05/2016 a 01/08/2017
Espécie / Linhagem	Canina – *Canis familiaris*
Nº de animais	40
Peso / Idade	Aproximadamente 6 kg / acima de 1 ano
Sexo	Machos e fêmeas
Origem	Criadores / Casuística do Serviço de Oftalmologia Veterinária

Jaboticabal, 04 de maio de 2016.

Profª Drª Lizandra Amoroso
Coordenadora – CEUA

Faculdade de Ciências Agrárias e Veterinárias
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LISTA DE ABREVIATURAS

AAR	acrônimo de “average absorption ratio”
AC	compacted chromatin
AgNOR	região organizadora nucleolar responsiva a íons prata
ARVO	“Association for Research in Vision and Ophthalmology”
AT	“total absorbance”
ATRX	helicase dependente do ATP
CCS	ceratoconjuntivite seca
CsA	ciclosporina A
CDKN2	gene inibidor 2A das quinases dependentes de cliclina
COBEA	**“**Brazilian College of Animal Experimentation”
EGF	fator de crescimento epidérmico
FL	filme lacrimal
GATA5	gene codificador do fator de transcrição que se liga a sequência [A/T]GATA [A/G] do DNA
GSTP1	gene glutationa S-transferase P1
IL	Interleucina
IOD	densidade óptica integrada nuclear
HCl	ácido clorídrico
HDACs	deacetilases de histonas
HP1	proteína heterocormatina 1
H3K4	lisina 4 da histona H3
H3H3K9	metilação da lisina 9 da histona H3
H3K9me	metil na lisina 9 da histona H3
H3K4me3	trimetil na lisina 4 da histona H3
H3K27me3	trimetil na lisina 27 da histona H3
H4K20me3	trimetil na lisina 20 da histona H4
MeCP2	proteína 2 de ligação ao metil-CpG
MGMT	O-6-metil-guanina-DNA-metiltransferase
MN	Membrana nictitante
MSH6	gene homólogo humano de 6 MutS
NORs	“Nucleolar Organizing Regions”
OD	densidade óptica
PAX5	gene homeodomínio box pareado
R^2	regressão linear quadrática
RB1	gene codificador da proteína do retinoblastoma 1
Sc%	fração de área nuclear
ST	“total area”
STT	“Schirmer Tear Test”
TBUT	“Tear Breakup Time”
TLS	teste lacrimal de Schirmer
TNF	fator de necrose tumoral
TP53	gene supressor de tumor
TRFL	tempo de ruptura do filme lacrimal
UA	unidades arbitrárias
VHL	gene supressor tumoral von Hippel-Lindau

ORGANIZAÇÃO ESTRUTURAL DA CROMATINA EM CÉLULAS EPITELIAIS DA CONJUNTIVA PALPEBRAL DE CÃES COM CERATOCONJUNTIVITE SECA, ANTES E APÓS TRATAMENTO LOCAL COM CICLOSPORINA A

RESUMO – Alterações na organização estrutural da cromatina estão sendo associadas ao desenvolvimento e à fisiopatologia de diversas afecções oftálmicas. Com o advento da epigenética, emergiu o conceito de que parte dessas alterações pode ser revertida por fármacos. Visando-se avaliar a organização estrutural da cromatina em células da conjuntiva palpebral de cães com ceratoconjuntivite seca (CCS), antes e após tratamento com pomada de ciclosporina A (CsA) 0,2%, a presente pesquisa foi dividida em duas fases. Na fase I estudaram-se núcleos de células epiteliais e de linfócitos colhidos da conjuntiva palpebral de cães com e sem CCS. Foram incluídos na pesquisa 64 olhos de 32 cães domésticos, distribuídos em dois grupos: grupo controle, composto por 16 cães saudáveis (32 olhos, valores de Schirmer ≥ 15 mm/min); grupo CCS, abrangendo 16 cães (32 olhos, valores de Schirmer ≤ 10 mm/min) com CCS bilateral nunca antes tratados com imunomoduladores, sem oftalmopatias concorrentes e livres de alterações sistêmicas. As células foram colhidas por citologia esfoliativa e coradas pela reação de Feulgen. Os seguintes parâmetros foram estudados por vídeo-análise de imagens: área nuclear, perímetro nuclear, fator de circularidade relativa do núcleo (RNRF), densidade óptica integrada (IOD = conteúdo de DNA), densidade óptica (OD = compactação de cromatina); e desvio padrão de valores densitométricos (SDtd = textura de cromatina). Os resultados mostraram que a CCS enseja alterações nos parâmetros nucleares das células epiteliais e dos linfócitos da conjuntiva palpebral. Comparativamente aos controles, as células epiteliais foram mais afetadas pela CCS (alterações em área, perímetro, RNRF, IOD, e OD) do que os linfócitos (alterações em OD, apenas). Tanto as células epiteliais quanto os linfócitos do grupo CCS apresentaram cromatina mais descompactada do que as células do grupo controle. Padrões aberrantes de cromatina, como a “snake-like-chromatin, comumente vistos em pacientes humanos, não foram detectados. Na fase II estudou-se se as alterações nucleares detectadas na fase I regrediriam após tratamento local com pomada de CsA 0,2% a intervalos regulares de 12 horas e lacrimomimético a base de ácido poliacrílico 0,2%, instilado localmente a cada 4 horas. O mesmo grupo controle composto por cães hígidos foi adotado e as células também foram colhidas por citologia esfoliativa aos 30 e 60 dias de tratamento. As preparações citológicas foram coradas pela reação de Feulgen ou submetidas ao bandamento AgNOR. Estudaram-se parâmetros de video-análise de imagens relacionados com a funcionalidade da cromatina, notadamente a fração de área nuclear coberta pela cromatina mais condensada (Sc%), a taxa média de absorbância (AAR), e a entropia. Após bandamento AgNOR, estudaram-se os tamanhos das regiões organizadores de nucléolo e as frações de áreas nucleares ocupadas por elas. Os resultados mostraram que o tratamento com CsA e lacrimomimético (30/60 dias) enseja remodelação cromatínica e desfaz parcialmente as alterações associadas à CCS.

Palavras-chave: citogenética, epigenética, imunomodulador, oftalmologia, olho seco, superfície ocular.

CHROMATIN STRUCTURE ORGANIZATION IN PALPEBRAL CONJUNCTIVAL EPITHELIAL CELLS FROM DOGS WITH SICCA KERATOCONJUNCTIVITIS, BEFORE AND AFTER LOCAL TREATMENT WITH CYCLOSPORINE A

ABSTRACT – Changes in the structural organization of chromatin are being associated with the development and pathophysiology of various ophthalmic conditions. With the advent of epigenetics, the concept emerged that part of these alterations can be reversed by drugs. To evaluate the structural organization of chromatin in palpebral conjunctival cells of dogs with keratoconjunctivitis sicca (KCS), before and after treatment with 0.2% cyclosporine A (CsA) ophthalmic ointment, the present research was divided into two phases. In stage I, epithelial cells and lymphocyte from the palpebral conjunctiva of dogs with and without KCS were studied. The study included 64 eyes of 32 domestic dogs, distributed into two groups: control group, composed of 16 healthy dogs (32 eyes, Schirmer values ≥ 15 mm / min); KCS group, formed of 16 dogs (32 eyes, Schirmer values ≤ 10 mm / min) with bilateral CCS never previously treated with immunomodulatory drugs, and without concurrent ophthalmic or systemic disorders. Cells were collected by brush cytology and stained by the Feulgen reaction. The following parameters were studied by video image analysis: nuclear area, nuclear perimeter, relative nuclear roundness factor (RNRF), integrated optical density (IOD = DNA content), optical density (OD = chromatin compaction). The results showed that KCS causes alterations in the nuclear parameters of the epithelial cells and the lymphocytes from the palpebral conjunctiva. Compared with the control, the epithelial cells were more affected by the disease (alterations in area, perimeter, RNRF, IOD, and OD) than lymphocytes (changes in OD only.) Both epithelial cells and lymphocytes from the KCS group showed more decompressed chromatin than cells from the control group. "Snake-like-chromatin commonly seen in human patients was not detected. In phase II it was studied whether the nuclear detected in stage I, regress after treatment with 0.2% CsA at regular intervals of 12 h and 0.2% polyacrylic acid-based artificial tears at regular intervals of 4h. The same control group composed of healthy dogs was adopted and cells were collected by brush cytology after 30 and 60 days of treatment. Cytological preparations were stained by the Feulgen reaction or submitted to the AgNOR banding. We studied video image analysis parameters related to the functionality of chromatin, notably the fraction of nuclear area covered by more condensed chromatin (Sc %), the average absorption ratio (AAR), and entropy. After AgNOR banding, the samples were studied for the sizes of the nucleolar organizer regions and the fractions of nuclear areas occupied by them. Results show that treatment with CsA and lacrimomimetic (30/60 days) leads to chromatin remodeling and partially reverses the chromatin changes elicited by KCS.

Key-words: cytogenetics, dry eye, epigenetic, immunomudulator, ocular surface, ophthalmology.

CAPÍTULO 1 – Considerações Gerais

1. INTRODUÇÃO

A ceratoconjuntivite seca (CCS) é uma desordem multifatorial, caracterizada por alterações quali-quantitativas na produção lacrimal, que compromete a homeostasia da superfície ocular e enseja intensa inflamação conjuntival. Em cães, na maioria dos casos (80%), trata-se de doença imunomediada cujos sinais clínicos variam em função do tempo de estabelecimento da enfermidade. O diagnóstico fundamenta-se nos sinais clínicos e em valores do teste lacrimal de Schirmer (TLS) $\leq$ 10 mm/min.

Estudos recentes sugerem que a fisiopatologia da CCS excede as manifestações macro e microscópicas observadas na superfície conjuntival. Emergem evidências de que ocorrem alterações em expressão gênica e na biossíntese de proteínas. Em pacientes humanos com CCS, as células epiteliais conjuntivais remodelam a cromatina, que, eventualmente, adquire formato de cobra ("snake-like chromatin") facilmente detectável por microscopia de luz comum. Não há relatos sobre a ocorrência de "snake-like chromatin" em cães. As possíveis remodelações cromatínicas e as alterações nucleares que acompanham a CCS canina não são conhecidas; portanto, estudá-las é importante, pois se trata de um dos últimos eventos a antecederem modificações transcricionais. Não raramente, alterações na cromatina estão associadas à gênese ou à progressão de síndromes autoimunes e de doenças oftálmicas. Conhecer a organização estrutural da cromatina pode informar sobre como as células reagem, com adaptação nuclear e metabólica, às alterações microambientais que ocorrem na conjuntiva acometida pela CCS. Ademais, pode direcionar novas estratégias terapêuticas, bem como ampliar entendimentos sobre mecanismos inesperados de ação dos imunomoduladores atualmente utilizados no tratamento da CCS.

A ciclosporina A (CsA), por exemplo, é um imunomodulador, com ação lacrimogênica, amplamente indicado para tratar pacientes com CCS. Ela age sobre

os linfócitos T e interrompe a síntese de interleucinas pró-inflamatórias. Todavia, há evidências de que a CsA também exerce efeitos sobre enzimas epigenéticas e os complexos remodeladores dependentes de ATP que agem sobre a estrutura da cromatina em células não-linfocíticas, modulando-os. Como não se sabe se a CsA induz ou modula mudanças na estrutura da cromatina de células epiteliais conjuntivais, o estudo igualmente contribuiria para uma melhor percepção relativamente aos eventos envolvidos no controle da CCS canina.

2. REVISÃO DE LITERATURA

2.1 Epitélio conjuntival

A superfície ocular compreende os epitélios da córnea, da esclera, do limbo e da conjuntiva, além do filme lacrimal (FL), cujas integridades são determinantes para a acuidade visual (ROLANDO; ZIERHUT, 2001). O tecido conjuntival é complexo e desempenha funções que auxiliam na homeostasia da superfície ocular (HOLLAND; MANNIS; LEE, 2015). Ele protege os tecidos moles da órbita e da pálpebra, atua ativamente na dinâmica lacrimal, provê células imunes à superfície ocular, e contribui para a movimentação ocular e a cicatrização de lesões corneais (GELATT, 2003; HOLLAND; MANNIS; LEE, 2015).

A conjuntiva é uma estrutura mucocutânea, percebida no exame clínico como uma membrana móvel, localizada na face interna das pálpebras superior e inferior, e na face bulbar e palpebral da membrana nictitante (MN). Ela também permeia a porção anterior do bulbo ocular, nas adjacências do limbo, onde se funde à cápsula de Tenon (JAKOBIEC; IWAMOTO, 1992). O epitélio conjuntival, em olhos saudáveis, é do tipo não queratinizado estratificado e secretor, formado, predominantemente, por seis ou mais camadas de células epiteliais cubóides dispostas ao redor de poucas células caliciformes, por linfócitos, por células de Langherans, e por melanócitos (SRINIVASAN et al., 1977; HOLLAND; MANNIS; LEE, 2015). As células epiteliais conjuntivais da camada apical mantêm permeabilidade seletiva à passagem de moléculas e de íons, e um complexo microvilosidades-glicocálice que facilita a aderência do FL (HOLLAND; MANNIS; LEE, 2015). Os linfócitos, quando

estimulados por antígenos, se reúnem para formarem folículos ativos, principalmente na face bulbar da MN (SLATTER, 2005).

As células caliciformes são do tipo secretoras apócrinas e, sob estímulos parassimpáticos, sintetizam mucinas e outras moléculas, que estabilizam o FL e fornecem uma superfície lisa e refrativa à córnea (GIPSON, 2004). Ademais, a células caliciformes removem “debris” e patógenos da superfície ocular, e contribuem com a imunidade local, pois sintetizam imunoglobulinas, lisozimas, e lactoferrina (PRYDAL et al., 1992; GIPSON; INATOMI, 1997; GIPSON; ARGÜESO, 2003). O conteúdo e a disposição espacial das células caliciformes conjuntivais variam entre as espécies (MOORE et al., 1987; MICALLI et al., 1997; GASSER et al., 2011; VOIGT et al., 2012; SEBBAG, 2016). Em cães, as células caliciformes concentram-se na face palpebral da MN e no fórnice conjuntival medial (MOORE et al., 1987; UMEDA et al., 2010). Em seres humanos, elas ocupam, principalmente, a prega ou dobra semilunar, que é um vestígio remanescente de MN, em formato de meia lua, no fórnice conjuntival medial (KESSING, 1966; ARENDS; SCHRAMM, 2004).

Sob condições patológicas, ocorrem alterações qualitativas e quantitativas, de maior ou menor cronicidade, na população celular total do epitélio conjuntival. Conjuntivites agudas, de etiologia viral ou bacteriana, por exemplo, são caracterizadas por infiltração neutrofílica (HOLLAND; MANNIS; LEE, 2015). Afecções conjuntivais crônicas, notadamente as imunomediadas (com exceções), são caracterizadas pelo aumento na população de linfócitos e pela presença de plasmócitos. Conjuntivites alérgicas ou imunomediadas, como as que acometem os cavalos e os gatos, são caracterizadas pela presença de eosinófilos (SLATTER, 2005; GELATT, 2003). Frente a certas ceratoconjuntivites, a conjuntiva sofre uma transição patológica designada de metaplasia escamosa, na qual o epitélio se torna queratinizado e perde parte de sua atividade secretora pela diminuição da população de células caliciformes (HOLLAND; MANNIS; LEE, 2015).

2.2 Filme lacrimal pré-corneal

O FL é o componente mais externo da córnea, responsável pela manutenção de uma superfície opticamente uniforme, necessária à adequada refração (HOLLAND; MANNIS; LEE, 2015). Ele também é responsável pela remoção de corpos estranhos e pela oxigenação e nutrição da córnea (DILLY, 1994; OHASHI, DOGRU; TSUBOTA, 2006). Por décadas admitiu-se que o FL tinha estrutura trilaminar, ou seja, era formado por três camadas distintas: a mucosa, subjacente ao epitélio corneal, composta principalmente de mucina secretada pelas células caliciformes conjuntivais; a aquosa, intermediária, produzida pelas glândulas lacrimal, da terceira pálpebra, e acessórias; e a lipídica, mais externa, secretada pelas glândulas sebáceas Meibômio e Zeiss (LAMBERT 1994; SAMUELSON 2007). Atualmente surgiram evidências de que as camadas do FL são interdependentes e se organizam em duas fases, a lipídica e a aquosa-mucosa (ROLANDO; ZIERHUT, 2001; JOHNSON; MURPHY, 2004; BUTOVICH, 2013). Há compartilhamento de moléculas entre as camadas do FL, notadamente de algumas mucinas (MUC1, 3, 4, 12, 13, e 16) que formam o glicocálice da superfície epitelial corneal (GIPSON et al., 1992).

Em seres humanos, a espessura média (± desvio padrão) do FL é de 3,4 (± 2,6) µm (LEVIN et al., 2011; WANG et al., 2006). Em animais domésticos pouco se conhece sobre a espessura do FL, porém, é consensual que alterações nesse parâmetro acompanham a gênese e a progressão de muitas enfermidades da superfície ocular.

A avaliação do FL envolve critérios quantitativos e qualitativos. Em veterinária, na avaliação quantitativa da camada aquosa, emprega-se, corriqueiramente, o Teste Lacrimal de Schirmer (TLS) tipo 1 ou 2 (GELLAT et al., 2003). Os valores fisiológicos de TLS 1, em cães saudáveis, variam entre 18,64 ± 4,47 mm/min e 23,90 ± 5,12 mm/min (OLLIVIER; PLUMMER; BARRIE, 2007). Em estudo envolvendo apenas cães de raças braquicefálicas, Lima e colaboradores (2011) observaram que o TLS 1 foi de 19,66 ± 7,30 mm/min no olho direito e de 21,97 ± 5,69 mm/min no olho esquerdo ($P > 0,05$). Na avaliação qualitativa do FL, emprega-se, como parâmetro fiável, o tempo de ruptura do filme lacrimal (TRFL), que informa sobre o tempo de aderência do FL à superfície corneal (OLLIVIER; PLUMMER; BARRIE, 2007). A avaliação do TRFL envolve o corante vital fluoresceína sódica, que deve ser

preferencialmente aplicado na região de conjuntiva bulbar dorsotemporal. Com auxílio de fonte de luz em filtro azul cobalto, o tempo para dissociação da fluoresceína sobre a superfície ocular é monitorado e cronometrado, em segundos (s). O valor fisiológico de TRFL, em cães saudáveis, foi estimado como sendo de 19,70 ± 5,00 s (OLLIVIER; PLUMMER; BARRIE, 2007; ANDRADE, 2008).

Na atualidade, estão sendo conduzidos estudos sobre a osmolaridade e os perfis moleculares do FL canino, visando-se, entre outros, à identificação de proteínas diferenciais, que, futuramente, poderão coadjuvar o diagnóstico e o tratamento de doenças oculares (WINIARCZYK et al., 2015).

2.3 Ceratoconjuntivite seca (CCS)

A CCS, também conhecida como síndrome de disfunção do sistema lacrimal ou doença do olho seco, é uma enfermidade que altera a homeostasia da superfície ocular e modifica o microambiente do epitélio conjuntival, culminando com ressecamento da córnea, desconforto e distúrbios visuais (HOLLAND; MANNIS; LEE, 2015). Trata-se de condição progressiva, que não raramente enseja resposta inflamatória grave secundária à deficiência de componentes do FL (GIULIANO; MOORE, 2007; HERRERA, 2008; GALERA et al., 2009; ANGÉLICO et al., 2011). A CCS pode ser quantitativa, caracterizada por deficiência na produção da camada aquosa do FL, e/ou qualitativa, caracterizada por alterações na camada lipídica do FL ou por evaporação excessiva da lágrima (GELATT, 2003; SLATTER, 2005).

Em cães, as manifestações clínicas que acompanham a CCS são variáveis e incluem blefaroespasmo, secreção mucoide, hiperemia conjuntival, ceratite ulcerativa, vascularização e pigmentação corneal (GUM et al., 2007).

A ocorrência de CCS é alta entre raças braquicefálicas, cuja configuração da face e características biométricas da órbita rasa, favorecem a evaporação da lágrima (SANCHES et al., 2007). O primeiro estudo epidemiológico sobre CCS canina, publicado em 1976, mostrou que a prevalência da doença era 0,4% (HELPER, 1976). Todavia, outro estudo, em 1998, mostrou que a prevalência em cães é de 35% (KASWAN et al., 1998). Estima-se que a incidência de CCS canina seja de 3,3% nos Estados Unidos, de 3,4% no Reino Unido, e de 5,5% no Brasil (BARROS,

1997). As fêmeas são mais acometidas pela doença, provavelmente porque suas glândulas lacrimais possuem parênquimas secretores (acinar e tubular) menores que os dos machos (BARROS, 1992; BARROS, 1997; CABRAL et al., 2005). Cães das raças Cocker Spaniel, Buldogue Inglês, West Highland White Terrier, Yorkshire Terrier, Shih Tzu, Lhasa Apso, Schnauzer, Boston Terrier, Cavalier King Charles Spaniel, Pug e Samoieda apresentam predisposição elevada à doença (SLATTER, 2005).

Dentre os fatores predisponentes de CCS canina congregam-se as anomalias congênitas, os distúrbios nutricionais (ex. hipovitaminose A) e os metabólicos (ex. hipotireoidismo, diabetes, hipoestrogenismo, hipo ou hiperadrenocorticismo), bem como doenças infecciosas (ex. cinomose, toxoplasmose e erliquiose) e tóxicas (ex. botulismo, astrágalo, beladona), certos fármacos (ex. buprenorfina, medetomidina, derivados de sulfas e atropina) e lesões iatrogênicas, idiopáticas, neoplásicas e neurogênicas (ex. síndrome da disautonomia) (MARGADANT et al., 2003; GELATT, 2003; CULLEN et al., 2005; BARNETT, 2006; WILLIAMS; PIECE, 2007). O diagnóstico de CCS baseia-se nos sinais clínicos, na presença de defeitos persistentes e de filamentos de muco na superfície ocular, e na quantidade e qualidade da lágrima avaliada ao TLS e o TRFL, respectivamente (BASTTISTELLA; KARA-JOSÉ, 1999; ANGÉLICO et al., 2011). Valores de TLS 1 inferiores a 10 mm/min são adotados para confirmação da enfermidade. Contudo, valores de TLS 1 entre 10 e 15 mm/min podem indicar CCS subclínica (BARNETT; SANSON, 1987; GIULIANO; MOORE, 2007).

Relativamente à etiologia e à fisiopatologia da CCS canina, achados à histopatologia sugerem, na maioria dos casos (80%), tratar-se de doença imunomediada caracterizada por inflamação exacerbada, com expressão elevada de linfócitos T “helper” e destruição de glândulas lacrimais (Fig. 1) (KASWAN; BOUNOUS; HIRSH, 1995; RORIG, 2009). A CCS canina imunomediada assemelha-se em muitos aspectos à CCS humana, que acomete, principalmente, mulheres na menopausa (WILLIAMS, 2008). Nos seres humanos, a CCS, geralmente, está associação à xerostomia (boca seca) e à artrite reumatoide, e decorre de uma síndrome autoimune grave, conhecida como Sjögren (WILLIAMS, 2008).

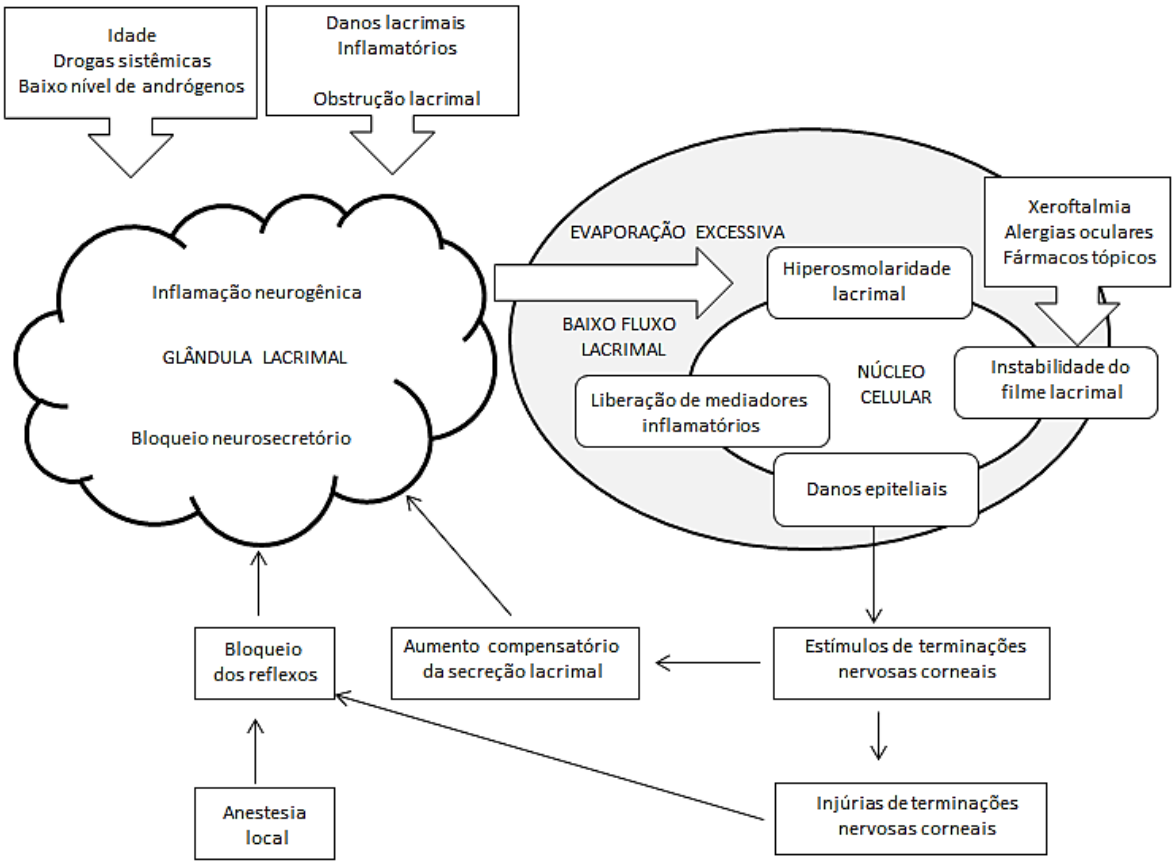

Figura 1. Mecanismo pelo qual a CCS estabelece um mecanismo de auto-destruição da glândula lacrimal, causando inflamação e danos à superfície ocular (RORIG, 2009, adaptado de DEWS, 2007).

As glândulas lacrimal e superficial da terceira pálpebra, em cães com CCS, desenvolvem graus variáveis de adenite crônica multifocal, caracterizada por atrofia dos ácinos, por infiltração plasmocitária e linfocítica, e por aumento na densidade de volume do tecido conjuntivo fibroso interacinar (SLATTER, 2005). A infiltração linfocítica que acomete as glândulas lacrimais das fêmeas é mais intensa (CABRAL et al., 2005). Relativamente à superfície conjuntival, um estudo envolvendo cães com cinomose e com CCS clínica detectou níveis elevados de citocinas pró-inflamatórias, notadamente de interleucinas (IL-1 e IL-6) e de fator de necrose tumoral alpha (TNF-alpha) (ALMEIDA, 2006). Stern e colaboradores (1998) propuseram que a inflamação, caracterizada por elevação nos níveis locais de citocinas pró-inflamatórias, é característica comum a todos os tipos de CCS. As citocinas pró-inflamatórias atuam nas imunidades inata e adaptativa, e favorecem o influxo de células inflamatórias (ALMEIDA, 2006).

Avanços recentes nos campos da biologia celular, da genética e da proteômica sugerem que a fisiopatologia da CCS excede as manifestações macro e microscópicas observadas na superfície conjuntival. Foi demonstrado que a lágrima de pacientes humanos com CCS, associada ou não à síndrome de Sjögren, apresenta menos lactoferrina e mais fator de crescimento epidérmico (EGF) do que a lágrima de indivíduos saudáveis (OHASHI et al., 2013). Há, também, evidências de que pacientes com CCS apresentam alterações na expressão dos genes que transcrevem RNAm para glicosiltransferases e glicoconjugados específicos da superfície epitelial conjuntival (MANTELLI et al., 2009). A análise proteômica da conjuntiva de pacientes com CCS mostrou que ocorre aumento na produção de anexina A-1, de anexina A-2, de alpha-enolase, de S100A8, de citoqueratina-1, de peroxirredoxina-2 e de inibidores da elastase leucocitária, concomitante à redução na síntese de galectina-3 e de lipocalina-1 (SORIA et al., 2017). Ademais, Narayanan e colaboradores (2003) observaram que o epitélio conjuntival com CCS expressa beta-defensinas, peptídeos antimicrobianos que participam da resposta imune inata e que podem afetar uma variedade de processos biológicos, tais como a proliferação celular, a produção de citocinas, a quimiotaxia, e a liberação mastocitária de histamina.

Mais recentemente, foi demonstrado que as células do epitélio conjuntival de pacientes com CCS possuem expressão gênica diferenciada (BRADLEY; EDWARDS; FULLARD, 2014). Também em pacientes humanos com CCS, as células epiteliais das conjuntivas palpebral e bulbar eventualmente podem apresentar-se com núcleos atípicos, caracterizados pela presença de cromatina aberrante, em formato de cobra (i.e., “snake-like chromatin”) (MARNER, 1980). Micronúcleos, bem como a fragmentação internucleossomal de DNA, que é característica de apoptose ou de morte celular programada, foram reportados em olhos humanos (DEWS, 2007). Não há, até o presente, relatos sobre a presença de “snake-like chromatin”, de micronúcleos, ou de outras aberrações cromatínicas, em núcleos interfásicos de células epiteliais conjuntivais de cães com CCS.

2.4 Cromatina

O DNA nuclear dos eucariotos está complexado com proteínas histônicas e não histônicas, formando a cromatina. A unidade fundamental (estrutura primária) da cromatina é representada pelos nucleossomos, que se organizam em cadeia para formar a fibra de 11 nm, conhecida como “colar-de-contas” (Fig. 2) (WOODCOCK; DIMITROV, 2001).

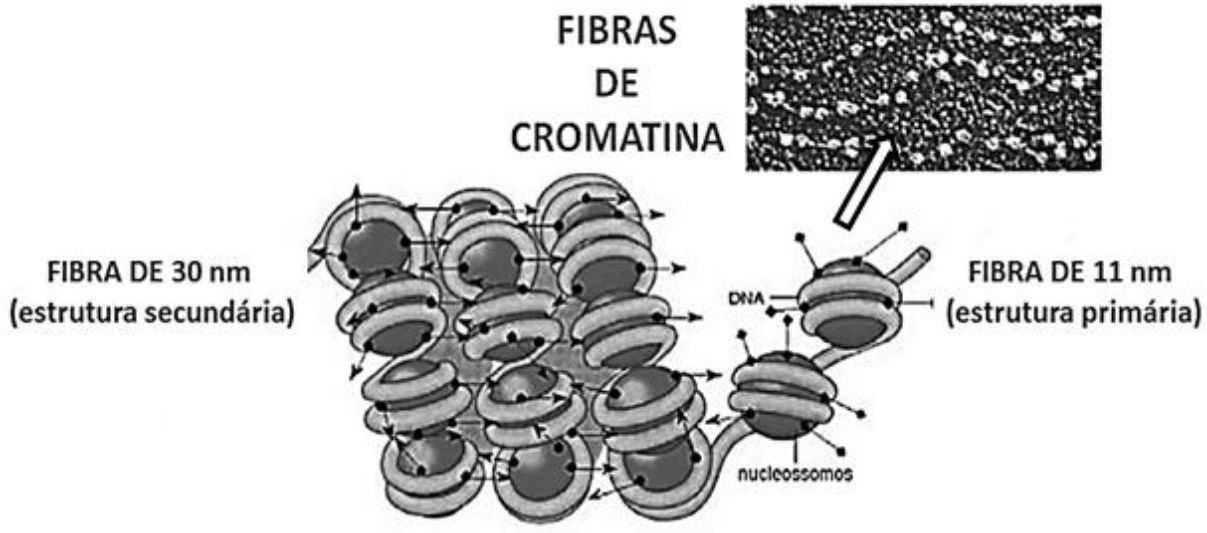

Figura 2. Estrutura básica da cromatina. A fibra de 11 nm consiste de DNA enrolado em octâmeros de histonas (nucleossomos) dispostos a intervalos regulares. O empacotamento dos nucleossomos cria uma fibra espiral, de 30 nm, que corresponde à estrutura secundária da cromatina (adaptado de http://sgi.bls.umkc.edu/waterborg/chromat/chroma09.html).

O nucleoide nucleossomal é formado por 147 pares de bases de DNA dispostos ao redor de um octâmero de histonas contendo duas unidades de H2A, de H2B, de H3 e de H4 (ALBERTS et al., 2008). Durante a expressão gênica, o DNA do nucleoide se solta, por milissegundos, do octâmero de histonas e interage com proteínas da forquilha de replicação e da maquinaria de transcrição (ALBERTS et al., 2008). A união dos nucleoides é feita por um DNA de ligação, cujo tamanho é variável (LUGER et al., 1997). Uma nona histona, H1, promove a compactação dos nucleoides, originando uma fibra de 30nm (i.e., estrutura secundária da cromatina) (BEDNAR et al., 1998; DORIGO et al., 2004; ALBERTS et al.,2008). Níveis superiores (ou supra) de organização cromatínica formam-se a partir de interações entre as fibras de 30 nm (CREMER; CREMER, 2001).

A cromatina supra-organizada a partir da fibra de 30 nm não é homogênea (estrutural e funcionalmente). Desde os estudos pioneiros de Heitz (1928; 1934), admite-se que ela pode se apresentar sob dois estados morfofuncionais de estereoarranjo, os quais são dinâmicos e coexistem, em diferentes proporções, nos núcleos celulares: a eucromatina e a heterocromatina.

A eucromatina é rica em genes. Considera-se que ela é descondensada e transcricionalmente ativa, uma vez que apresenta espaçamento irregular entre seus nucleossomos (ELGIN; GREWAL, 2003). A heterocromatina é altamente condensada e consiste em arranjos nucleossomais regulares, contendo elevada proporção de sequências repetitivas transcricionalmente inativas, intercaladas por poucos genes, o que impede que nucleases acessem o DNA (GREWAL; MOAZED, 2003). A característica principal da heterocromatina é a capacidade de se propagar, reprimindo a expressão gênica ao longo de um domínio (fenômeno conhecido como efeito de posição). Todavia, durante alguns processos, como a diferenciação de alguns tipos celulares, a heterocromatina é requerida no controle da expressão gênica (LU et al., 2000; YASUHARA; WAKIMOTO, 2006; FINNEGAN, 2011). Dentre muitas funções, a heterocromatina protege a integridade do genoma, inibe a recombinação de elementos dispersivos do DNA repetitivo, facilita a coesão de cromátides irmãs e direciona a segregação de cromossomos (HENIKOFF, 2000; BERNARDI et al., 2000; HALL et al., 2003).

Os estereoarranjos de cromatina são mutáveis graças aos processos dinâmicos de remodelação, que envolvem complexos enzimáticos dependentes de ATP e enzimas que agem sobre o DNA e as caudas das histonas. Ao remodelar, a cromatina pode acumular alterações que contribuem para o desenvolvimento e a progressão de doenças, a depender de uma série de eventos inseridos no recente campo da epigenética.

2.5 Remodelação cromatínica

Organismos eucariotos possuem uma variedade de complexos de remodelação cromatínica, que utilizam energia liberada pela hidrólise de ATP para quebrar o nucleossomo e permitir que regiões gênicas promotoras se liguem aos

fatores transcricionais. O complexo remodelador mais estudado é o SWI/SNF, que ao ser recrutado por fatores transcricionais promove deslizamento nucleossomal e expõe regiões do domínio TATA, onde o complexo de pré-iniciação da transcrição pode se ligar (NASIPAK et al., 2015).

Em muitos casos, os eventos responsáveis pela remodelação cromatínica são epigenéticos e mediados por enzimas. Destacam-se a metilação de DNA, as modificações em histonas (acetilação, metilação, fosforilação, ubiquitinação, sumoilação, ADP ribosilação, citrulinação, propionilação, glicosilação e n-nitrosação) e os RNAs de interferência (ALLIS et al., 2007; GOLDBERG et al., 2007; BURGESS; ZHANG, 2010; ZHANG et al., 2012).

O termo epigenética foi originalmente atribuído a eventos biológicos não explicáveis por princípios genéticos. Conrad Waddington (1942), a quem foi dado crédito de tê-lo cunhado, definiu epigenética como "o ramo da biologia que estuda as interações causais entre os genes e seus produtos, os quais trazem o fenótipo para o ser". Representa uma ciência de vanguarda e refere-se às marcações herdáveis (por exemplo, heterocromatização) na expressão gênica (i.e., assinaturas epigenéticas), sem mudanças na sequência primária do DNA.

Cada linhagem celular contém assinaturas epigenéticas específicas, que representam o epigenoma e ajudam a estabelecer os estados funcionais da cromatina. O epigenoma é lábil e diversos estímulos podem modificá-lo, o que, às vezes, ocasiona danos à célula, suscitando o desenvolvimento de doenças e de síndromes (EGGER et al., 2004).

Modificações no epigenoma e na supra-organização da cromatina podem ser iniciadas por estímulos internos, que envolvem alterações em genes mantenedores das assinaturas epigenéticas. Em seres humanos, por exemplo, mutações no gene *DNMT3b* (DNA-citosina-5-metiltransferase-3-beta) ensejam hipometilação de DNA, com descompactação e afrouxamento de cromatina, e estão relacionadas à gênese da síndrome de imunodeficiência - instabilidade centromérica - anomalias faciais (síndrome ICF) (MATARAZZO et al., 2008) e da neoplasia escamosa da superfície ocular (PRASAD et al., 2009). Mutações no gene *ATRX* (helicase dependente do ATP) alteram os níveis de metilação do DNA ribossômico em regiões repetitivas e

subteloméricas do cromossomo Y, causando alfa-talassemia (síndrome ATR-X, caracterizada por retardo mental) (DELAUNOY et al., 2006). No diabetes tipo I, ocorre redução nos níveis de expressão de histonas deacetilases (HDACs) e aumento na dimetilação da lisina 4 da histona H3 (H3K4) dos linfócitos do pâncreas, o que pode estar relacionado com alterações na imunidade e com a destruição das células beta (COOPER; EL-OSTA, 2010).

Estímulos microambientais podem também atuar como efetores de alterações na cromatina e no epigenoma celular, como a exposição aos metais pesados, aos pesticidas, ao tabaco, aos hidrocarbonetos policíclicos aromáticos e a certos fármacos, bem como mudanças hormonais, radiação, infecções virais e bacterianas, privação de nutrientes, restrição hídrica e inflamação, entre outros (SZYF; McGOWAN; MEANEY, 2007; DELAGE; DASHWOOD, 2008). Células de tumores cujas causas envolvem fatores microambientais, por exemplo, sofrem remodelação cromatínica mediada pela perda global das assinaturas epigenéticas trimetil na lisina 4 da histona H3 (H3K4me3) e na lisina 20 da histona H4 (H4K20me3), concomitante ao ganho das assinaturas metil na lisina 9 da histona H3 (H3K9me) e trimetil na lisina 27 da histona H3 (H3K27me3) (HAMAMOTO et al., 2004; VIRE et al., 2006; KONDO et al., 2007). Modificações em proteínas não histônicas reconhecedoras de marcas epigenéticas, como a proteína HP1 (proteína heterocromatina 1) que detecta metilação na lisina 9 da histona (H3H3K9), ensejam progressão tumoral (RODRÍGUEZ-PAREDEZ; ESTELLER, 2007).

Alterações em assinaturas epigenéticas contribuem para a progressão de doenças neurogênicas, como Alzheimer, Huntington, autismo, esquizofrenia, esclerose múltipla, epilepsia, e Parkinson (URDINGUIO; SANCHEZ-MUT; ESTELLER, 2009; PLAZAS-MAYORCA; VRANA, 2010). Elas, também, estão associadas com a fisiopatologia de doenças e de síndromes autoimunes, como lúpus eritomatoso, artrite reumatoide, escleroderma, e Sjögren (MILLINGTON, 2008; BROOKS et al., 2010; TRENKMANNet al., 2010), esta última desencadeando destruição de tecidos glandulares e manifestações oftálmicas similares às da CCS canina.

Importantes afecções oftálmicas cursam com mudanças no epigenoma e na cromatina. Por exemplo, a depleção experimental de *DNMT1* (DNA-metiltransferase-1) ocasiona perda de fotorreceptores (RHEE et al., 2012). Olhos com degeneração macular apresentam padrões elevados de metilação de DNA, o que silencia a expressão de alguns genes (WEI et al., 2012). Células epiteliais lenticulares de olhos com catarata compactam cromatina e apresentam hipermetilação de DNA nas ilhas de citosina-guanina (CpG) do promotor CRYAA (alfa cristalino A) (ZHOU et al., 2012). Opacidades de cápsula posterior e as vitreorretinopatias proliferativas estão associadas com alterações na proteína 2 de ligação ao metil-CpG (MeCP2) (ZHOU et al., 2011).

Células do retinoblastoma possuem regiões promotoras hipermetiladas nos genes *MSH6* (homólogo humano de 6 MutS), *PAX5* (homeodomínio box pareado), *GATA5* (codificador do fator de transcrição que se liga a sequência [A/T]GATA [A/G] do DNA), *TP53* (gene supressor de tumor), *VHL* (supressor tumoral von Hippel-Lindau), *GSTP1* (glutationa S-transferase P1), *MGMT* (O-6-metil-guanina-DNA-metiltransferase), *RB1* (codificador da proteína do retinoblastoma 1) e *CDKN2* (inibidor 2A das quinases dependentes de cliclina), e aumento na expressão de RNAs do cluster miR-17/92 (HE et al., 2013).

Modelos experimentais de uveorretinite autoimune acumulam alterações em RNAs de interferência, como o miR-142-5p, o miR-21 e o miR-182 (KUTTY et al., 2010). A ambliopia experimental, em ratos, está associada com alterações nos graus de acetilação e deacetilação de histonas (SILINGARDI et al., 2010). Após lesões no nervo óptico, ocorrem aumentos nas atividades das HDACs 2 e 3 e redução nos níveis de acetilação da histona H4 nas células ganglionares da retina (PELZEL; SCHAMP; NICKELLS, 2010). Células endoteliais da retina de ratos com hiperglicemia induzida por estrepzotocina apresentam aumento nas expressões de HDACs 1, 3 e 8, concomitante à redução na atividade de acetiltransferase específica da histona H3 (ZHONG; KOWLURU, 2010), podendo culminar em compactação de cromatina. Modificações epigenéticas em histonas e em RNAs de interferência foram observadas em células epiteliais conjuntivais da superfície ocular com inflamação crônica (HE et al., 2013). Córneas de ratos diabéticos induzidos por aloxana, com hiperglicemia de moderada a severa, apresentam redução nos níveis de acetilação

da histona H3, concomitante com alterações cromatínicas em células epiteliais e em ceratócitos (HERENCIA BUENO, 2017).

Características importantes permitem distinguir modificações epigenéticas de modificações genéticas. Dentre elas, os efeitos de posicionamento, a habilidade em modificar territórios gênicos (não um único gene) e a reversibilidade dos eventos envolvidos (FEINBERG, 2011). Sobre a reversibilidade, há evidências de que muitas alterações cromatínicas induzidas por vias microambientais nocivas regridem espontaneamente, após cessão do estímulo (MORAES et al., 2005). Ademais, estudos recentes mostram que certos fármacos, alguns deles tradicionais e de emprego consagrado, como o ácido valpróico (anticonvulsionante usado no tratamento de epilepsia) e a zebularina (anti-neoplásico), além da tricostatina A, do ácido anacárdico, da 5-azacitidina, da cúrcuma, do garcinol, do BIX-01294, do E72, do UNC321, do UNC0638, da 3-deazaneoplanocina e do vorinostat, agem sobre enzimas epigenéticas e podem desfazer alterações na estrutura da cromatina (FELISBINO; TAMASHIRO; MELLO, 2011; HEERBOTH et al., 2014).

O tratamento da CCS, salvo exceções, envolve o emprego de lágrimas artificiais (colírios lubrificantes) e de imunomoduladores como o tacrolimus, o sirolimus, o pimecrolimus e a ciclosporina A (CsA) (OFRI et al., 2009; PIGATTO et al., 2009). Lágrimas artificiais aumentam a estabilidade do filme lacrimal, diminuem a evaporação da lágrima e podem reduzir a expressão de moléculas juncionais na membrana plasmática das células epiteliais, favorecendo a proliferação celular (HOLLAND; MANNIS; LEE, 2015). Já os imunomoduladores, ou seus derivados, podem afetar, direta ou indiretamente, o epigenoma causando remodelação cromatínica, que aliada aos efeitos farmacodinâmicos, parece contribuir para o controle de doenças imunomediadas. Mecanismos epigenéticos explicariam porque organismos de mesma espécie ou de espécies diferentes apresentam respostas terapêuticas díspares ou manifestações adversas, quando expostos a um fármaco. Foi, também, proposto que a CsA afeta a atividade mitocondrial e previne a apoptose das células epiteliais conjuntivais (DURNIAN et al., 2007).

2.6 Ciclosporina A (CsA)

A CsA é um decapeptídico cíclico produzido pelo fungo *Tolypocladium inflatum gams* (BRUNTON; CHABNER; KNOLLMANN, 2011). Trata-se de um imunossupressor, com ação lacrimogênica, que age preferencialmente sobre os linfócitos T. Ela não causa alterações importantes na imunidade humoral, portanto, não compromete a ação de vacinas que estimulam a resposta protetora humoral (LIU et al., 2007). A introdução de CsA, na prática médica, inaugurou a era moderna dos transplantes de órgãos. Antes da CsA, a maioria dos transplantes de coração e de fígado era mal sucedida (ABBAS; LICHTMAN; PILLAI, 2012). Com a CsA, o tempo médio de sobrevida de aloenxertos é de cinco anos (ABBAS; LICHTMAN; PILLAI, 2012).

A CsA está sendo amplamente indicada para o tratamento de doenças imunomediadas, como a artrite reumatoide, a psoríase e a dermatite atópica (CARDENAS et al., 1999). Em animais com CCS a CsA na forma de pomada, diluída a diferentes concentrações, é utilizada há mais de duas décadas (GRAHN; STOREY, 2004; RIBEIRO et al., 2008), combinada com lágrimas artificiais. Comumente emprega-se a pomada à intervalos regulares de 12 horas e as concentrações variam de 0,2% a 2%. Devido às suas qualidades lipofílicas e hidrofóbicas, sua diluição pode ser conseguida em óleos vegetais, o que dispensa a adição de conservantes na formulação (BARNETT; SANSON, 1987).

Os mecanismos farmacodinâmicos da CsA em linfócitos T estão ilustrados na figura 3. Sumariamente, a CsA se liga a proteína intracelular ciclofilina-1 e inibe a atividade da calcineurina serina/treonina fosfatase, o que resulta em interrupção ou em redução na síntese de citocinas, notadamente de interleucina-2 (BRUNTON; CHABNER; KNOLLMANN, 2011).

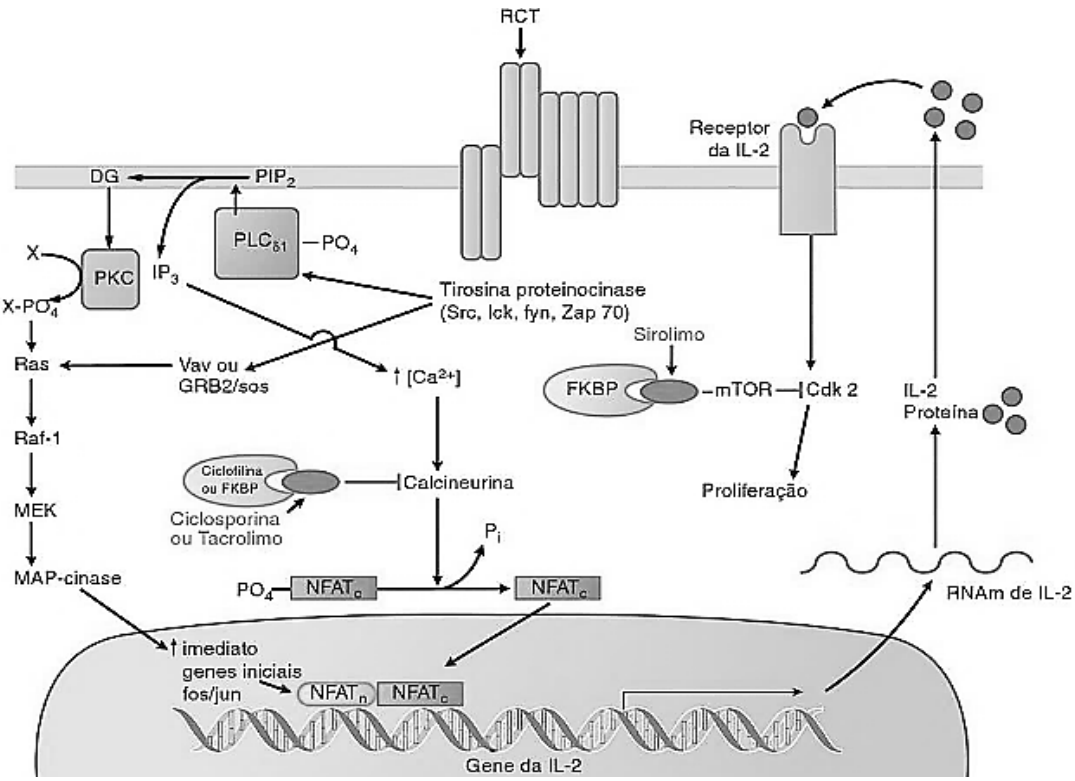

Figura 3. Mecanismos de ação da ciclosporina A (CsA), do tacrolimus e do sirolimus, nos linfócitos T. Relativamente à CsA, ela se liga à ciclofilina-1 e forma um complexo, que inibe a calcineurina e impede o influxo do fator nuclear das células T ativadas, para dentro do núcleo. O fator nuclear das células T ativadas é necessário para a transcrição de interleucina-2 e de outras citocinas associadas ao crescimento e à diferenciação celular (Copyright Lippincot Willians & Wilkins. http://lww.com.).

A calcineurina está também presente no citoplasma de linhagens celulares não linfocíticas, como as epiteliais e as miocíticas. Há evidências de que sua inibição funcional interfere na remodelação e nos estereoarranjos supraorganizacionais da cromatina, por bloquear a ação da subunidade catalítica do complexo remodelador SWI/SNF (NASIPAK et al., 2015). Quando prolongada, a inibição de calcineurina parece favorecer o desenvolvimento de tumores (SPINOSA; GÓRNIAK; BERNARDI, 2006), por mecanismos epigenéticos pouco elucidados. De fato, alguns autores reportaram que há associação entre uso de CsA e desenvolvimento de tumores conjuntivais (DURNIAN et al., 2007). Todavia, também há autores que utilizam CsA, combinada à outros fármacos, no controle da progressão de tumores oculares, devido suas propriedades antiangiogênicas (TUNC, 2006). Desconhecem-se casos de CCS canina que após instituição de terapêutica com CsA desenvolveram neoplasias oculares. Durnian e colaboradores (2007) propuseram que os riscos do uso de CsA na prática oftálmica são aceitáveis, na ausência de opções terapêuticas

melhores. Há, sem dúvida, necessidade de se discutirem questões de malignidade celular associada ao uso do fármaco e ao bloqueio de calcineurina.

Relativamente às funções da calcineurina, ela atua como fator de transcrição de citocinas associadas ao crescimento e à diferenciação celular. Sua ativação ocorre por vias dependentes de cálcio e envolve calmodulina (BRUNTON; CHABNER; KNOLLMANN, 2011), uma proteína que se liga ao cálcio, formando o complexo calmodulina-cálcio quinase II, o qual parece estar associado com fosforilação de histonas H3 (AWAD et al., 2013). Há evidências de que a calmodulina desempenha função chave na ativação de um dos complexos de remodelação da cromatina, o que explicaria o fato dela estar presente em todos os eucariotos e apresentar domínio catalítico altamente conservado (LAI et al., 2009).

3. HIPÓTESES

Duas hipóteses alicerçam a presente investigação. A primeira (Capítulo 2) é a de que células epiteliais e linfócitos da conjuntiva palpebral de cães com CCS apresentam alterações supraorganizacionais na cromatina, que afetam a morfologia e a funcionalidade nuclear. A segunda (Capítulo 3) é a de que os mecanismos de ação do tratamento da CCS canina, com 0,2% CsA e lágrima artificial, envolvem remodelação de cromatina e mudanças em eventos nucleares correlatos.

4. OBJETIVOS GERAIS

Estudar, por videoanálise de imagens, a supraorganização cromatínica e os parâmetros de morfofuncionalidade nuclear em células epiteliais e em linfócitos da conjuntiva palpebral inferior de cães com e sem CCS; avaliar se eventuais alterações nucleares que acompanham a progressão da CCS canina, notadamente mudanças no estado de compactação e na textura de cromatina em células epiteliais conjuntivais, regridem após 30 e 60 dias de tratamento com CsA 0,2% e lágrima artificial.

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Capítulo 2 – Artigo Científico*

Nuclear parameters and chromatin remodeling in epithelial cells and lymphocytes from the palpebral conjunctiva of dogs with keratoconjunctivitis sicca

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Running title: Chromatin remodeling in KCS conjunctiva

*Aceito para publicação no Periódico “Veterinary Ophthalmology” em 22 de janeiro de 2018

Abstract

Objective To study parameters related to nuclear morphology and chromatin remodeling in epithelial cells and lymphocytes from the inferior palpebral conjunctiva of dogs with and without keratoconjunctivitis sicca (KCS).

Animals studied Thirty-two dogs (64 eyes) were included in the study. Based on the tear production measured by Schirmer tear test 1, the dogs were distributed into control and KCS groups.

Procedures Epithelial cells and lymphocytes were collected by conjunctival brush cytology, fixed on glass slides, and subjected to the Feulgen reaction, a topochemical method specific for DNA/chromatin. Feulgen-stained cells were studied by microscopy and video image analysis to establish nuclear size (area and perimeter) and shape (relative nuclear roundness factor = RNRF), DNA content (ploidy), and compaction and texture of chromatin.

Results Conjunctival samples in the KCS group showed infiltration of inflammatory and immune cells. Micronuclei, snake-like chromatin, aberrant chromosomes, and goblet cells were not detected. Compared with the controls, cells on the conjunctival surface of dogs with KCS showed altered nuclei. Conjunctival epithelial cells were more affected by KCS (changes in nuclear size, shape, DNA content, and chromatin compaction) than lymphocytes (changes in chromatin compaction, only). Significant chromatin decompaction was observed in both conjunctival epithelial cells and lymphocytes.

Conclusions Our results show that KCS promotes chromatin remodeling in epithelial cells and lymphocytes on the conjunctival surface of dogs. The changes described in this study are different from those reported for conjunctival cell nuclei of human KCS patients.

Key words: autoimmune disease, cell nucleus, conjunctival cytology, dry eye, ocular surface, tear.

INTRODUCTION

Keratoconjunctivitis sicca (KCS), more commonly known as dry eye syndrome, is a multifactorial disease of the tear film and ocular surface that results in signs of discomfort, tear film instability, intense inflammation, and visual disturbance.[1-4] It may be quantitative, characterized by a deficiency in the production of the aqueous layer of the tear film, or qualitative, characterized by changes in the lipid layer of the tear film with rapid evaporation of tears.[3] KCS commonly affects dogs and is defined as a Schirmer tear test 1 (STT1) reading of less than 10 mm/ min concomitant with pathological changes in the ocular surface.[3,5] Many cases of canine KCS are immunemediated.[3,6,7] With regard to the pathophysiology of KCS, recent advances in the fields of cell biology and genetics suggest that it goes beyond the histopathological manifestations seen in the lacrimal gland and the ocular surface.[2,3,8-13] There is evidence that the metabolism of cells on the surface of the palpebral conjunctiva is also severely altered by the disease.[2] In human patients, KCS has been closely associated with changes in interphase nuclear morphology of palpebral/bulbar conjunctival epithelial cells, and with the appearance of snake-like chromatin, whose clinical and biological significances remain under investigation.[4,14-16] In dogs, the effects of KCS on the nuclear morphology and the chromatin of cells in the conjunctival epithelium have not been established yet. Apparently, snake-like chromatin or other aberrant chromatin patterns do not occur as they are easily identifiable by light microscopy, and to date, no report has been published. Studies evaluating nuclear and chromatin parameters in normal and KCS conjunctival cells are needed to enhance our understanding of the pathophysiology of KCS. An increased understanding of the cell nuclei in the palpebral conjunctival epithelium affected by KCS can potentially help veterinary ophthalmologists to improve diagnosis and management of canine patients with KCS, and may also be beneficial in the future when designing new pharmacological strategies intended to cure or relieve patient's symptoms and ocular surface disease.[2] The nucleus is a large cellular structure that defines the metabolism and functions of the cell.[17] A normal cell maintains its nucleus within a genetically defined size, but an altered cell often exhibits changes in nuclear size and shape.[18] The major component of the nucleus is

chromatin, a malleable fiber that results from the assembly of DNA with histones and other nucleoproteins.[1,19,20] The degree of DNA affinity for histones organizes the genome into either open/relaxed (euchromatin) or compacted chromatin (heterochromatin) and thus regulates the accessibility of genes for transcription, replication, and repair.[19] The process of interaction between DNA and histones is dynamic and mediated by ATP-dependent nucleosome remodeling complexes and by epigenetic enzymes, which are up- or downregulated by microenvironmental stimuli.[17,19-22] There is emerging evidence that changes in the microenvironment surrounding the cells elicit the chromatin to remodel from a more compacted to a more relaxed state and vice versa.[22-24] Remodeling alters the functionality of chromatin, the expression of genes, and the synthesis of proteins, and can contribute to the progression of diseases and/ or syndromes.[19,22,23] Senile cataract, diabetic retinopathy, macular degeneration, and autoimmune uveoretinitis are examples of diseases associated with remodeling of chromatin in ocular cells.[24-29] Nuclear size and shape, DNA content (ploidy), and chromatin remodeling can be studied in cytological preparations using video image analysis and the Feulgen reaction.[20,30] Video image analysis in Feulgen-stained preparations is recommended to evaluate defined cell populations.[20] In addition to reporting on supraorganizational and functional parameters of the chromatin fiber (compaction and texture), the method provides information on the geometry of the cell nucleus and allows quantification of DNA ploidy by generating an equivalent cytometric of chromosomal ploidy.[20,30] The Feulgen reaction is a stoichiometric DNA-staining technique that consists of two steps.[20,30,31] In the first step, the DNA is submitted to acid hydrolysis to split off the purine bases from the double-stranded DNA. The result is an apurinic acid presenting free aldehyde groups at the C1 position of the deoxyribose.[20,30] In the second step, a Schiff base binds to the aldehyde groups and produces a magenta color whose intensity is proportional to the DNA content.[20,30] The objective of this research was to study video image analysis parameters related to nuclear morphology (size and shape), DNA content, and chromatin remodeling (ie changes in compaction and texture of chromatin) in epithelial cells and lymphocytes collected by brush cytology from the inferior palpebral conjunctiva of dogs with KCS. Nuclei in

cells from the palpebral conjunctiva of dogs without KCS were also studied and used as controls.

MATERIAL AND METHODS

Ethics

This study adhered to the ARVO Statement for Use of Animals in Ophthalmic Vision and Research. All procedures involving dogs followed the Ethical Principles of Animal Experimentation established by the Brazilian College of Animal Experimentation (COBEA) and were performed according to protocols approved by Ethics Committee on Animal Use (CEUA) of the FCAV/Unesp (protocol number 6.329/16). All owners of dogs that met the criteria for inclusion were informed as to the importance and objective of the study and those who agreed to have their animals involved provided a statement of informed consent.

Animals

Sixty-four eyes of 32 brachycephalic and mesocephalic dogs aged 2-13 years (Table 1) were included in the study. All dogs were free of systemic diseases, as revealed by fisical, hematological (complete blood count), and biochemical (creatinine and alanine aminotransferase) examinations. Eyes were examined using STT1 (Schirmer strip, Ophthalmos SA, Sao Paulo, Brazil), slit-lamp biomicroscopy (Kowa, Tokyo, Japan), applanation tonometry (Tonopen XL, Reichert, NY, USA), indirect ophthalmoscopy (Eyetec, Sao Carlos, Brazil), fluorescein stain (Fluorescein strips, Ophthalmos SA), and tear breakup time (TBUT).

Based on the tear production measured by STT1, the dogs were distributed into control and KCS groups (Table 1). The control group consisted of 16 dogs (n = 32 eyes) with normal values of tear production (STT1 ≥ 15 mm/min). The KCS group consisted of 16 dogs (n = 32 eyes) presenting bilateral KCS (STT1 values ≤ 10 mm/min),3 without any other associated disease. None of the animals included in the study had a history of neoplasms of any type or were using antibiotics or immunomodulatory drugs. The study groups were homogeneous for skull shape, age, and sex ($P > .05$ for all) (Table 1).

Table 1. Demographic characteristics and tear production data of the dogs that composed the groups of this study

Groups	Characteristics							
	Skull shape	Breed	Age	Sex	STT1 (mm/min)		TBUT (sec)	
					RE	LE	RE	LE
Control								
1	brachycephalic	English Bulldog	5	M	30	30	2	2
2	brachycephalic	English Bulldog	8	M	30	25	2	2
3	brachycephalic	English Bulldog	4	F	25	19	3	3
4	brachycephalic	English Bulldog	3	F	25	30	7	10
5	brachycephalic	English Bulldog	2	F	27	29	2	3
6	brachycephalic	English Bulldog	2	F	30	30	1	2
7	brachycephalic	English Bulldog	4	F	30	25	2	2
8	brachycephalic	English Bulldog	4	M	31	35	3	4
9	mesocephalic	Beagle	13	M	24	24	15	20
10	mesocephalic	Beagle	13	M	27	24	12	20
11	mesocephalic	Beagle	13	M	28	30	20	22
12	mesocephalic	Beagle	13	M	16	21	20	20
13	mesocephalic	Beagle	13	M	25	24	15	15
14	mesocephalic	Beagle	13	M	24	22	20	25
15	mesocephalic	Beagle	13	F	22	24	18	15
16	mesocephalic	Beagle	13	F	23	24	10	12
KCS								
17	brachycephalic	Shih Tzu	6	F	0	3	0	0
18	brachycephalic	Shih Tzu	10	F	0	0	0	0
19	brachycephalic	Shih Tzu	11	M	5	4	0	0
20	brachycephalic	Shih Tzu	11	M	1	0	0	0
21	brachycephalic	Shih Tzu	2	M	3	3	3	3
22	brachycephalic	Shih Tzu	6	M	4	8	8	8
23	brachycephalic	Lhasa Apso	9	M	0	0	0	0
24	mesocephalic	Daschund	6	F	9	9	6	6
25	mesocephalic	Yorkshire	7	F	0	0	0	2
26	mesocephalic	Cocker Spaniel	8	F	7	2	0	0
27	mesocephalic	West Higland White	13	F	0	0	0	0
28	mesocephalic	Maltese	11	F	0	0	13	0
29	mesocephalic	Saint Bernard	3	F	7	6	3	12
30	mesocephalic	Undefined breed	7	F	0	0	0	0
31	mesocephalic	Undefined breed	12	M	4	5	0	0
32	mesocephalic	Bull Terrier	7	M	4	9	0	0

M, male; F, female; RE, right eye; LE, left eye. Ages are presented in years.
According to the Wilcoxon test, the study groups were homogeneous for skull shape ($P = 0.763$), age ($P = 0.571$), and sex ($P = 0.546$).

At present, dogs in the KCS group remain on treatment with topical 0.2% cyclosporine A ophthalmic ointment. Treatment was started immediately after the disease was diagnosed.

Conjunctival brush cytology

Brush cytology was performed as previously described.[32-34] Cells were harvested from the inferior palpebral conjunctival epithelium because it is easy to access, with less risk of accidentally damaging the cornea. The eyes were anesthetized with one drop of 0.5% proxymetacaine (Anestalcon, Alcon, Sao Paulo, Brazil), and the inferior palpebral conjunctiva was carefully pulled down and scraped 4 times using a sterile cervical cytobrush (Kolplast Ltda, Itupeva, Brazil). The smears with all cellular material were spreads on glass slides (Knittel, Braunschweig, Germany) and then the cytological preparations were fixed for 10 min in a mixture of 70% ethylic alcohol (Ecibra, Sao Paulo, Brazil), glacial acetic acid (Synth, Sao Paulo, Brazil), and 10% buffered formalin (Dinâmica, Sao Paulo, Brazil); 3:1:1, vol/ vol/vol.

Feulgen reaction

Cytological preparations from the conjunctival surface were subjected to the Feulgen reaction, a topochemical method specific for DNA/chromatin.[20,30,31] Acid hydrolysis was performed in 4 M HCl for 40 minutes at 25°C for the generation of deoxyribose in an aldehyde form.[20] The hydrolysis reaction was quenched by rapid treatment with cold 0.1 M HCl. The cytological preparations were then exposed to a reactive Schiff base (Merck, Darmstadt, Germany) for 40 minutes in the dark, rinsed in sulfurous water (mixture of distilled water, 1 M HCl, and 10% sodium metabisulfite; 18:1:1, vol/vol/vol), air-dried, cleared in xylene (Synth, Sao Paulo, Brazil), and mounted with natural Canada balsam (Vetec, Rio de Janeiro, Brazil).[20,35,36] All staining and subsequent steps were performed in parallel for the cytological preparations to minimize variations in the experimental conditions and to reduce the possibility of systematic errors.[20]

Video image analysis

Feulgen-stained nuclei were analyzed under an Olympus BX-53 light microscope (Tokyo, Japan) equipped with a Plan Neofluar 40/0.75 objective, optovar 1.6, condenser 0.90, and an interferential filter (Edmund Optics, Barrington, NJ, USA) to obtain monochromatic light of 546 nm. A 100-W halogen lamp and a luminous-filter diaphragm (transmitted light) at its maximal opening were used to provide Köhler illumination.[20] With help of a high-resolution video camera, images were transmitted to a computer where they were digitized and studied using ImageJ software (http://imagej.nih.gov/ij/;National Institutes of Health, Bethesda, MD, USA). The spatial calibration of the software was made with the ruler of the microscope.

Nuclear images digitized in color were converted to binary data (ImageJ > Image > Adjust > 8-bit) containing zero to 255 gray levels. The microscope operating conditions, with special attention to the intensity of the light emitted by the halogen lamp, were the same for all cytological preparations studied. Only separate and intact cells were studied. Thus, it was possible to measure 640 epithelial cell nuclei and 100 lymphocyte nuclei from each group. Overlapping or unfocused nuclei, and the nuclear debris, were not analyzed.

Video image parameters consistent with the objective of this study were: nuclear area (square micrometer); nuclear perimeter (micrometer); relative nuclear roundness factor (RNRF = (nuclear perimeter / 2π) / (nuclear area / π)) that described the nucleus shape (circularity); optical density (OD = average absorbance) that described the degree of chromatin compaction; standard deviation of gray average in pixels per nucleus (SDtd, this defined the contrast between areas of greater and lesser chromatin compaction); and integrated optical density (IOD) that measured the DNA content in arbitrary units (AUs).[20,23,36]

DNA ploidy classes related to DNA biosynthesis and cell cycle phases were established from relative frequency histograms of the IOD values.[20,23,36] The lymphocytes were used as diploid reference standards for ploidy.[20,23,36]

Surface plot graphs (ImageJ > Analyze > Surface plot) were constructed to represent the compaction states of chromatin based on the topography of different bitmap pixel values from the nuclear images. Prior, pseudocolors (i.e., false colors) were applied (ImageJ > Image > Lookup table > 6 Shades).

Data analysis

All data were tested for statistical normality using the Kolmogorov-Smirnov test. Tear and nuclear parameters obtained for the control and KCS groups were compared using the Mann-Whitney nonparametric test because the distributional assumptions required for parametric testing were not satisfied. The Spearman's correlation (r) was used as a measure of association between the study variables. Differences with P < .05 were considered significant, with 95% confidence interval (95% CI). All calculations and comparisons were performed using the MINITAB 12TM (Minitab, State College, San Diego, CA, USA) and the MEDCALC (version 9.3.6.0, MedCalc, Mariakerke, Belgium) software programs.

RESULTS

Tear production

The median values of STT1 were 25.00 mm/min (range, 16–31) for the control group and 2.90 mm/min (range, 0–9) for the KCS group ($P < 0.001$). In relation to the TBUT, the median values were 10.00 sec (range, 1–25) for the control group and 1.9 sec (range, 0–13) for the KCS group ($P < 0.001$).

Visual inspection by light microscopy of Feulgen-stained samples

Figure 1 corresponds to the Feulgen-stained nuclei in epithelial cells and lymphocytes from the inferior palpebral conjunctiva of dogs with and without KCS, after conversion of color images to binary. All nuclei were interphase and exhibited varying intensities of mean gray levels, reflecting different states of chromatin compaction. Differences in nuclear morphology (size and shape) between the control and KCS groups could not be detected visually, that is, without the aid of ImageJ software. Micronuclei, apoptotic bodies, snake-like chromatin, and aberrant chromosomes were not detected. The samples in the KCS group showed intense infiltration of inflammatory and immune cells (Figure 2). Goblet cells were not found in any of the Feulgen-stained samples.

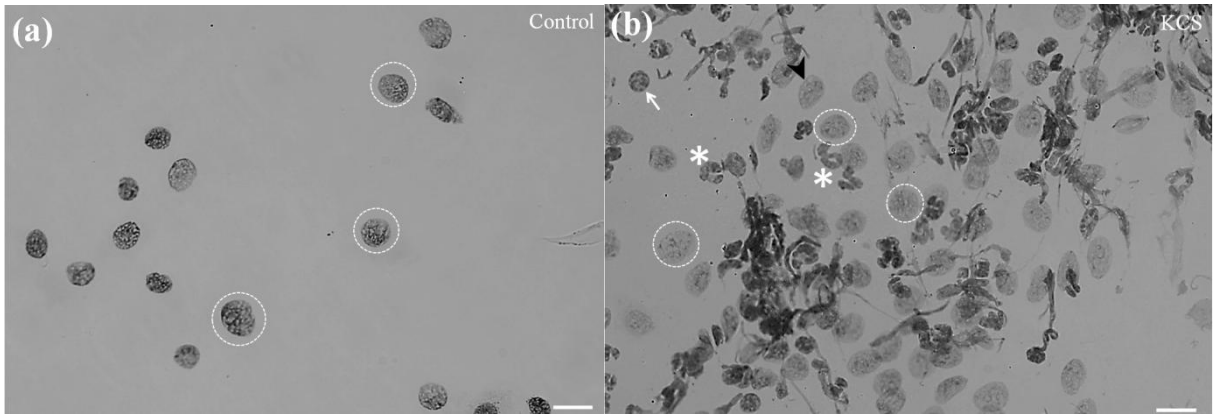

Figure 1. Feulgen-stained nuclei in epithelial cells and lymphocytes from the inferior palpebral conjunctiva of dogs. (a) control sample, after conversion of color image digital to binary in the ImageJ software; (b) KCS sample. The circles in the images (a) and (b) delimit some conjunctival epithelial cell nuclei. The arrow in the image (b) points to a lymphocyte nucleus, and the asterisks denote some areas of inflammatory cells. Bar = 20 micrometers

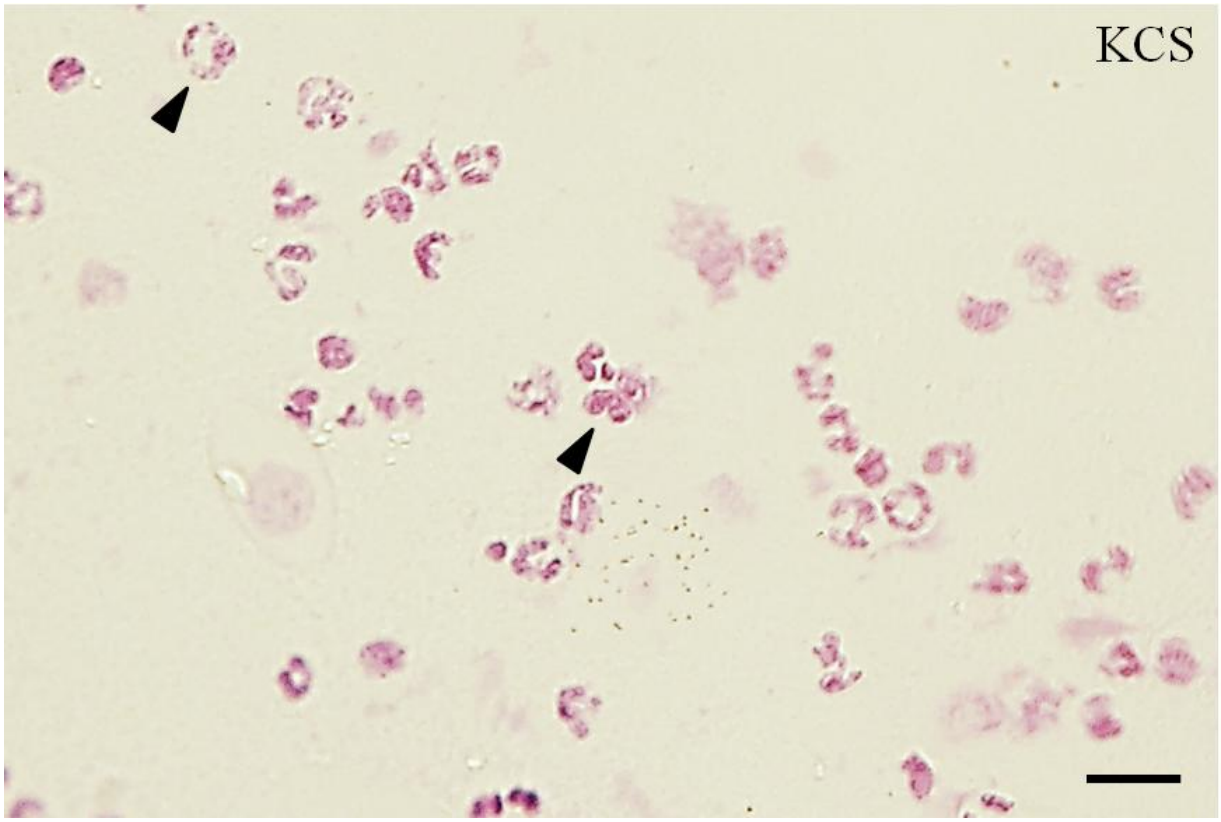

Figure 2. Feulgen-stained nuclei (arrowheads) in inflammatory cells from the inferior palpebral conjunctiva of a dog with KCS. The most abundant inflammatory cells were neutrophils. Bar = 20 micrometers.

Nuclear size and shape

Figure 3 corresponds to the illustration of the segmentation masks overlaid on nuclei images for calculating the size (area and perimeter) and the shape (RNRF) in the Image J software. Nuclei in conjunctival epithelial cells of KCS dogs presented increased values of area, perimeter, and RNRF, compared to nuclei in conjunctival epithelial cells of control dogs (Table 2) ($P < .001$ for all). No differences in areas ($P = .690$), perimeters ($P = .666$), and RNRFs ($P = .574$) of lymphocyte nuclei were observed between the control and KCS groups.

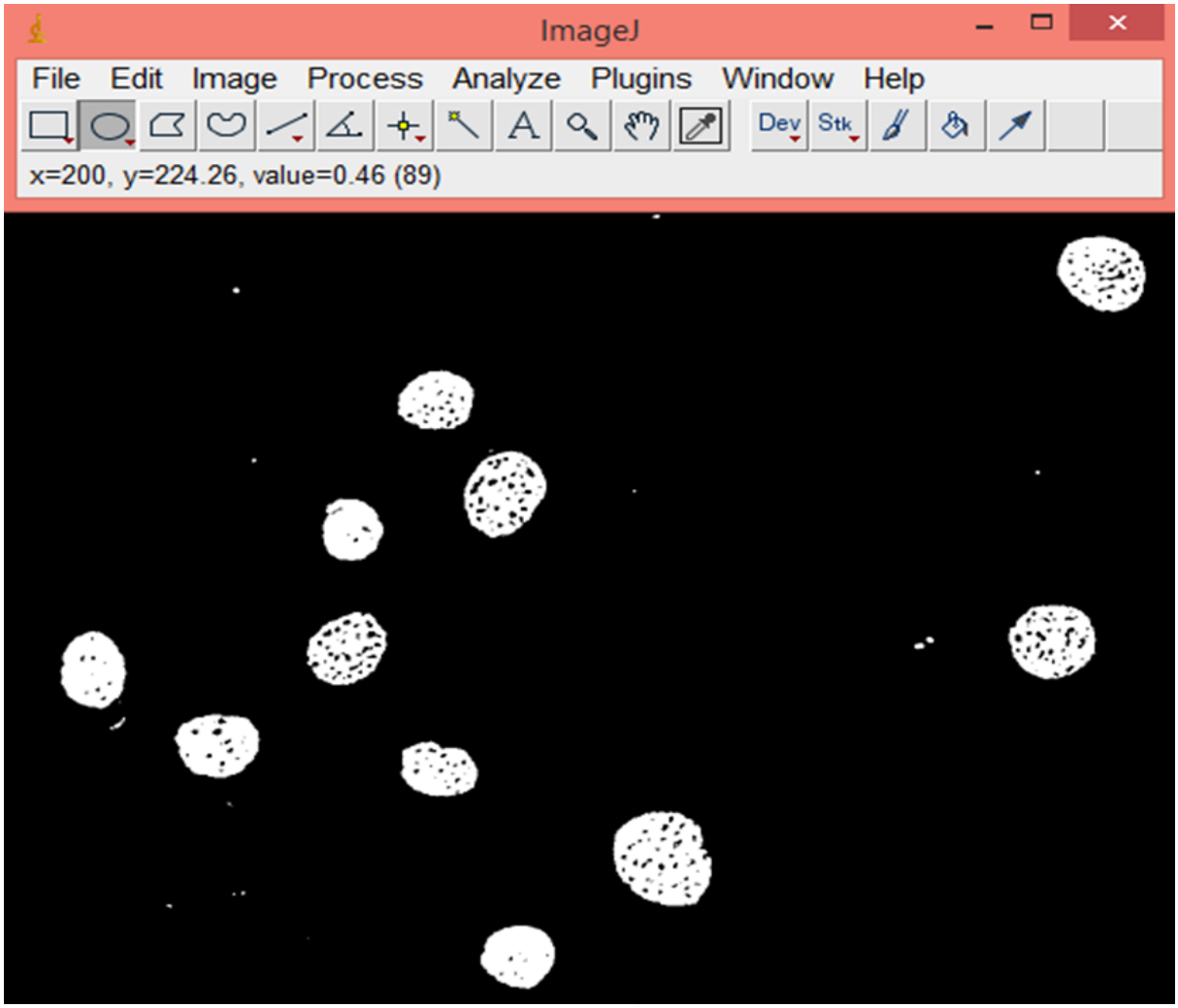

Figure 3. Illustration of the segmentation masks overlaid on cell images for calculating nuclear area (square micrometer) and perimeter (micrometer), and RNRF, in the ImageJ software. Cell nuclei that appeared deformed or overlapping, and the nuclear debris, were excluded from the evaluation.

Table 2. Video image analysis parameters related for nuclear size and shape of the epithelial cells and the lymphocytes from the palpebral conjunctiva of dogs with and without keratoconjunctivitis sicca (KCS)

Parameters	Conjunctival epithelial cells										
	Control (n = 640)			95% CI		KCS (n = 640)			95% CI		
	Min	Max	Median	Lower	Upper	Min	Max	Median	Lower	Upper	*P*-value
Area (μm^2)	33.612	152.571	79.694	78.189	81.963	34.327	179.980	90.418	88.282	92.735	< 0.001
Perimeter (µm)	27.826	54.753	40.841	40.392	41.290	25.133	59.241	42.636	42.187	43.085	< 0.001
RNRF	0.166	0.521	0.248	0.244	0.254	0.215	0.670	0.429	0.422	0.435	< 0.001
	Lymphocytes										
	Control (n = 100)			95%CI		KCS (n = 100)			95%CI		
	Min	Max	Median	Lower	Upper	Min	Max	Median	Lower	Upper	*P*-value
Area (μm^2)	12.265	91.633	53.326	49.556	57.327	15.898	93.143	52.143	48.870	56.088	= 0.690
Perimeter (µm)	17.054	44.431	34.109	31.865	35.006	20.196	43.533	33.660	31.865	34.558	= 0.666
RNRF	0.234	0.746	0.319	0.309	0.343	0.233	0.635	0.322	0.310	0.336	= 0.574

Max, maximum value; Min, minimum value; n, number of nuclei measured; RNRF, relative nuclear roundness factor; 95% CI, 95% confidence interval of the median; μm^2, square micrometer; µm, micrometer.
Differences were significant when $P < 0.05$ (Mann-Whitney non-parametric test).

Ploidy levels

Fig. 4 corresponds to the frequency histograms (%) of IOD values (DNA content) obtained for epithelial cells and lymphocytes in the KCS and control groups. Ploidy levels of nuclei in conjunctival epithelial cells were established by comparison with IOD values obtained for lymphocytes, which typically are diploid cells arrested in different cell cycle phases. While the nuclei in conjunctival epithelial cells presented a multimodal distribution of IOD values (Fig. 4a and b), the lymphocyte nuclei had a limited Feulgen-DNA content range (Fig. 4c and d). The median IOD values were 54.043 AU (range, 21.448–108.454) vs. 57.638 AU (range, 23.666–115.572) ($P < 0.001$) for conjunctival epithelial cells, and 44.209 AU (range, 16.301–55.516) vs. 43.466 AU (range, 15.921–55.280) ($P = 0.624$) for lymphocytes, in control group vs. KCS group respectively.

A sort manipulation (Minitab 12™ software) of the IOD values allowed us to detect that 47.1% and 51.7% of the conjunctival epithelial cells in the control and KCS groups, respectively, had more nuclear DNA than diploid lymphocytes; that is, a large part of the conjunctival epithelial cells studied had near-tetraploid nuclei and were synthesizing DNA. There is the statistically significant difference in the proportion of DNA-synthesizing cells between the control and KCS groups ($P < 0.001$).

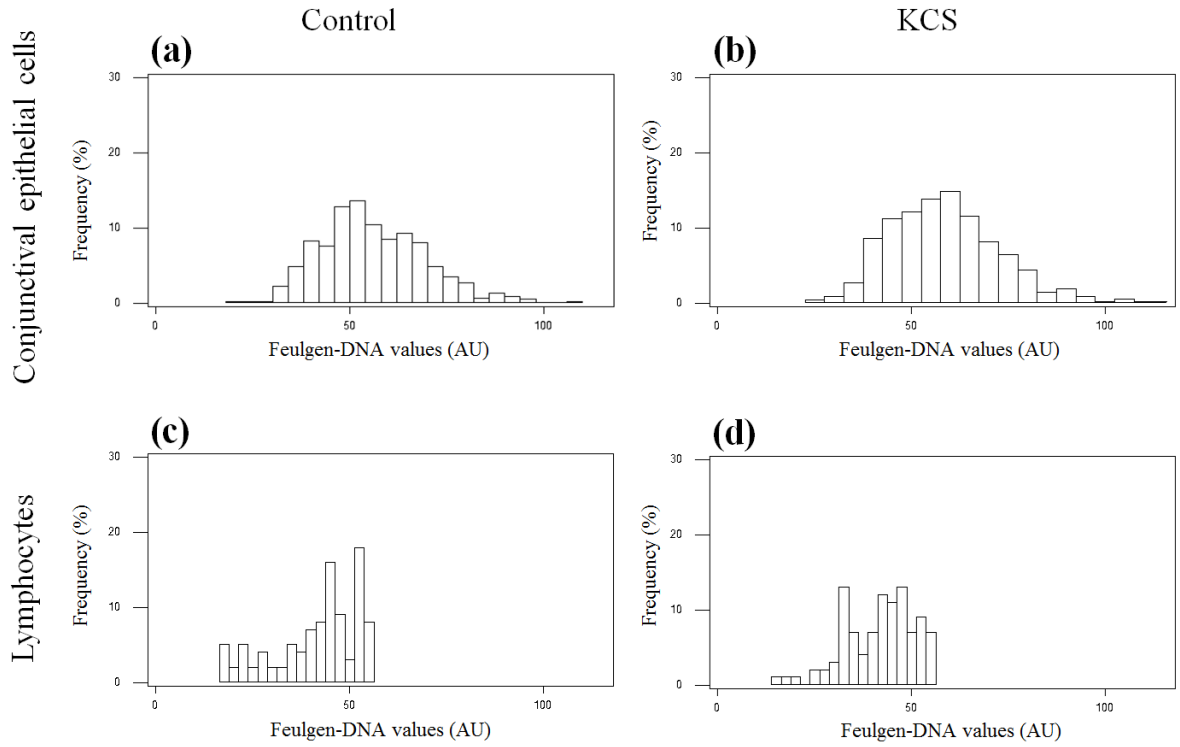

Figure 4. Frequency histograms of IOD values, in arbitrary units (AU). (a) epithelial cells from the inferior palpebral conjunctiva of dogs without KCS (controls); (b) epithelial cells from the inferior palpebral conjunctiva of dogs with KCS; (c), lymphocytes from the inferior palpebral conjunctiva of dogs without KCS; (d), lymphocytes from the inferior palpebral conjunctiva of dogs with KCS. Note that while the nuclei in conjunctival epithelial cells presented a multimodal distribution of IOD values, the lymphocyte nuclei had a limited Feulgen-DNA content range (15.921–55.280 AU).

Compaction and texture of chromatin

Assessments by video image analysis revealed that nuclei in epithelial cells and lymphocytes in the KCS group have less compacted chromatin (low OD values) compared to nuclei in epithelial cells and lymphocytes in the control group (Table 3). No differences in the SDtd values (chromatin texture) obtained for conjunctival epithelial cells ($P = .556$) or for lymphocytes ($P = .375$) were observed between the control and KCS groups. In both groups, the OD and SDtd values for lymphocytes were higher than those for conjunctival epithelial cells ($P < .001$).

Surface plot graphs (Figure 5) revealed details related to compaction state and arrangements of chromatin. Three sets of supraorganized chromatin were observed as follows: The first set (green pseudocolor) has least packed chromatin, characterized by OD values below 0.660 AU; the second (red) has moderately compacted chromatin, with OD values between 0.661 and 0.800 AU; and the third

(black pseudocolor) has highly compacted chromatin, with OD values above 0.801 AU.

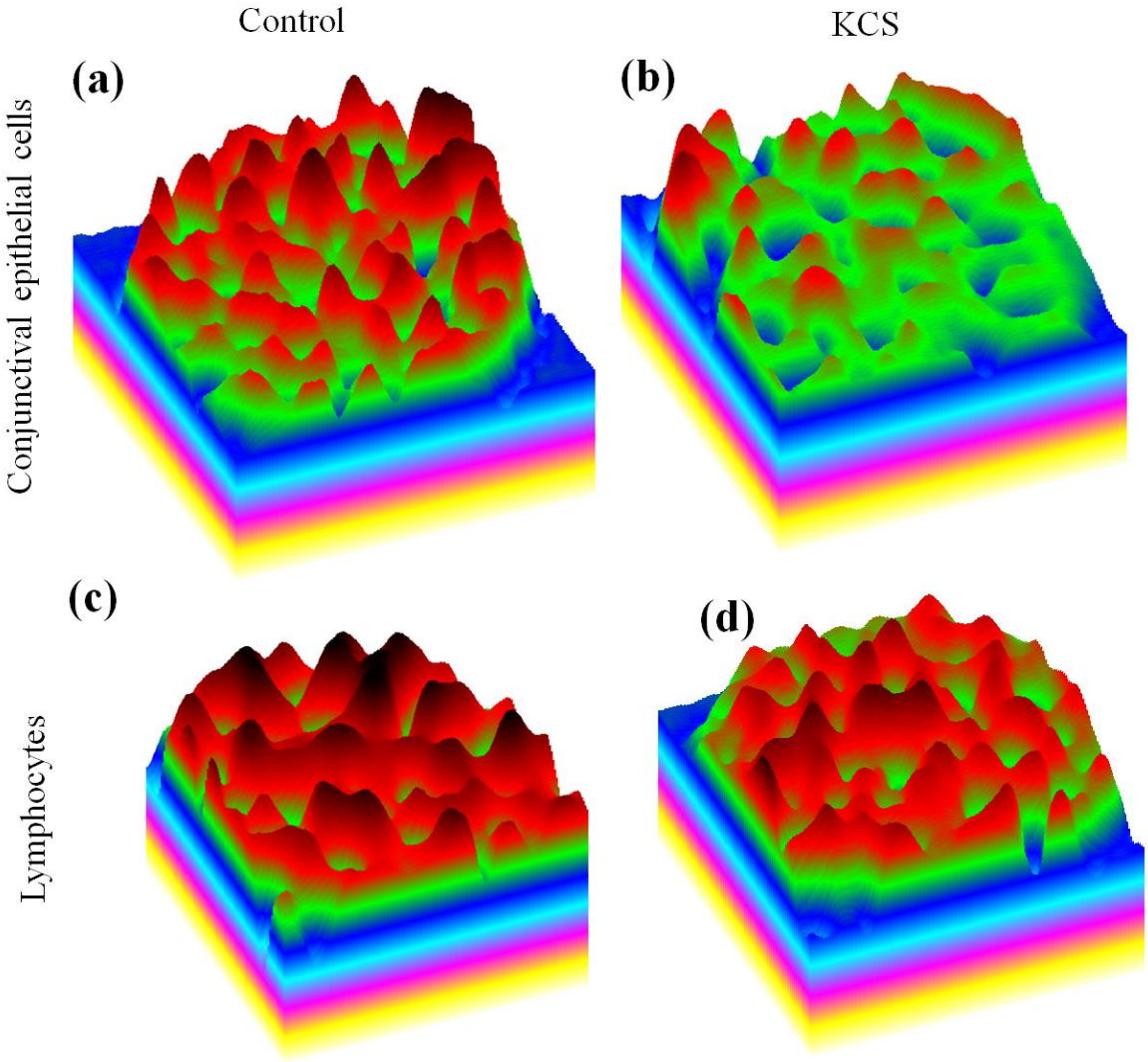

Figure 5. Surface plot graphs representative of the compaction states of chromatin. (a) epithelial cells from the inferior palpebral conjunctiva of dogs without KCS (controls); (b) epithelial cells from the inferior palpebral conjunctiva of dogs with KCS; (c), lymphocytes from the inferior palpebral conjunctiva of dogs without KCS; (d), lymphocytes from the inferior palpebral conjunctiva of dogs with KCS. Up to three sets of supraorganized chromatin could be observed. The first set (green pseudocolor) has least compacted chromatin, the second (red) has moderately compacted chromatin, and the third (black pseudocolor) has highly compacted chromatin.

Table 3. Video image analysis parameters related for chromatin supraorganization of the epithelial cells and the lymphocytes from the palpebral conjunctiva of dogs with and without keratoconjunctivitis sicca (KCS)

Parameters	Conjunctival epithelial cells										
	Control (n = 640)			95% CI		KCS (n = 640)			95% CI		
	Min	Max	Median	Lower	Upper	Min	Max	Median	Lower	Upper	*P*-value
OD (AU)	0.549	0.914	0.663	0.653	0.675	0.547	0.876	0.634	0.627	0.642	< 0.001
SDtd (AU)	0.002	0.127	0.022	0.018	0.025	0.005	0.082	0.023	0.022	0.024	= 0.556
	Lymphocytes										
	Control (n = 100)			95%CI		KCS (n = 100)			95%CI		
	Min	Max	Median	Lower	Upper	Min	Max	Median	Lower	Upper	*P*-value
OD (AU)	0.695	1.353	0.844	0.799	0.840	0.684	1.120	0.819	0.823	0.864	= 0.016
SDtd (AU)	0.016	0.280	0.073	0.066	0.084	0.028	0.251	0.071	0.064	0.077	= 0.375

AU, arbitray units; Max, maximum value; Min, minimum value; n, number of nuclei measured; OD, optical density; SDtd, standard deviation of gray average in pixels per nucleus (i.e., chromatin texture); 95% CI, 95% confidence interval of the median. Differences were significant when $P < 0.05$ (Mann-Whitney non-parametric test).

Associations between compaction states of chromatin and STT1

Spearman's correlation analyses revealed significant positive associations of nuclear OD values with STT1 in both the control and KCS groups: control = [(conjunctival epithelial cells: *r*, 0.789; 95% CI, 0.482–0.924; *P* = 0.002) (lymphocytes: *r*, 0.591; 95% CI, 0.345–0.843; *P* = 0.001)]; KCS = [(conjunctival epithelial cells: *r*, 0.769; 95% CI, 0.441–0.916; *P* = 0.002) (lymphocytes: *r*, 0.633; 95% CI, 0.302–0.856; *P* = 0.000)].

DISCUSSION

We studied the size and shape of nuclei, DNA content, and compaction and texture of chromatin in epithelial cells and lymphocytes collected by brush cytology from the palpebral conjunctiva of dogs with and without KCS. Goblet cell nuclei were not found, probably because they are not distributed throughout the conjunctival surface. In dogs, goblet cells are predominantly located on the palpebral surface of the nictitating membrane and on the medial conjunctival fornix.[37,38]

With regard to tear production, only STT1 values were used as criteria for inclusion of dogs into the study. TBUT was not considered because of the high variability of values in the control group. Besides, the normative values of TBUT in this study were lower than those reported by Ollivier et al.[39] and Kobashigawa et al.,[40] probably due to characteristics of the dogs evaluated. Here, half of the dogs had a brachycephalic skull conformation, which makes the eye protruded and favors tear evaporation. Eye examination ruled out the possibility that the reduced TBUT values, in the control group, are related to qualitative changes in the tear film.

Age, sex and skull shape were also selection criteria for inclusion of dogs into the study since they affect the values of STT1.[39–41] A rigorous screening of animals was necessary to ensure experimental homogeneity among the studied groups and thus avoid biases that could compromise the interpretation of the results.

Cell nuclei were evaluated by video image analysis and following Feulgen reaction, which is the preferred technique for in situ analyses of chromatin/DNA.[30,31] Compared with other topochemical techniques, the Feulgen reaction better preserves nuclear morphology.[20] The video image analysis parameters evaluated in this study

are the main descriptors of the nuclear phenotype of a cell and are closely related to the control of gene expression and cellular functionality.[36] Changes in these parameters accompany the progression of many diseases and have been explored for different clinical purposes, such as establishing malignancy and prognosis of tumor type.[31] In addition, knowing the effects of diseases on nuclear size and shape, DNA content, and chromatin supraorganization of different cell types can contribute to the optimization of the design of advanced therapeutic strategies, such as the use of drugs to reverse epigenetic changes in the genome.

Our results revealed that KCS modified parameters of nuclear morphology and high-order organization of chromatin in cells on the palpebral conjunctival surface of dogs. Interestingly, epithelial cells were more affected by KCS (changes in nuclear size, shape, DNA content, and chromatin compaction) than lymphocytes (changes in chromatin compaction, only).

Nuclear size and shape are important geometric parameters associated with cell integrity and functionality.[18,42] It is likely that changes in the size of the cell nucleus, as seen in this study, result from alterations in vesicular transport of proteins within the endoplasmic reticulum, and in nuclear envelope formation.[18,42,43] Changes in nuclear shape result from the reorganization of the nuclear lamina and are indicative of changes in nuclear stiffness and/or chromatin supraorganization and functionality.[42,44] In contrast to the results of this study, other investigators[15] found no change in the nuclear size and shape of conjunctival epithelial cells in human KCS patients. However, compositional changes in the nuclear lamina of conjunctival cells from human patients with KCS have already been observed and there is evidence that a lamin A/C deficiency in epithelial cells contributes to the progression of the disease.[15]

Video image analysis of Feulgen-stained nuclei is a technique comparable to flow cytometry for the detection of DNA content.[20,30] In this work, larger nuclei with high DNA content (i.e., near-tetraploid nuclei) were observed in both study groups and are typical of cells synthesizing DNA.[20] DNA synthesis (rise in ploidy) is an event that precedes mitosis and the proliferation of cells involved for example in the homeostasis of self-renewing adult tissues,[20,45] such as the conjunctival epithelium. Compared with the control group, the KCS group showed a high proportion of

epithelial cells synthesizing DNA (51.7%), which indicates changes in tissue metabolism. Our results are in line with those of Gao et al.,[46] who found reduced apoptosis of conjunctival epithelial cells in KCS dogs. In general, apoptosis and DNA synthesis are inversely correlated parameters, that is, when one decreases the other increases.

In KCS, it is possible that increases in DNA synthesis and nuclear ploidy represent protective mechanisms of palpebral conjunctival epithelial cells against microenvironmental stress and physiologically aversive stimuli on the ocular surface. An increase in nuclear ploidy levels as a response to tissue injury was previously observed in conjunctival epithelial cells of human patients with conjunctival neoplasms treated with mitomycin-C or radiation.[47] Cell nuclei in cornea, tendon, heart, and liver of diabetic animals also undergo a rise in ploidy as a response to hyperglycemic stimulus and oxidative stress.[23,48-51] In addition, a significant increase of DNA content has been reported for cells from different tissues under inflammatory or hypoxia conditions.[45,52,53] Inflammation is one of the main features of KCS and was observed in all KCS eyes of this study. Also, KCS leads to hypertonicity of tears with subsequent hypoxia of the ocular surface.[3]

The main finding of this study was that KCS promoted chromatin remodeling in epithelial cells and lymphocytes on the conjunctival surface of dogs. Remodeling is an event that precedes changes in gene expression and that modifies the supraorganizational state and the functionality of the chromatin fiber. The pattern of chromatin remodeling found in dogs appears to be different from that reported for human patients and did not culminate in the compaction of chromatin into a long snake-like or stick-like structure.[4,14–16,54] However, it is necessary to ponder that snake-like chromatin has been observed mainly in cells in the superior palpebral and bulbar conjunctiva, and we studied only cells in the inferior palpebral conjunctiva.

Textural contrast between nuclear areas with more and less compacted chromatin was not affected by KCS, as revealed by comparative analyses of the SDtd values. This result indicates that the KCS did not cause preferential effects on areas of heterochromatin (transcriptionally inactive chromatin) or euchromatin (transcriptionally active chromatin) of the cell nuclei studied. Chromatin decompaction (reduction in OD), as observed in epithelial cells and lymphocytes from the palpebral

conjunctiva of KCS dogs, was a widespread nuclear event that may be indicative of an increase in the transcriptional activity. In agreement with this, chromatin decompaction has often been implicated in the activation of molecules and in the occurrence of cell events that contribute to the etiology and pathogenesis of diseases.[23,55] There is evidence for example that chromatin decompaction increases lymphocyte responsiveness to microenvironmental stimuli, contributing to pathological inflammation in severely injured tissues.[55] Also, studies have shown that cells on the conjunctival surface of KCS patients increase the expression of genes and proteins related to defense and inflammatory responses.[9-11] For example, conjunctival epithelium in KCS eyes expresses beta-defensins which are antimicrobial peptides absent in the normal conjunctival epithelium.[11]

The remodeling of chromatin from a more compacted to a relaxed state, as seen in KCS (vs. control), involves epigenetic modifications, such as histone acetylation, which may occur in response to microenvironmental stimuli and have been implicated in the pathogenesis of human autoimmune diseases such as systemic lupus erythematosus and Sjögren's syndrome.[56,57] Sjögren's syndrome is a chronic autoimmune epithelitis that affects salivary and lacrimal glands, resulting in a clinical phenotype of dry eye very similar to that of canine KCS.[58] Although the etiology of canine KCS has not been investigated in this study, it is believed that many cases are immune-mediated.

In dogs, the degree of chromatin remodeling of the conjunctival cells seems to correlate with the severity of KCS, since the Spearman's correlation analyses revealed that animals with lower values of STT1 had more relaxed chromatin (< OD values). The association between chromatin compaction and tear production reinforces the concept that external microenvironment stimuli can act as effectors of chromatin remodeling in cells on the conjunctival surface.

In conclusion, our results showed that KCS promotes changes in nuclear morphology and chromatin of epithelial cells and lymphocytes on the conjunctival surface of dogs. The changes described in this study, particularly in chromatin, are different from those reported for conjunctival cell nuclei of human KCS patients and are indicative of increased transcriptional activity.

ACKNOWLEDGEMENTS

This manuscript is a part of the Master's thesis of Daniela Moura dos Santos and the scientific initiation project of Marcella Rosa Filezio. Funding was provided by the Sao Paulo Research Foundation – FAPESP (Proc. 2012/17308-5), and the National Council for Scientific and Technological Development–CNPq (Proc.467289/2014-0).

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CAPÍTULO 3 – Artigo Científico[1]

Effects of cyclosporin A combined with artificial tear substitute on the nuclear phenotypes of conjunctival epithelial cells in dogs with keratoconjunctivitis sicca

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Running title: Conjunctival epithelial cell nuclei in treated KCS

De acordo com as normas do periódico "Veterinary Ophthalmology". Disponível em http://onlinelibrary.wiley.com/journal/10.1111/(ISSN)1463-5224/homepage/ForAuthors.html

Abstract

Objective In a previous study we observed that epithelial cells in the conjunctiva of dogs with keratoconjunctivitis sicca (KCS) accumulate chromatin changes suggestive of increased transcriptional activity. The present study aims to evaluate whether these changes regress or disappear after institution of therapy with 0.2% cyclosporin A (CsA) and artificial tears.

Animal studied Sixteen dogs (32 eyes) free of ocular disorders and 16 dogs (32 eyes) with KCS evaluated at the diagnosis time and after 30 and 60 days of treatment.

Procedures Eyes were evaluated for the presence of clinical signs of KCS and for tear production. Preparations of cells collected from the canine palpebral inferior conjunctiva by brush cytology were subjected to the Feulgen reaction to detect nuclear DNA or impregnated with silver ions to detect nucleolar organizing regions (NORs). With the aid of light microscopy and video image analysis, the material was evaluated for the lymphocyte content and for the chromatin supraorganization and the intranuclear distribution of NORs in conjunctival epithelial cells.

Results The protocol used to treat KCS was associated with a significant reduction in clinical signs of keratitis but failed to restore the tear production in 15 dogs. Treated KCS samples showed less lymphocytic infiltration than did untreated KCS samples. The chromatin changes observed in epithelial cells of KCS dogs regressed but did not disappear after 60 days of treatment. Analysis of the NORs associated with ribosomal DNA biogenesis showed no difference in epithelial cell kinetics between the control and KCS samples after treatment.

Conclusions Chromatin changes observed in epithelial cells in the palpebral conjunctiva of dogs with KCS regress but do not disappear after 60 days of treatment with CsA and artificial tears.

Key words: chromatin, dry eye, cell metabolism, ocular surface, autoimmune disease, tear.

INTRODUCTION

Canine keratoconjunctivitis sicca (KCS), also known as dry eye disease, is a multifactorial disorder of tears and ocular surface, usually defined as a Schirmer tear test 1 (STT1) reading less than 10 mm/min concomitant with pathological changes in the cornea and conjunctiva.[1–4] Knowledge of the cellular pathophysiology of canine KCS has increased considerably due to recent advances in the fields of microscopy and imaging, among others.[5–7]

By using an approach that combined advanced light microscopy and video image analysis, we have recently demonstrated that canine KCS is associated with changes in the compaction degree of the chromatin of epithelial cells in the inferior palpebral conjunctiva.[7] Chromatin is the macromolecule that stores the genetic information and directs the transcriptional activities of a cell,[8] and the chromatin supraorganization pattern found in epithelial cells on the conjunctival surface of KCS dogs is suggestive of increased transcriptional activity.[7]

There is evidence that chromatin changes in epithelial cells in the conjunctiva with KCS correlate to the severity of the disease and result from the stress and the aversive stimuli that occur in the damaged ocular surface.[7] However, new studies are needed to validate this evidence, especially since many cases of canine KCS are immune-mediated and the predisposing factors to autoimmune diseases also lead to changes in the high-order chromatin structure of different cell types.[7] Altered chromatin supraorganization may both contribute to the development of pathological manifestations in the ocular surface with KCS and represent mechanisms of cellular adaptation to the conjunctival microenvironment altered by the disease. However, the implications of chromatin changes to the vital functions of epithelial cells in the KCS conjunctiva, including biosynthesis of protein and proliferation, have not been established yet; knowledge in this respect is important and may help to improve treatments. Microenvironment-mediated deleterious changes in the chromatin of a cell may or may not regress after the pharmacological blockade or the interruption of an aversive external stimulus.[7–9]

Information on the repercussion of chromatin changes to the cell function and on the reversibility of disease-related chromatin changes can be obtained *in situ* from

cytological preparations following appropriate topochemical methods. A valid way to access the implications of chromatin changes in the biosynthetic and proliferative processes of epithelial cells in the KCS conjunctiva is to study the intranuclear distribution of nucleolar organizing regions (NORs), which are argyrophilic segments of DNA expressing genes involved in the assembly of one of the ribosomal particles.[9] To establish whether changes in the chromatin of conjunctival epithelial cells in KCS patients regress ou disappear after interrupting or reducing external pathological stimuli, such as inflammation, a reliable way is to study nuclear phenotypes related to chromatin/DNA functionality of these cells before and after treatment to restore tear production, reduce lymphocytic infiltration, and decrease inflammatory processes.

Routinely the clinical treatment of canine KCS involves local use of tear substitutes and 0.2% cyclosporin A (CsA).[10-12] Tear substitutes increase the stability of the tear film, prevent tear evaporation, remove pathogens from the ocular surface, and stimulate the proliferation of corneal and conjunctival epithelial cells.[13] CsA is an immunomodulatory drug that has a lacromimetic action and that inhibits the serine/threonine phosphatase activity of calcineurin, interrupting the lymphocytic infiltration and the synthesis of proinflammatory cytokines, notably interleukin-2.[14-17] Although CsA has affinity for T lymphocytes, inhibiting the activation of these cells, there is evidence that it also affect the conjunctival epithelial cells, influencing the mitochondrial activity and the apoptotic mechanisms.[18] It is not known whether CsA exerts direct effects on epithelial cell chromatin in the conjunctiva with KCS. However, there is evidence that prolonged use of CsA is associated with DNA damage and the development of tumors in humans, including conjunctival tumors, although no occurrence in dogs has been reported in the literature.[19] Aside from that, conjunctival epithelial cells also have calcineurin, which acts as a growth factor and whose activation occurs by pathways involving calmodulin, a molecule that plays a key role in the activation of the remodeling complexes that elicit changes in chromatin structure.[20]

The contribution of this study was to evaluate the nuclear phenotypes related to chromatin functionality and the distribution of NORs in conjunctival epithelial cells of dogs with KCS before and after treatment. We investigated whether nuclear and

chromatin changes that accompany the progression of canine KCS regress after 30 and 60 days of treatment with 0.2% CsA and artificial tear substitute.

MATERIAL AND METHODS

Ethics

This study adhered to the ARVO Statement for Use of Animals in Ophthalmic Vision and Research. All procedures involving dogs were performed according protocols approved by the Ethics Committee on the Use of Animals in Research of the FCAV/Unesp (protocol No. 329/16) and followed the guidelines established by the Brazilian College of Animal Experimentation (COBEA). All dog owners included in the study provided an informed consent statement.

Animals and experimental design

Sixty-four eyes of 32 dogs belonging to the brachycephalic or mesocephalic breeds, aged over one year, and free of systemic diseases, as revealed by clinical, hematological (complete blood count), and biochemical (creatinine and alanine aminotransferase) exams, were evaluated using Schirmer tear test 1 (STT1) (Schirmer strip, Ophthalmos SA, Sao Paulo, Brazil), slit lamp biomicroscopy (Kowa, Tokyo, Japan), applanation tonometry (Tonopen XL®, Reichert, NY, USA), indirect ophthalmoscopy (Eyetec, Sao Carlos, Brazil), fluorescein stain (Fluorescein strips, Ophthalmos SA), tear breakup time (TBUT), and response to menace reflex.

The dogs were distributed into KCS and control groups. The KCS group consisted of 16 dogs (32 eyes), 7 with a brachycephalic skull shape and 9 with a mesocephalic skull shape, male (n = 7) or female (n = 9), aged between 2 and 13 years (mean ± standard deviation, 8.06 ± 3.15), presenting bilateral KCS characterized by STT1 values ≤ 10 mm/min,[2,7] without comorbidities. The control group control consisted of 16 dogs (32 eyes), 8 with a brachiocephalic skull shape and 8 with a mesocephalic skull shape, male (n = 9) or female (n = 7), aged between 2 and 13 years (8.50 ± 4.83), presenting STT1 values ≥ 15 mm/min,[2,7] without any

ocular disorder. None of the dogs included in the study had a history of neoplasms of any kind or were using immunomodulatory drugs or antibiotics. The study groups were homogeneous for skull shape ($P = 0.76$, Wilcoxon test), sex ($P = 0.54$), and age ($P = 0.57$).

As treatment, eyes in the KCS group are receiving local 0.2% CsA ophthalmic ointment (Optimmune, Intervet Schering-Plough, Sao Paulo, Brazil) at regular intervals of 12 h and 0.2% polyacrylic acid-based artificial tears (Vidisic®, Bausch & Lomb, Rochester, NY, USA) at regular intervals of 4 h.

The dogs remain under clinical follow-up. For the present study, data on clinical signs (discharge, conjunctival hyperemia, and corneal edema and vascularization), blink-to-threat reflex, and tear production of KCS eyes, as well as the conjunctival epithelial cells, were collected at the time of diagnosis and 30 and 60 days after initiation of treatment.

Collection of epithelial cells of the inferior palpebral conjunctiva

Epithelial cells were collected by brush cytology from the inferior palpebral conjunctival surface, as previously described.[7] The ocular surface was anesthetized with one drop of 0.5% proxymetacaine (Anestalcon®, Alcon, Sao Paulo, Brazil) and the inferior palpebral conjunctiva was pulled down and scraped using a sterile cervical cytobrush (Kolplast Ltda, Itupeva, Brazil). The cells were spreads on glass slides (Knittel, Braunschweig, Germany) and fixed for 10 min in a mixture of 70% ethylic alcohol (Ecibra, Sao Paulo, Brazil), glacial acetic acid (Synth, Sao Paulo, Brazil), and 10% buffered formalin (Dinâmica, Sao Paulo, Brazil); 3:1:1, vol/vol/vol.[7]

Chromatin/DNA topochemistry

To detect chromatin/DNA, cytological preparations were subjected to the Feulgen reaction, which consisted of acid hydrolysis in 4 M HCl (Vetec, Rio de Janeiro, Brazil) for 45 min and subsequent exposure of the hydrolyzed material to a reactive Schiff base (Merck, Darmstadt, Germany) for 40 min.[7] All steps of the Feulgen reaction were done in an air-conditioned laboratory at a constant

temperature of 25°C. Following the staining, the samples were washed in sulfurous water (mixture of distilled water, 1 M HCl, and 10% sodium metabisulfite; 18:1:1, vol/vol/vol), cleared in xylene (Synth, Sao Paulo, Brazil), and mounted with natural Canada balsam (Vetec, Rio de Janeiro, Brazil).

Silver-impregnated NOR (AgNOR) banding

To detect NORs, cytological preparations were incubated at 37°C for 30 min, in a mixture containing two parts of a 50% aqueous solution of silver nitrate (Synth) and a part of colloidal gelatin (Sigma-Aldrich, St. Louis, MO, USA) dissolved in a 1% aqueous solution of formic acid (Synth).[9] The samples were then washed in distilled water, air dried, and mounted with natural Canada balsam.

Relative frequency of lymphocytes

Feulgen-stained samples were studied for presence of lymphocytes whose frequencies (%) were estimated for a total population of 500 conjunctival surface cells evaluated from five microscopic fields randomly chosen in each cytological preparation. The evaluations were conducted under light microscopy by a single blinded examiner.

Video image analysis

Conjunctival epithelial cell nuclei (n = 110) subjected to the Feulgen reaction or to the AgNOR banding were analyzed under an Olympus BX53 microscope (Tokyo, Japan), equipped with UPLAN Neofluar 100/0.75 objective, optovar factor 2, 100-W halogen lamp, Köhler illumination, 0.90 condenser, and a interferential filter (Edmund Optics, Barrington, NJ, USA) to obtain monochromatic light of 546 nm.[7,9,21] With a high-resolution video camera (Olympus), the nuclear images were transmitted from the microscope to a computer, where they were digitized, converted to 8-bits, and studied using ImageJ software (National Institute of Health, Bethesda, MS, USA). The operating conditions of the microscope, with particular attention to the intensity of the light emitted by the halogen lamp, were the same for all samples

studied. The spatial calibration of the video image analysis system was done with a micrometer ruler for microscopy (Laxco Inc. Bothell, WA, USA), and the threshold technique used for segmentation of the cell nuclei was semiautomatic. Overlapping, damaged or unfocused nuclei were excluded from the evaluations. Separate and intact nuclei were examined from three microscopic fields chosen at random from each cytological preparation. All assessments by video image analysis were conducted by two experienced examiners who were masked to study groups.

Feulgen-stained nuclei (n = 100) were studied for total area (S_T), in square micrometers; fraction of area (Sc%) showing absorbance above a selected cutoff point (> 0.66), which correspond to the more compacted chromatin; total absorbance (A_T), which reports on chromatin compaction states; absorbance of the area covered by the more compacted chromatin (Ac); total integrated optical density (IOD, in arbitrary units (AU)), which reports on Feulgen-DNA content; and average absorption ratio (AAR = $(Ac/Sc\%)/(A_T/S_T)$), a dimensional parameter that expresses how many times the absorbance of more compacted chromatin exceeds that of the entire nucleus.[22] The value of the cutoff point (absorbance > 0.66) used to discriminate the more condensed chromatin was established from preliminary studies involving the control group samples.

Surface plot graphs (ImageJ > Analyze > Surface plot) were constructed to represent the compaction states of chromatin based on the topography of different bitmap pixel values from the nuclear images. Prior, pseudocolors (i.e., false colors) were applied (ImageJ > Image > 8-bit > Lookup table > Royal > Apply LUT).[7,9]

Scatter diagrams relating AAR to Sc% were plotted as previously proposed.[22,23] These diagrams allow for the discrimination of the position of points that correspond to specific nuclear phenotypes.

The size of NORs (in square micrometer) and the fractions of the nuclear area occupied by them were quantified in the nuclei (n = 50) subjected to AgNOR banding. Points with absorbance values below 0.020 were considered backgrounds and removed from the nuclear images. An absorbance value above 0.62 was used as the cutoff point to discriminate AgNOR-positive nuclear areas after preliminary tests performed using the control group samples.[9]

Statistical analyses

All data were tested for statistical normality using the Kolmogorov-Smirnov test. Kruskal-Wallis test and Friedman test with Dunn's post hoc test for multiple comparisons were used for statistical analyses because the distributional assumptions required for parametric testing were not satisfied. The Spearman's coefficient (*r*) was used as a measure of association between the study variables. Differences with $P < 0.05$ were considered significant, with 95% confidence interval (95% CI). All calculations were performed using the Minitab 12™ (Minitab, State College, San Diego, CA, USA) and the MedCalc (version 9.3.6.0, MedCalc, Mariakerke, Belgium) software programs.

RESULTS

Clinical features of the eyes in the KCS group before and after treatment

At diagnosis time, the eyes in the KCS group presented intense mucopurulent secretion, moderate conjunctival hyperemia, diffuse corneal edema, corneal vascularization (Fig. 1 a), and negative menace reflex. Resolution or attenuation of the clinical signs of KCS was only observed 60 days after initiation of treatment, with dogs showing a positive menace reflex (Fig. 1 b).

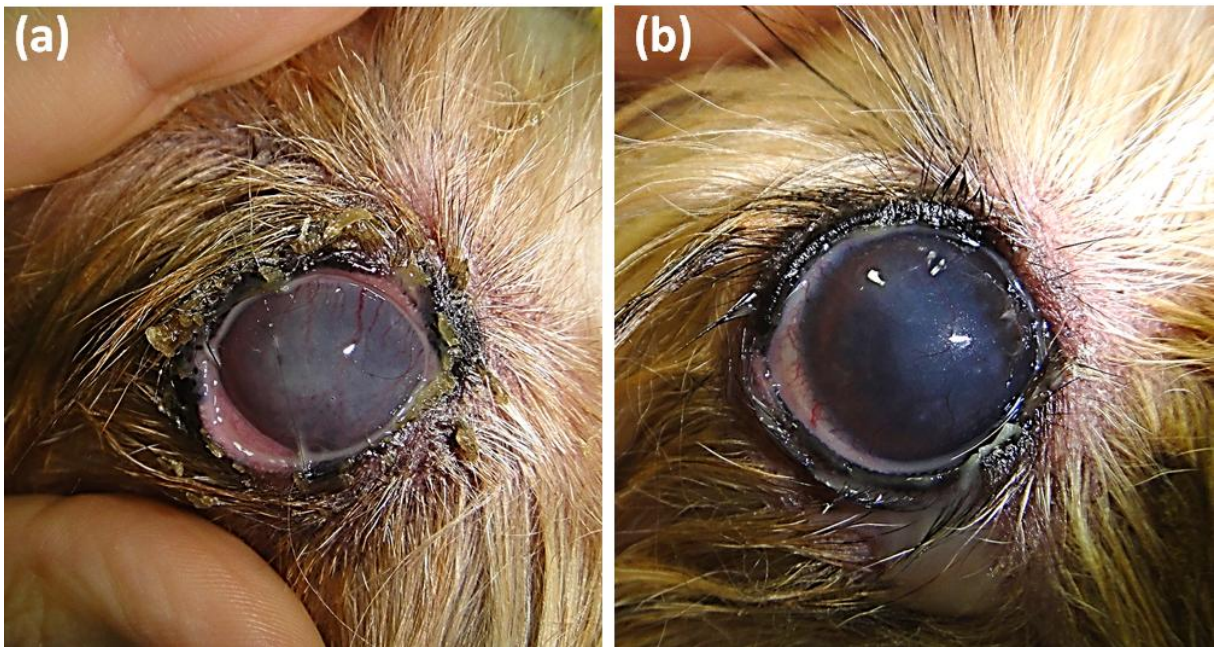

Figure 1. Clinical ocular feature of a patient in the KCS group, at diagnosis (a) and after 60 days of treatment with 0.2% cyclosporin (b). At the time of diagnosis, the eye presented intense mucopurulent secretion, moderate conjunctival hyperemia, diffuse corneal edema, corneal vascularization, and negative menace reflex. Sixty days after initiation of treatment, a decrease in mucoid secretion, regression of conjunctival hyperemia and corneal edema, reduction of vascularization, and a positive menace reflex were observed.

Tear production

The values of STT1 (mm/min) and TBUT (sec) obtained for both control and KCS groups are shown in figure 2. Compared with the control eyes, the eyes in the KCS group presented reduced median values of STT1, both before and after initiation of treatment (Fig. 2 a) ($P < 0.05$). No differences in median TBUT values were observed between control eyes and those with KCS treated for 30 or 60 days (Fig. 2 b) ($P > 0.05$).

Although the protocol used for treating the KCS has resulted in a significant increase in the median values of STT1 ($P < 0.05$ vs. diagnosis time), boxplot graphs (Fig. 2 a) revealed a high dispersion and heterogeneity in the variance of the data set, evidencing that the treatment failed to improve the tear production. Eighteen of 32 eyes (56.25%) with KCS maintained STT1 values below 15 mm/min (normative value) after 60 days of treatment.

The TBUT values obtained in this study were highly heterogeneous (Fig. 2 b), even in the control group, probably because half of the dogs included in the study had a brachycephalic skull conformation which favors tear evaporation.

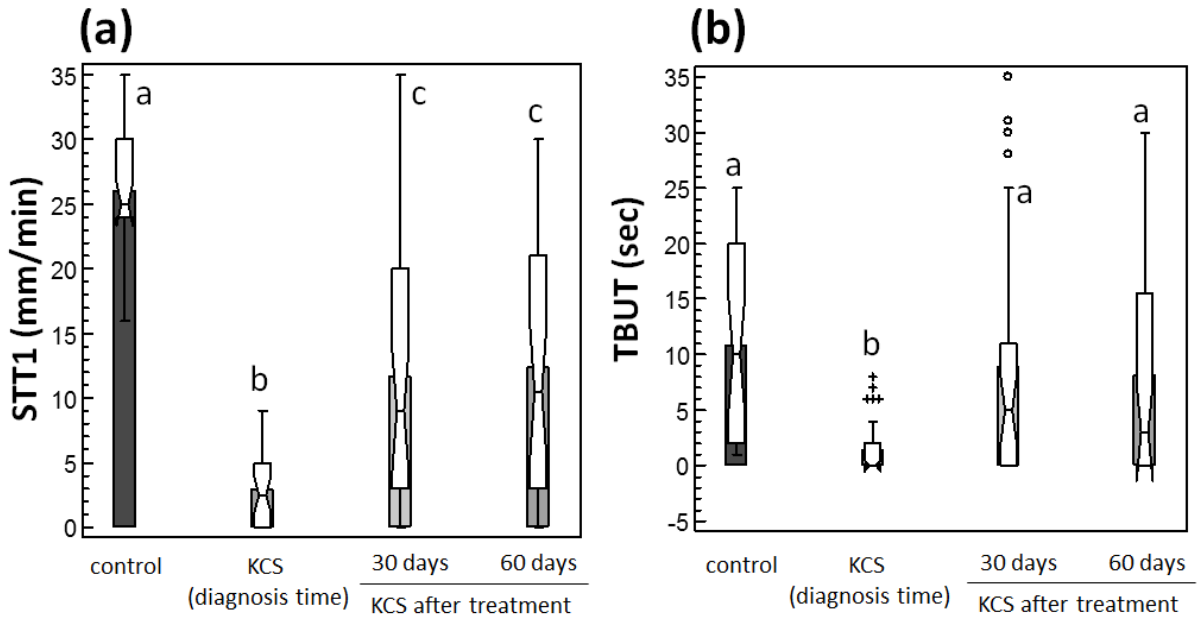

Figure 2. Boxplot graphs, combined with bar graphs of median values, with 95% CI for median (error bars), related to tear production parameters in dogs free from ophthalmic disorders (controls) and in dogs with KCS, before and after 30 and 60 days of treatment. In (a), STT1 values (mm/min). In (b), TBUT values (sec). Different letters represent the statistical differences. $P < 0.05$ was considered significant.

Relative frequency of lymphocytes

Lymphocyte frequencies were 7.38% (range, 2.41–13.15%) for control group samples, 72.59% (range, 48.66–91.38%) for samples from the KCS group collected at the diagnosis time, 44.23% (range, 17.06–52.72%) for samples from the KCS group collected 30 days after the initiation of treatment, and 19.53% (range, 7.14–31.12%) for samples from the KCS group collected 60 days after the treatment.

Video image analysis parameters for Feulgen-stained nuclei

Color digital images of Feulgen-stained nuclei were converted for gray levels (8-bits) and used for establishing nuclear phenotypes of conjunctival epithelial cells. After the binarization of the images, cell nuclei showed varying intensity of mean gray

values, corresponding to different supraorganizational patterns (compaction and texture) of the chromatin. Only a digital image of a control sample is displayed (Fig. 3 a) because differences of nuclear phenotypes among the groups studied can only be discernible by software-assisted image analysis, and not visually. Fig. 3 b displays conjunctival epithelial cell nuclei after image treatment that allows us to discriminate the areas of more compacted chromatin.

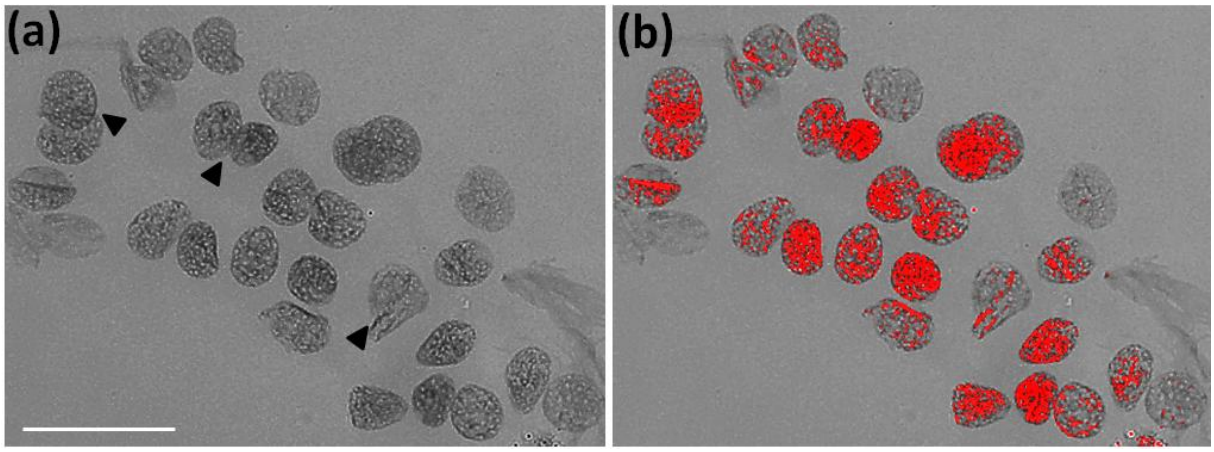

Figure 3. Digital images of Feulgen-stained nuclei (control sample). In (a), cell nuclei converted to the binary system, using ImageJ software. Note that the nuclei have varying intensities of mean gray levels, which correspond to different patterns of compaction and chromatin texture. In (b) the same nuclei of (a), after application of threshold masks (red) for selection of the areas containing the more condensed chromatin. Overlapping, damaged or unfocused nuclei (arrowheads) were excluded from the evaluations. Bar = 50 µm.

Details of chromatin compaction could be observed from representative surface plots (Fig. 4 a–d) constructed after the application of pseudocolors to the nuclear images. Compared with the control nuclei, the nuclei in the KCS group, both before and after treatment, showed extensive areas of less compacted chromatin (pink pseudocolor) and reduced areas of more compacted chromatin (blue pseudocolor).

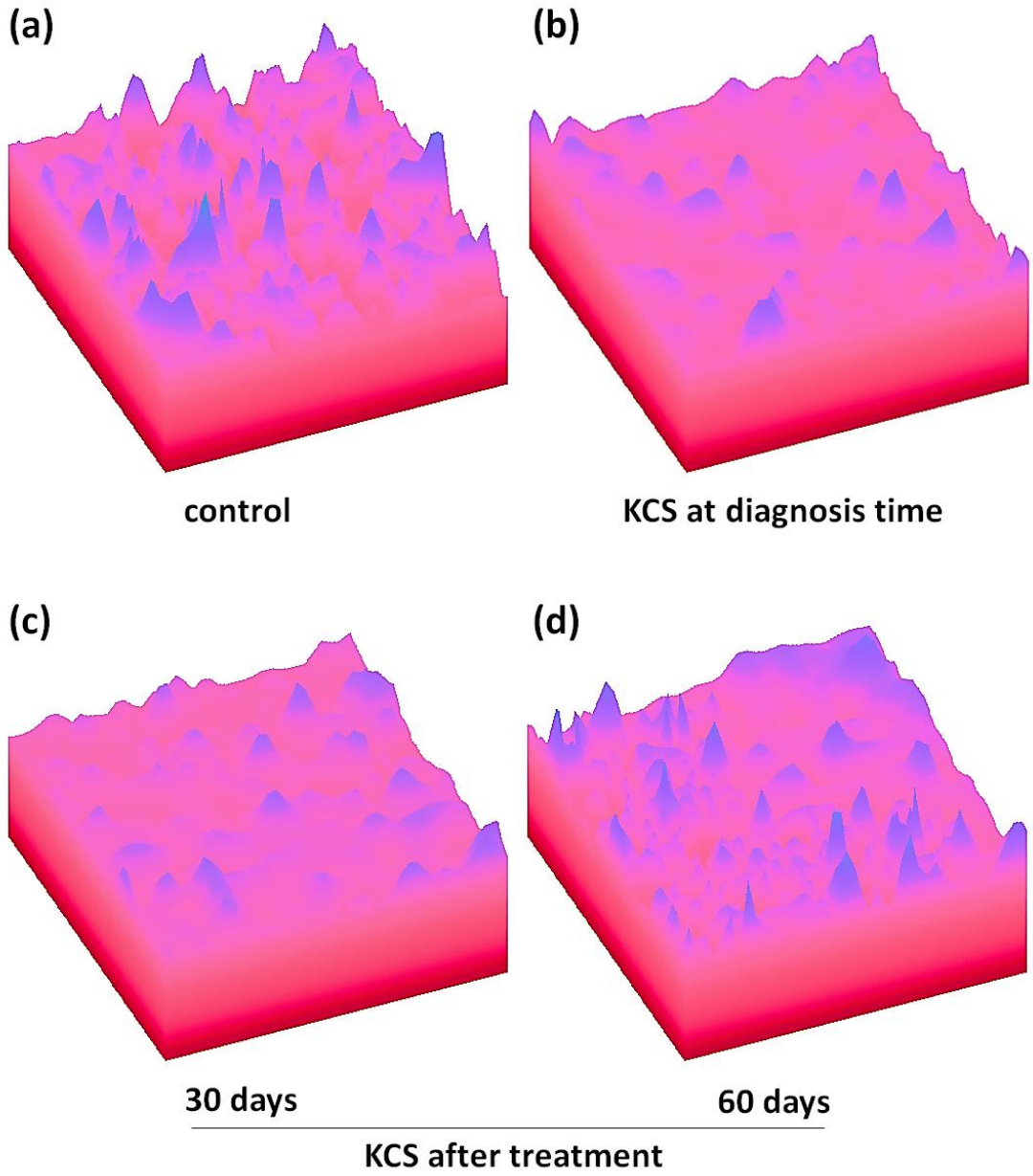

Figure 4. Surface plot graphs representative of the compaction states of chromatin. (a) control nuclei; (b) conjunctival epithelial cell nuclei in the KCS group at diagnosis time; (c), KCS nuclei after 30 days of treatment; (d), KCS nuclei after 60 days of treatment. Pink pseudocolor corresponds to the nuclear areas of less compacted chromatin, and blue pseudocolor corresponds to the areas of more compacted chromatin.

Statistical comparisons between nuclear parameters obtained by video image analysis for conjunctival epithelial cells of the different samples that composed this study are presented in Table 1.

Table 1. Video image analysis parameters obtained for Feulgen-stained nuclei

Parameters	Study samples											
							KCS after treatment					
	Control			KCS (at diagnosis time)			30 days			60 days		
	Median	Q1	Q3	Median	Q1	Q3	Median	Q1	Q3	Median	Q1	Q3
Sc%		15.61	79.16	6.14[b]	1.75	18.25	9.60[c]	7.29	53.27	22.30[d]	5.13	55.13
S_T (μm^2)	183.79[a]	163.92	224.49	218.02[b]	170.67	289.54	148.26[c]	119.78	169.85	194.61[a]	159.17	232.68
AAR	1.05[a]	1.02	1.05	1.12[b]	1.10	1.16	1.05[a]	1.03	1.07	1.05[a]	1.03	1.09
Ac	0.68[a]	0.66	0.69	0.71[b]	0.66	0.72	0.69[a]	0.66	0.70	0.68[a]	0.66	0.69
A_T	0.65[a]	0.63	0.65	0.59[b]	0.58	0.61	0.62[c]	0.61	0.63	0.63[c]	0.31	0.65
IOD (AU)	128.93[a]	106.24	151.41	132.87[b]	100.18	173.51	91.89[c]	74.68	106.44	123.86[a]	106.00	150.44

Results are expressed as median and interquartile intervals (Q1 and Q3).
Different letters in the same line represent the statistical differences. $P < 0.05$ was considered significant.
Comparisons were performed using the Kruskal-Wallis test and the Friedman test with Dunn's post hoc test.

Conjunctival epithelial cell nuclei in samples of KCS dogs treated for 30 days were smaller in size (< S_T values) and had less DNA (< IOD values) than the cell nuclei in controls or in samples of dogs with KCS untreated ($P < 0.05$ for all) (Table 1). No differences in S_T and IOD were observed among the nuclei in samples of KCS patients treated for 60 days and those in control samples ($P > 0.05$); both samples had smaller nuclei and less DNA when compared to untreated KCS samples (Table 1).

Nuclei in samples of KCS dogs, both before and after treatment, presented reduced A_T values, compared to the nuclei in the control samples ($P < 0.05$ for all) (Table 1).

Figure 5 correspond to the scatter diagram of the Sc% values as a function of AAR, which allows to discriminate specific nuclear phenotypes (related to chromatin functionality) of conjunctival epithelial cells.

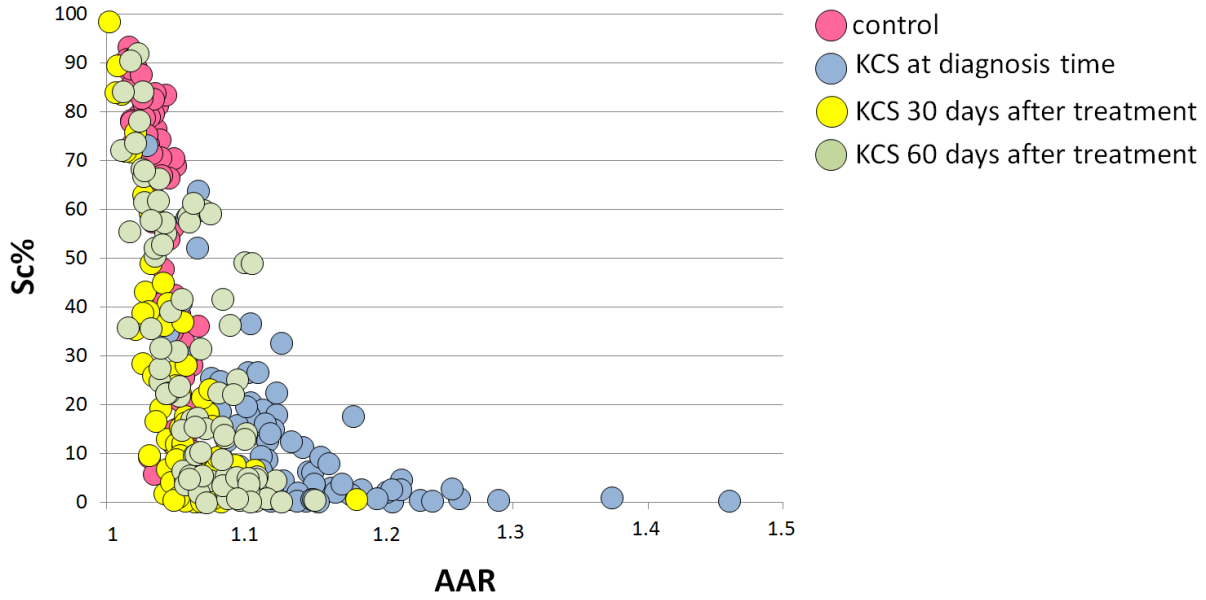

Figure 5. Scatter plot of Sc% values (nuclear fraction of chromatin covered plus condensed) as a function of AAR values (dimensional parameter that expresses how many times the absorbance of nuclear areas with more condensed chromatin exceeded that of the entire nucleus).

At the time of diagnosis, dogs in the KCS group presented conjunctival epithelial cells with loose chromatin, characterized by a significant reduction of the Sc% values concomitant with an elevation of AAR values ($P < 0.05$ vs. controls) (Fig. 5) (Table 1). A sort manipulation (Minitab 12™ software) of the data allowed us to

detect that 20.96% of the conjunctival epithelial cell nuclei of dogs with untreated KCS (diagnosis time) had AAR values above 1.2, whereas all nuclei of the other samples studied had AAR below this value. There are no differences between the AAR values obtained for cell nuclei of the KCS dogs treated for 30 or 60 days and those observed for control nuclei ($P > 0.05$) (Table 1).

Treatment with 0.2% CsA and tear substitute elicited changes in the nuclear phenotypes of the conjunctival epithelial cells of the KCS dogs, resulting in increase of Sc% values ($P < 0.05$ vs. KCS at time of diagnosis), i.e., the extent of areas covered by more compacted chromatin. We have calculated that the frequencies of nuclei that had at least half of their S_T occupied by more compacted chromatin (absorbance > 0.66) were 51.83% for the control group, 0.9% for the samples of dogs with untreated KCS, 13.09% for the samples of KCS dogs treated for 30 days, and 28.73% for samples of KCS dogs treated for 60 days. It was also calculated that the frequency of fully loosened chromatin nuclei, presenting less than 2% of S_T occupied by more compacted chromatin, were 2.40% for the control group, 25.92% for the samples of dogs with untreated KCS, 20.23% for the samples of KCS dogs treated for 30 days, and 11.49% for samples of KCS dogs treated for 60 days.

The AAR and Sc% values observed for nuclei in both the control and untreated KCS samples were significantly influenced by the STT1 values, as revealed by the Spearman's coefficient analysis (Table 2). However, surprisingly, the AAR and Sc% values for conjunctival epithelial nuclei of dogs with KCS treated for 30 or 60 days had no association with STT1 values (Table 2). AAR and Sc% values have not association with TBUT values ($P > 0.05$).

In all groups studied, Sc% values, but not AAR values, had a negative association with the presence of lymphocytes, that is, the less lymphocyte in the samples, the greater the Sc% values of the conjunctival epithelial cell nuclei (Table 2).

Table 2. Correlations of Sc% and AAR with tear production (STT1) and lymphocyte frequency in the studied samples

Samples	Correlations involving Sc%			
	STT1		Lymphocyte frequency	
	r	*P*	*r*	*P*
Control	0.781	<0.01	–0.813	<0.01
KCS at diagnosis time	0.643	<0.01	–0.745	<0.01
KCS after 30 days of treatment	0.029	=0.31	–0.791	<0.01
KCS after 60 days of treatment	0.071	=0.10	–0.781	<0.01
	Correlations involving AAR			
	STT1		Lymphocyte frequency	
	r	*P*	*R*	*P*
Control	0.761	<0.01	0.029	=0.31
KCS at diagnosis time	0.591	<0.01	0.033	=0.34
KCS after 30 days of treatment	0.023	=0.31	0.031	=0.33
KCS after 60 days of treatment	0.051	=0.09	0.029	=0.31

P < 0.05 was considered significant (Spearman's coefficient analysis).
TBUT was not included in the Table because the statistical analysis revealed that it has no influence on Sc% and AAR values.

Distribution of NORs

Figure 6 (a and b) corresponds to images of conjunctival epithelial cell nuclei subjected to AgNOR banding, illustrating differences in NOR content between control samples and those of dogs with untreated KCS. Assessments by video image analysis (Fig. 6 c and d) revealed that nuclei in the samples of dogs with untreated KCS have larger NORs, occupying larger area fractions, compared to nuclei in the samples of control dogs ($P < 0.01$) Treatment with 0.2% CsA and tear substitute reduced the size of NORs as well as the fraction of nuclear area occupied by them. No differences in size and distribution of NORs were observed between control samples and those with KCS treated for 30 or 60 days ($P > 0.05$).

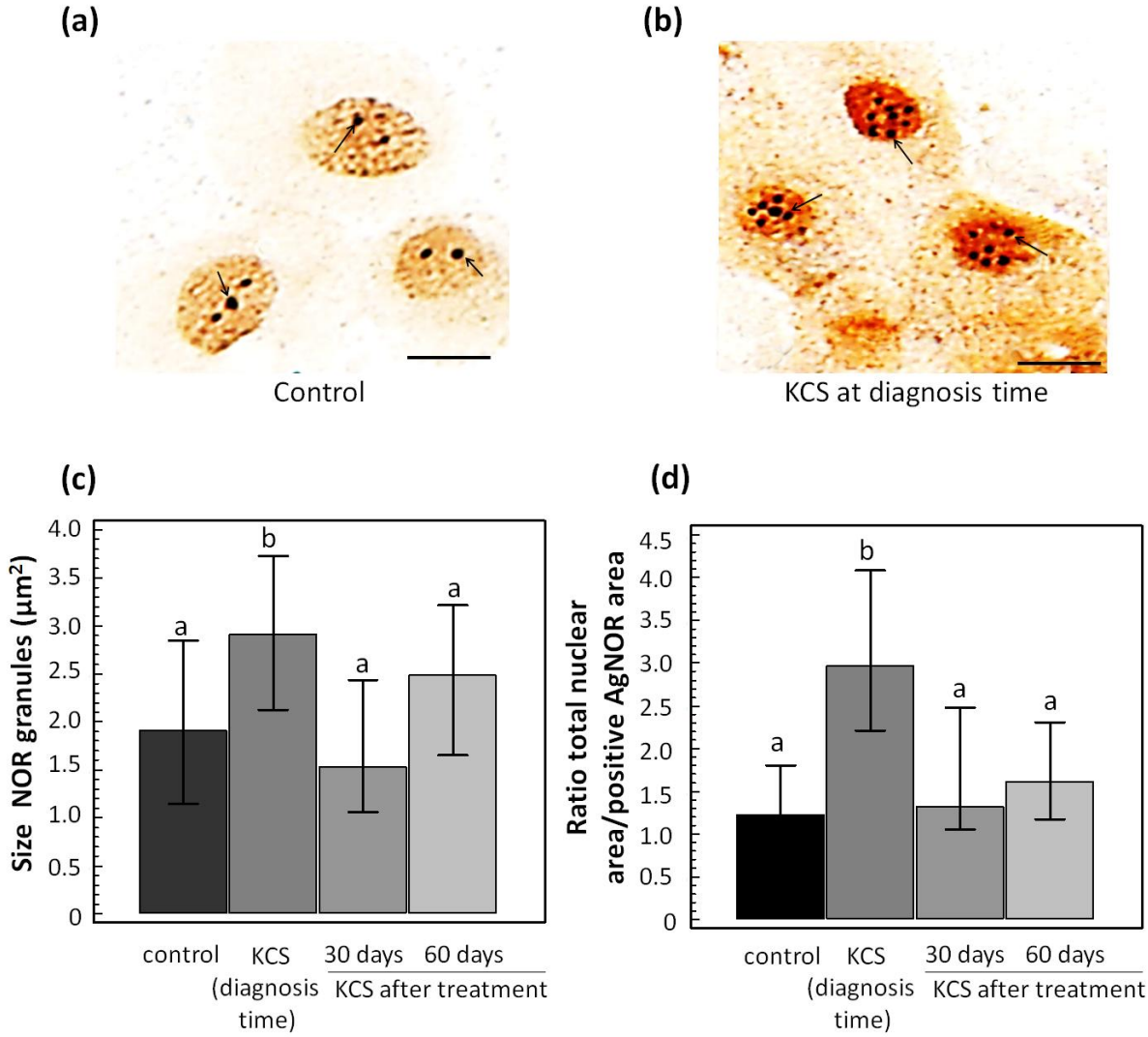

Figure 6. (a) and (b), photomicrographs of conjunctival epithelial cell nuclei of control and KCS dogs, respectively, after AgNOR banding. The dark brown granules within the cell nuclei correspond to NORs (heads). Bar = 10 mm. (c) and (d), graphs of median values, with interquartile intervals (error bars), related to the size of positive AgNOR granules and fractions of the nuclear area occupied by NORs. Different letters represent the statistical differences. $P < 0.05$ was considered significant.

DISCUSSION

Canine KCS is a disease, usually immune-mediated, associated with changes in the anatomy or function of some of the components of the lacrimal functional unit.[24] The most striking clinical features of KCS are the inflammation of the ocular surface, including the adnexa, and the reduction or absence of tear production, which makes the eye predisposed to lesions.[25]

In our service, 0.2% CsA ophthalmic ointment combined with a tear substitute is the first choice to treat canine KCS due to its immunomodulatory and lacromimetic

properties, as well as its efficacy and safety as evidenced by previous studies.[26–28] Ophthalmic CsA act specifically on suppressing T helper lymphocytes (CD4+) and even when used for long periods, at concentrations of 0.2% to 2%, it does not reach significant blood concentrations.[29]

Interestingly, in the present study, 56.25% of the dogs treated with 0.2% CsA and with artificial tears for 60 days had no significant increase in tear production, with STT1 values below those considered normal for dogs.[30,31] This poor result may be related to the clinical characteristics of the dogs that composed the KCS group and suggest that CsA in concentrations higher than that used in this study (0.2%) may be a better option to treat dogs diagnosed with a total absence of tears and/or presenting a brachycephalic skull conformation which favors the tear evaporation.

Although the therapeutic protocol used in this study was not 100% effective in restoring the tear production of dogs in the KCS group, it resulted in a significant regression of all clinical signs of KCS that, in addition to being detected by ophthalmic evaluations, was perceived and reported by the dog owners. Probably the regression of the clinical signs of KCS, despite the low values of tear production, occurred due to the anti-inflammatory action of 0.2% CsA and its scientifically proven ability in reestablishing the functionality of goblet cells.[32] The use of artificial tears is also a contributing factor to the regression of KCS signals as it helps remove pathogens and proinflammatory cytokines from the ocular surface and stimulates the proliferation of epithelial cells.

It is widely recognized that KCS-related ocular surface inflammation is mediated by lymphocytes.[33] Based on earlier immuno-histopathological evaluations[34], proposed that human patients with both Sjögren syndrome-related as well as non-Sjögren syndrome KCS have identical conjunctival inflammation manifested by T lymphocytes infiltrates, which suggest that clinical symptoms of KCS may be dependent on T lymphocyte activation. In this context, our results suggest a relationship between the presence of lymphocytes on the inferior palpebral conjunctival surface and clinical symptomatology of dogs in the KCS group. Samples of conjunctival cells collected before treatment showed high lymphocyte content (72.95%); however, treatment of KCS resulted in a significant reduction of the number of lymphocytes (44.23% at 30 days and 19.53% at 60 days), which can be

attributed to the modulating effect of CsA and apoptosis of lymphocytes, with subsequent reduction of inflammatory processes.[33]

Conjunctival samples to evaluate the frequency of lymphocytes and nuclei of the epithelial cells were collected by brush cytology, a technique that is easy to perform and causes little discomfort to the patient.[35–37] Cells were collected from the inferior palpebral conjunctival surface because it is easy to access, with less risk of damaging the cornea.[7] Brush cytology resulted in preparations with high content of cells with pre-preserved morphology and few overlapping cells, as reported by other authors who also used this method for collecting conjunctival cells.[38–41] The cell types observed in the control and KCS samples are those reported by different authors in previous studies,[28,38–41] no atypical morphology was detected.

Conjunctival cytological preparations were submitted to the Feulgen reaction[21,42,43] which is the gold standard method for detecting nuclear DNA and used to evaluate nuclear phenotypes of conjunctival epithelial cells of dogs with KCS before and after treatment. We evaluated whether chromatin changes in conjunctival epithelial cells of dogs with KCS regress or disappear after institution of therapy that disrupts or blocks aversive processes on the ocular surface with KCS. The results obtained in this study for dogs of the KCS group prior to treatment replicated those from a previous study[7] where KCS was associated with chromatin loosening, suggestive of increased transcriptional activity, and with increase in Feulgen-DNA content (DNA ploidy) in conjunctival epithelial cells of dogs. Also, our results, based on the Sc% x AAR scatter plot, revealed that while the epithelial cells of the KCS conjunctiva decompress much of their chromatin (< Sc% vs controls), they increase the state of chromatin compaction in specific nuclear territories (> AAR vs. controls), probably repressing the expression of genes whose identity remains to be made in later studies.

Chromate loosening and increase in DNA ploidy levels are adaptive events that can occur in different cell types of injured tissues and appear to increase the cell response to microenvironmental stimuli.[44–47] While increased ploidy results from DNA synthesis, chromatin loosening results from a remodeling process involving ATP-dependent remodeling complexes, epigenetic enzymes, transcription factors, and modifications at cell-nuclear matrix adhesion sites and in gene activity.[48]

Remodeling of chromatin has been associated with the development of several diseases including ocular disorders so that many studies have been conducted in scope.[49–52] One of the crucial issues involving changes in the structure of cell chromatin in diseased tissues concerns reversibility. Certain changes in chromatin structure, particularly those mediated by changes in environmental stimuli, regress rapidly after pharmacological intervention for normalization of tissue homeostasis. However, there are persistent chromatin alterations, particularly those related to genetic predisposition to autoimmune diseases, whose reversal may not be achieved with traditional drugs, and it is necessary in this case to seek new therapeutic strategies, including epigenetic drugs. Undoing diseases-related chromatin changes can help restore the function of an injured cell or tissue, thereby improving clinical prognoses.[48]

The results of this study suggest that changes in chromatin supraorganization (compaction and texture) and DNA ploidy in epithelial cells of the palpebral conjunctiva of dogs with KCS regress in a time-dependent manner after institution of treatment with 0.2% CsA and artificial tears. After 60 days of treatment, the epithelial cells presented AAR values similar to those presented by healthy canine conjunctival epithelial cells, suggesting that those nuclear territories of genes that were highly repressed in cells collected at the time of diagnosis resumed their supraorganizational configuration.

Treatment with 0.2% CsA and tear substitute resulted in increase of median Sc% values ($P < 0.05$ vs. KCS at time of diagnosis), i.e., the extent of areas covered by more compacted chromatin. However, our calculations revealed that only 13.09% of the cells in the KCS group treated for 30 days and 28.73% of the cells in the KCS group treated for 60 days had the most frequent nuclear phenotype presented by the cells of the control group (more than 50% of the nuclear area occupied by more compacted chromatin). Thus, the pharmacological therapy administered in KCS patients in this study was not able to reestablish the nuclear phenotypes of conjunctival epithelial cells in terms of functionality of chromatin.

Based on our findings, we believe that stimuli associated with chromatin decompaction in cells of the untreated KCS group are multiple, and treatment with CsA was not enough to resolve all of them as demonstrated by clinical evaluations.

Although STT1 values are positively associated with the functional status of chromatin compaction in epithelial cells of normal and KCS conjunctiva, it appears that tear production is not an independent factor affecting cell nuclei. Apparently, associations between STT1 and chromatin compaction state depend on the symptomatology of the patient. In those eyes whose tear production was reduced but signs of keratitis were absent, STT1 values had no significant association with chromatin function states in epithelial cells.

The lymphocyte content in the conjunctiva was an important factor associated with the chromatin functional state of the conjunctival epithelial cells. The treated KCS samples that presented epithelial cells with nuclear phenotypes closer to those of the control were those that had less lymphocytic infiltration. This finding suggests that humoral and inflammatory responses mediated by lymphocytes are important stimuli that modify the supraorganizational pattern of chromatin in conjunctival epithelial cells. In addition, it is possible that the presence of lymphocytes and cytotoxicity of the mediators expressed by them interfere with signaling pathways that regulate the metabolism of conjunctival epithelial cells; there is evidence that the presence of lymphocytes compromises the differentiation of epithelial cells.[53]

A direct mechanism by which the pharmacological therapy used in this study, most notably CsA, may have acted on conjunctival epithelial cell nuclei, leading to chromatin remodeling and regression of chromatin alterations may be through blocking of intracellular cyclophilin. To activate cyclophilin, CsA is actively transported via P-glycoprotein pumps, and expression of genes transcribing mRNA to specific glycosyltransferases and glycoconjugates is severely compromised on the conjunctival surface of patients with KCS.[54] Calcineurin acts as a transcription factor and binds to calmodulin,[17] forming a macromolecular complex that phosphorylates histone H3 (one of the proteins that binds to DNA to form the chromatin),[55] making chromatin open to transcriptional processes. Since calcineurin is inhibited by CsA, certain modifications that may lead to chromatin loosening do not occur.

Although treatment with 0.2% CsA and artificial tears did not restore the functional states of chromatin supraorganization of epithelial cells in KCS dogs to close to those observed in controls, it was associated with a significant restoration of cell kinetics, as revealed by the analyzes of samples subjected to AgNOR banding.

Studies in AgNOR represent a valuable method of evaluating cell kinetics, as it reports on ribosomal biogenesis, protein biosynthesis, and cell migration and proliferation.[56–60] Before treatment, cells in the KCS group presented large NOR granules with extensive nuclear distribution, which suggests an intense metabolic cellular activity and corroborates with results of DNA ploidy and chromatin supraorganization, both suggestive of increased transcriptional activity. Metabolic cell demand is directly related to proinflammatory stimuli. Metabolic cell demand is directly related to proinflammatory stimuli. Many growth factors and cytokines released during inflammatory events may stimulate epithelial cells to synthesize growth and defense proteins.[60–64]

In conclusion, the results of the present study replicated those from a previous study showing that KCS is associated with changes in chromatin supraorganization of epithelial cells in the canine palpebral conjunctiva. The present study showed that accumulated chromatin changes in conjunctival epithelial cells of dogs with KCS regress in a time-dependent manner, but do not disappear, after 30 and 60 days of treatment with conventional therapy, involving immunomodulatory drug and artificial tears, which aims to reduce inflammatory processes and improve tear production. Although the treatment used in the present study did not reverse the altered nuclear phenotypes of conjunctival epithelial cells with KCS, it was associated with restoration in cellular kinetics related to ribosomal biogenesis and protein biosynthesis.

ACKNOWLEDGEMENTS

This manuscript is a part of the Master's thesis of Daniela Moura dos Santos and the scientific initiation project of Marcella Rosa Filezio. Funding was provided by the Sao Paulo Research Foundation – FAPESP (Proc. 2012/17308-5), and the National Council for Scientific and Technological Development – CNPq (Proc. 467289/2014-0). We thank MSD for providing cyclosporine ophthalmic ointment Optimmune® to the patients included in this research. We would also like to show our gratitude to the "Big Bones" English bulldog kennel for the kind contribution.

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