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Original Article
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Dry-eye syndrome in Shih Tzu dog: Ocular surface clinical and morphological aspects in the different stages of keratoconjunctivitis sicca[1]

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ABSTRACT.- Fayad A.R., Lobo T.V., da Silva R.H., Neves C.A., Arnold E., Lima A.M.V. & Moura V.M.B.D. 2023. **Dry-eye syndrome in Shih tzu dog: Ocular surface clinical and morphological aspects in the different stages of keratoconjunctivitis sicca.** *Pesquisa Brasileira Veterinária 43:e07270, 2023.* Setor de Patologia Animal, Universidade Federal de Goiás, Rodovia Goiânia – Nova Veneza Km 8, Campus Samambaia, Goiânia, GO 74001-970, Brazil. E-mail: andre_fayad@hotmail.com

This study analyzed clinical and cyto-histomorphological parameters of the ocular surface of Shih Tzu dogs, non-carriers and carriers of quantitative keratoconjunctivitis sicca (KCS) at different stages. Thirty-five eyes from 23 male and female Shih Tzu dogs between two and eight years were evaluated in four groups: control group (CG – without KCS), mild KCS group (KCS1), moderate KCS group (KCS2), and severe KCS group (KCS3). Most clinical variables among KCS carrier groups worsened at the more advanced stages of the disease, with a negative correlation between the Schirmer tear test (STT-1) and tear film break-up time (TBUT). Squamous metaplasia, lymphoplasmacytic inflammatory infiltrate, and decrease in conjunctival goblet cells on histopathological examination comprised disease severity parameters. Quantitative KCS non-carried Shih Tzu dogs have qualitative and tear film distribution changes. The cytomorphological exam is limited to evaluating the inflammatory infiltrate and quantifying conjunctival goblet cells. However, intermediate epithelial cells were higher in healthy eyes compared to eyes with KCS in Shih Tzu dogs. Also, moderate and severe KCS carrier Shih Tzu dogs have qualitative dysfunction of the tear film. KCS in Shih Tzu dogs is common and chronic and may be responsible for the loss of vision of these animals. Early identification of the disease and routine evaluation can improve these dogs' quality of life and ocular health.

INDEX TERMS: Dog, cornea, conjunctiva, dry eye syndrome, Shih Tzu, keratoconjunctivitis sicca.

RESUMO.- [**Síndrome do olho seco em Shih Tzu: aspectos clínicos e morfológicos da superfície ocular nas diferentes fases da ceratoconjuntivite seca.**] Este estudo analizou os parâmetros clinicos e cito-histomorfológicos da superfície ocular de cães da raça Shih Tzu, portadores e não portadores de ceratoconjuntivite seca quantitative (CCS) em diferentes estágios. Trinta e cinco olhos de 23 cães da raça Shih Tzu, machos e fêmeas, entre as idades de dois e oito anos foram avaliados em quatro grupos: grupo controle (GC – sem CCS), grupo CCS inicial (CCS1), grupo CCS moderada (CCS2) e grupo CCS severa (CCS3). A maioria das variáveis clínicas dentro os grupos portadores de CCS pioraram a medida que os estágios da doença avançam, com correlações negativas entre o teste lacrimal de Schirmer (TLS) e o tempo de ruptura do filme lacrimal (TRFL). Metaplasia escamosa, infiltrado inflamatório linfoplasmocítico e diminuição das células caliciformes no exame histopatológico compreenderam parâmetros de severidade da doença. Cães Shih Tzu não portadores de CCS quantitativa tem alterações qualitativas e na distribuição do filme lacrimal. O exame citomorfológico é limitado para a avaliação do infiltrado inflamatório e quantificação de células caliciformes. Entretanto, células inermediárias epiteliais são mais presents nos olhos saudáveis quando comparados aos olhos portadores de CCS em Shih Tzus. Também, Shih

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Tzus portadores de CCS nos estágios moderados e severos possuem disfunções qualitativas da lágrima. A CCS em Shih Tzu é comum, crônica e pode ser responsável pela perda de visão dos animais. A identificação precoce da doença e avaliações rotineiras podem aumentar a qualidade de vida e a saúde ocular nesses cães.

TERMOS DE INDEXAÇÃO: Cão, ceratoconjuntivite seca, córnea, conjunctiva, Shih Tzu, síndrome do olho seco.

INTRODUCTION

Keratoconjunctivitis sicca (KCS) is a disease of the ocular surface that affects humans and other animal species, being frequent in dogs. It is a severe chronic disease characterized by a quantitative and/or qualitative tear film deficiency, resulting in dryness and chronic inflammation of the conjunctiva and cornea, which can progress to blindness (Giuliano 2013). Quantitative KCS is characterized by a reduction in the aqueous portion of the tear film, which may have an iatrogenic, congenital, infectious, traumatic, neurogenic, neoplastic, pharmacological, metabolic, and immune-mediated origin. Autoimmune dacryoadenitis is the most common cause of quantitative KCS in dogs, with the dog being used as an experimental model for the study of autoimmune KCS in humans due to its similar pathophysiology (Barabino et al. 2004).

The diagnosis and classification of the quantitative KCS stage are determined according to the Schirmer tear test (STT-1), with values of 14-11mm/min determining a mild KCS, 10-6mm/min determining a moderate KCS, and equal to or lower than 5mm/min determining a marked KCS (Giuliano 2013). However, data on the disease characteristics according to its stage in dogs are limited despite this classification (Balicki et al. 2011, Madruga et al. 2018). Clinical characteristics observed in KCS include corneal dryness, conjunctival hyperemia, pigmentary keratitis, tear film instability, conjunctival mucous secretion, corneal neovascularization, and ulcerative keratitis. However, these signs vary according to the disease severity (Giuliano 2013).

In medicine, STT-1 is considered a limited examination for analyzing the tear film because it only evaluates the quantitative portion of the tear. Thus, tests such as meniscometry, tear film break-up time (TBUT), meibometry, and vital dyes are used to evaluate tear quality (Williams & Griffiths 2017). In contrast, diagnostic aid exams in veterinary medicine have been advocated to increase the diagnostic quality and targeting of KCS treatment in dogs (Williams & Griffiths 2017, Radziejewski et al. 2018, Haeussler Jr. 2019). Cyto-histomorphological analyses of the ocular surface of KCS carrier animals demonstrate conjunctival inflammatory infiltrate, reduced goblet cell density, and squamous metaplasia (Marek & Podhorska 1995, Balicki et al. 2011, Radziejewski et al. 2018). Moreover, studies that evaluated the ocular microbiota of dogs of different breeds with the disease indicate a higher contamination rate and opportunistic bacteria (Oria et al. 2013, Pereira et al. 2019a).

A genetic predisposition to the development of quantitative KCS is suggested in brachycephalic dogs, including those of the Shih Tzu breed (Giuliano 2013, Arnold et al. 2014). Studies carried out in dogs of this breed that had normal STT-1 values have demonstrated that the Tear Film Break-Up Time, corneal sensitivity, and goblet cell density are lower than those described for dogs without ocular alterations (Lima et al. 2011, Kobashigawa et al. 2015). Given this particularity, questions have been raised about the evolution and progression of ocular lesions in quantitative KCS carrier Shih Tzu dogs. Furthermore, little literature deals with clinical and morphological changes of KCS in dogs, considering the different disease stages (Balicki et al. 2011, Williams & Griffiths 2017, Madruga et al. 2018). In this context, this study aimed to investigate the clinical characteristics of the ocular surface and the cyto-histomorphological characteristics of the conjunctiva of Shih Tzu dogs, non-carriers and carriers of quantitative KCS at different stages.

MATERIALS AND METHODS

Study local. Twenty-three dogs were evaluated at a Veterinary Ophthalmology Service of Veterinary Hospital at "Universidade Federal de Goiás" (UFG) and veterinary hospitals of Goiânia and region. The study has been approved by "Cômite de Ética no Uso de Animais" (CEUA) of UFG, and the owners of the dogs used in this study were informed about the experimental protocols. They signed an informed consent form authorizing their animal use in the research.

Selection of animals and groups. Thirty-five eyes from 23 male and female Shih Tzu dogs aged between two and eight were evaluated. The animals were submitted to anamnesis and clinical examination to verify the degree of hydration, mucosal color, capillary filling time (CFT), pulse, cardiac and pulmonary auscultation, heart rate (HR), and respiratory rate (*f*). All evaluations were carried out in a single moment, with each eye considered an experimental unit. The eyes were distributed into groups according to STT-1 values. Thus, ten eyes with STT-1≥15mm/min were included in the control group (CG – eyes without quantitative KCS), eight with STT-1 of 14-11mm/min composed the KCS1 group (eyes with mild KCS), seven with STT-1 of 10-6mm/min in the KCS2 group (eyes with moderate KCS), and ten eyes with STT-1≤5mm/min in the KCS3 group (eyes with severe KCS). Dogs with other ophthalmic diseases or those submitted to topical ocular medication within 30 days of the evaluation were excluded from the study.

After classifying the groups according to STT-1 value, each eye was subjected to inspection of the conjunctiva and cornea using a slit lamp biomicroscope, TBUT, fluorescein test, conjunctival sample collection for cytomorphological analysis, Rose Bengal test, and tissue sample collection for histomorphological evaluation of the conjunctiva.

Schirmer tear test (STT-1) and clinical evaluation of the ocular surface. The STT-1 test was performed by bending the end of the Schirmer strip (Ophthalmos®) and placing it in the middle portion of the inferior conjunctival sac. The length of the wetted strip was measured, in millimeters, after one minute, according to the manufacturer's recommendations. The variables evaluated by conjunctiva inspection were hyperemia, discharge, and edema, while cornea inspection was pigmentation and neovascularization, according to criteria adapted from Balicki et al. (2011) (Table 1).

TBUT and fluorescein tests. A drop of fluorescein dye eye drops (Ophthalmos, São Paulo/SP, Brazil) was instilled in the dorsolateral region of the sclera of the examined eye. Then, the eyelid was manually occluded for 10 seconds. After opening the eyelids, the dye was observed on the corneal

surface using a biomicroscope with a cobalt blue filter (Kowa, Tokyo, Japan), with the time until the visualization of the first dry spot on the cornea being timed (Ollivier et al. 2007). A light spot was positioned in front of the cornea after cleaning with 0.9% NaCl, to evaluate the fluorescein impregnation on the corneal surface.

Cytomorphological analysis. Conjunctival samples for cytomorphological analysis were obtained two minutes after the ocular instillation of anesthetic eye drops based on 0.5% proxymetacaine (Anestalcon, Alcon Laboratórios do Brasil Ltda). For this, the conjunctiva of the lateral third of the lower eyelid was exposed manually, and rolling movements were performed over the conjunctiva using an interdental brush (Bitufo, Itupeva, São Paulo, Brazil). The obtained material was transferred to histological glass slides by gently rolling the brush over the surface. Subsequently, the slides were fixed in methanol, subjected to staining using the rapid panoptic method, and evaluated under an optical microscope coupled to an image capture system (Leica DM 750/ICC50E, LAS EZ). The conjunctival epithelial and inflammatory cells identified by microscopy were counted following a continuous zig-zag pattern until completing 500 cells, and thus the frequency of each cell type was determined. Basal (CECb), intermediate (CECi), superficial (CECs), columnar (CECc), and keratinized conjunctival epithelial cells (CECk), as well as conjunctival goblet cells (GC), were considered. Neutrophils, eosinophils, lymphocytes, plasma cells, and macrophages were considered among the inflammatory cells. The leukocyte morphology and the presence of cocci or rods were also evaluated (Bauer et al. 1996, Borges et al. 2012).

Table 1. Criteria adopted to variables conjunctival hyperemia, discharge, edema, corneal pigmentation and neovascularization

Bulbar conjunctival hyperemia and palpebral conjunctival hyperemia	
0	Absent
1	Mild hyperemia
2	Moderate hyperemia
3	Accentuated hyperemia
Conjunctival discharge	
0	Absent
1	Small amount in the conjunctival sac
2	Present in the medial corner
3	Present along the eyelid margin
4	Present along the eyelid margin and scabs in the peripalpebral area
Conjunctival edema	
0	Absent
1	Mild edema
2	Moderate edema
3	Accentuated edema
Corneal pigmentation and neovascularization	
0	Absent
1	One affected quadrant
2	Two affected quadrant
3	Three affected quadrant
4	Four affected quadrant

Adapted from Balicki (2011).

Rose Bengal test (RBT). A drop of Rose Bengal dye eye drops (Ophthalmos) was instilled in the dorsolateral region of the sclera of each examined eye, considering the previous topical anesthesia performed for the cytomorphological examination. Then, a light spot was positioned in front of the cornea after cleaning with 0.9% NaCl, to show dye impregnation on the conjunctival and corneal surfaces (Ollivier et al. 2007).

Histomorphological analysis. Conjunctival samples for histomorphological analysis were collected by performing an ocular surface anesthesia protocol with a drop of anesthetic eye drops based on 0.5% proxymetacaine (Anestalcon, Alcon Laboratórios do Brasil Ltda, São Paulo/SP, Brazil) every 30 s, completing six applications (Slatter 2005). Subsequently, the middle inferior conjunctival fornix was fixed with Adson forceps, and a fragment of approximately 5mm was excised using conjunctival scissors, being then fixed in 10% buffered formalin for 24 h and subjected to histological processing and embedding in paraffin. Then, 5µm sections were made from the paraffin blocks, stretched over histological slides and stained with hematoxylin and eosin (HE) and periodic acid-Schiff (PAS). The sections were evaluated under an optical microscope coupled to an image capture system (Leica DM 750/ICC50E, LAS EZ). The sections stained with HE was used for counting neutrophils, eosinophils, lymphocytes, macrophages, and plasma cells and evaluating the variables edema, hyperemia, and squamous metaplasia. Moreover, the sections stained with PAS were used to count GC. Cell counts were performed in five fields at 40x magnification. The tutor of an animal from the KCS2 group did not authorize the performance of the conjunctival biopsy procedure.

Statistical analyses. Analysis of variance with Tukey's post hoc test was used to compare parametric data with a normal distribution (STT-1, TBUT, CECi, and neutrophils in cytomorphology and variables of the histomorphological evaluation). The Kruskal-Wallis test was used to compare the variables with free distribution (CECb, CECs, CECc, CECk, GC, lymphocytes, plasma cells, eosinophils, and macrophages) and qualitative variables (conjunctival hyperemia, discharge, and edema and corneal pigmentation and neovascularization). The considered significance level was 5% ($p<0.05$). The description of the data was performed for binomial variables. Spearman's correlation test ($-1 \leq r \leq 1$) was used to evaluate the correlation between variables, considering a 1% ($p<0.01$) significance level. The interpretation of results was performed as described by Mukaka (2012), in which positive or negative r values >0.9 indicate a very strong correlation, 0.7-0.9 strong correlation, 0.5-0.7 moderate correlation, 0.3-0.5 weak correlation, and 0-0.3 negligible correlation.

RESULTS

The means of STT-1 and other clinical variables evaluated in the dog eyes from each group are shown in Table 2.

TBUT showed no difference between the eyes of CG and KCS1. Furthermore, the TBUT of the eyes of CG was higher than that of KCS2 and KCS3 groups, while the TBUT of KCS1 was higher than that of KCS3. Moreover, there was a strong correlation (r=0.73) between STT-1 and TBUT.

The clinical evaluation of the conjunctiva showed no difference between the eyes of CG and KCS1 groups regarding the palpebral conjunctival hyperemia. On the other hand, the eyes from KCS2 and KCS3 groups had a higher degree of hyperemia than those from CG. Among the groups of eyes with KCS, there was a difference between KCS1 and KCS3. In contrast, the bulbar conjunctival hyperemia was higher in the eyes from KCS3 than CG. However, no difference was observed between the eyes from the KCS groups for this variable. Conjunctival discharge was higher in the eyes of KCS groups compared to CG. Also, the KCS3 group had higher conjunctival discharge than KCS1 when considering only the groups of eyes with KCS. Importantly, the edema was not found in the eyes of the studied groups. Moreover, bulbar hyperemia, palpebral hyperemia, and conjunctival secretion negatively correlated with STT-1 and TBUT. Furthermore, a positive correlation was observed between conjunctival secretion and bulbar hyperemia (r=0.55) and between conjunctival secretion and palpebral hyperemia (r=0.69).

The clinical evaluation showed that the cornea of Shih Tzu dogs with KCS3 has higher pigmentation than those with KCS2 and KCS1 and those with eyes that do not have KCS (CG). Corneal pigmentation was also higher in the eyes of KCS2 than CG's. However, no difference was observed in the corneal pigmentation between KCS1 and CG. The neovascularization was higher in eyes with KCS3 than those with KCS1 and KCS2. Also, the three groups of eyes with KCS had higher corneal neovascularization than those from CG.

The corneal pigmentation and neovascularization showed a moderate to strong negative correlation between STT-1 and TBUT. Furthermore, a positive correlation was found between corneal pigmentation and palpebral conjunctival hyperemia (r=0.64) and between corneal pigmentation and conjunctival secretion (r=0.71). Moreover, corneal neovascularization was positively correlated with bulbar (r=0.53) and palpebral conjunctival hyperemia (r=0.75) and with conjunctival discharge (r=0.79). A strong positive correlation (r=0.88) was also found between corneal pigmentation and neovascularization.

Rose Bengal dye impregnation in the conjunctiva and cornea occurred at different frequencies in eyes from all groups. The KCS2 and KCS3 groups showed dye impregnation in all corneas. The studied eyes did not show impregnation for the fluorescein dye, regardless of the group.

Conjunctival cytomorphology showed basal (CECb), intermediate (CECi), superficial (CECs), keratinized (CECk), columnar epithelial cells (CECc), and conjunctival goblet cells (GC). Regarding morphology, CECb were small, round to oval, with a large and central nucleus and a high nucleus-to-cytoplasm ratio. CECi were oval to polyhedral, with a round, central, or slightly peripheral nucleus and a lower nucleus-to-cytoplasm ratio than CECb. On the other hand, CECs had a polyhedral morphology, a small, round, and central nucleus, and a low nucleus-to-cytoplasm ratio. CECk were large, polygonal, with clear angles, pyknotic or absent nucleus, and sometimes with cytoplasmic melanin granules. CECc exhibited a cuboid or columnar morphology, with a round and peripheral nucleus and basophilic apical cytoplasm. Furthermore, GC was round, with an intermediate size, eccentric and flattened nucleus, and a low nucleus-to-cytoplasm ratio, being arranged alone or together with CECc groups (Fig.1-6).

Eye samples from CG had a higher frequency of CECi than eyes from the KCS1, KCS2, and KCS3 groups (Fig.7). In contrast, no difference was observed between the studied groups regarding the frequency of other types of conjunctival epithelial cells. A positive correlation was also found between the frequency of CECi and STT-1 (r=0.61) and TFBT (r=0.52). The frequency of CECi was negatively correlated with bulbar (r=−0.51) and palpebral conjunctival hyperemia (r=−0.53), conjunctival secretion (r=−0.52), and pigmentation (r=−0.67) and corneal neovascularization (r=−0.66).

The cytomorphological analysis also identified neutrophils, lymphocytes, plasma cells, eosinophils, and macrophages. The frequency of neutrophils in the conjunctival samples was higher in the eyes from the KCS2 and KCS3 groups than that of CG. No difference was observed between the groups of eyes with KCS regarding the frequency of neutrophils. A negative correlation was also observed between the frequency of neutrophils and STT-1 (r=−0.46), TBUT (r=−0.60), and the frequency of CECi (r=−0.70). Palpebral conjunctival hyperemia (r=0.44), conjunctival secretion (r=0.53), and pigmentation (r=0.54) and corneal neovascularization (r=0.61) were positively correlated with the frequency of neutrophils.

A higher frequency of lymphocytes was observed in the conjunctiva of eyes from KCS1, KCS2, and KCS3 than that of the eyes from CG. However, no difference was observed in the lymphocyte frequency between groups with KCS. The KCS1 and KCS2 groups had a higher frequency of macrophages

Table 2. Mean and standard deviations of the Schirmer tear test (STT-1) and tear film break-up time (TBUT); median palpebral and bulbar conjunctival hyperemia, conjunctival discharge, neovascularization, and corneal pigmentation scores; and frequencies of impregnation by Rose Bengal in the cornea (RBT cornea) and conjunctiva (RBT conjunctiva) of the eyes of Shih Tzu dogs from the control group (CG) and with mild (KCS1), moderate (KCS2), and severe (KCS3) keratoconjunctivitis sicca (KCS)

Clinical variables	CG	KCS1	KCS2	KCS3
STT-1 (mm/min)	22.6±2.69^{a}	12.12±1.26^{b}	8.57±1.39^{c}	2.3±1.55^{d}
TBUT (seconds)	13±3.68^{a}	9±5.07^{ab}	6.42±4.01^{bc}	3.6±1.85^{c}
Bulbar conjunctival hyperemia	0^{a}	0^{ab}	0^{ab}	1^{b}
Palpebral conjunctival hyperemia	0^{a}	0^{ab}	1^{bc}	2^{c}
Conjunctival discharge	0^{a}	2^{b}	3^{bc}	3^{c}
Corneal neovascularization	0^{a}	1^{ab}	2^{b}	4^{c}
Corneal pigmentation	0^{a}	2^{b}	2^{b}	4^{c}
RBT cornea (%)	40	75	100	100
RBT conjunctiva (%)	20	12.5	28.6	100

^{a,b,c,d} The difference between the mean values of columns with different overwritten is statistically significant (p>0.05).

in the conjunctival samples than that of the eyes from the CG and KCS3 groups, with no difference between groups regarding the frequency of eosinophils and plasma cells in the cytomorphological evaluation of the conjunctiva.

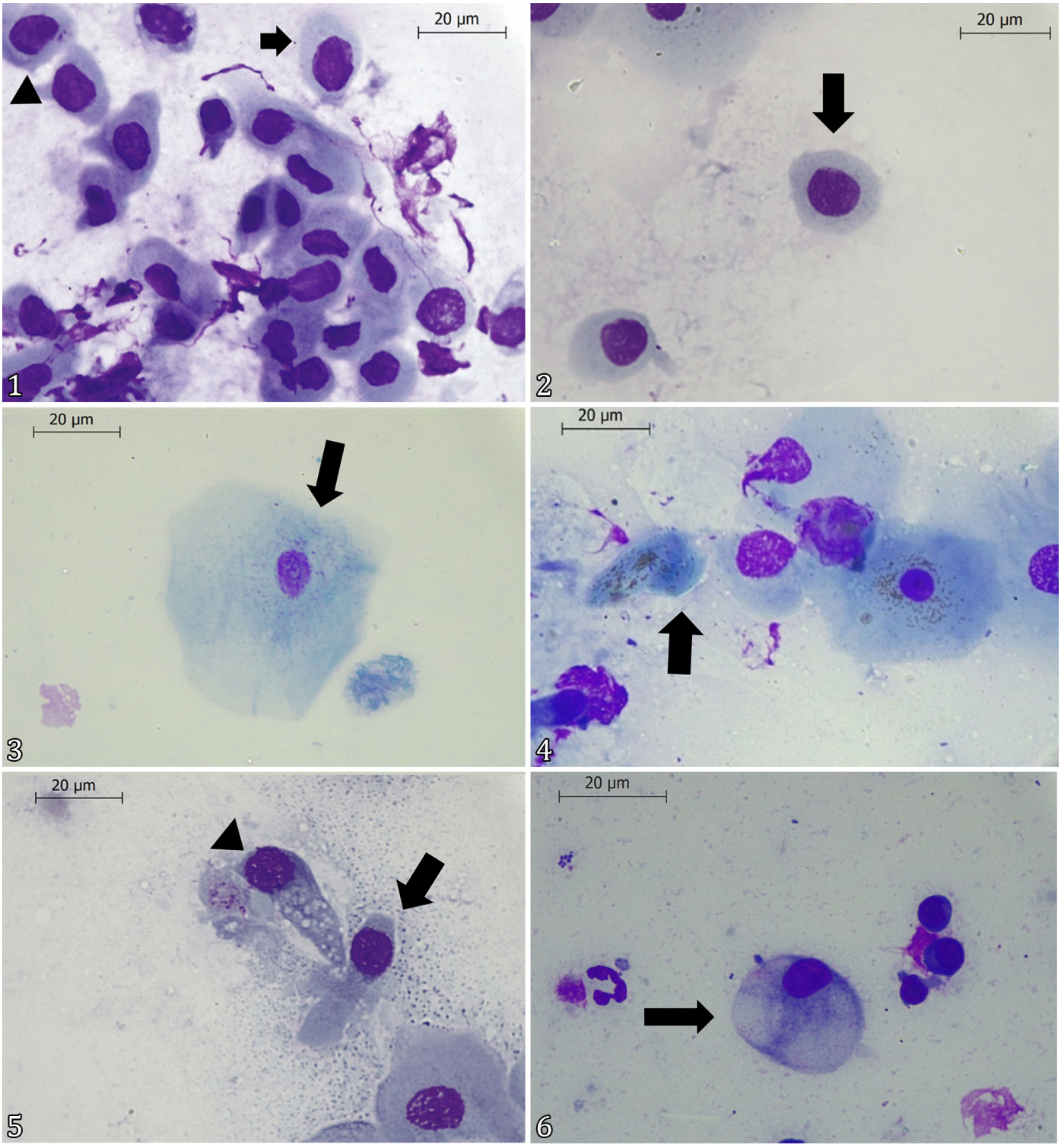

Fig.1-6. Photomicrographs of cytological samples of the conjunctiva of normal Shih-Tzu dogs with mild KCS (KCS1), moderate KCS (KCS2) and severe KCS (KCS3). (**1**) Basal epithelial cells in KCS1 dogs. Small size, round nucleus and high nucleus ratio:cytoplasm (arrows). (**2**) Intermediate epithelial cells in CG dogs. Round morphology, slightly larger in size than basal cells (arrow). (**3**) Superficial epithelial cell in KCS2 dog. Large, polyhedral, central, and low nucleus:cytoplasm (arrow) ratio. (**4**) Keratinized epithelial cell in KCS3 dog. Polygonal form, anucleated and with cytoplasmic melanin granules (arrow). (**5**) Columnar epithelial cells in KCS1 dogs. Elongated morphology, peripheral oval nucleus and apical cytoplasm (arrows). (**6**) Goblet cell in CG dog. Round to oval shape, peripheral and flattened nucleus, low nucleus:cytoplasm (arrow) ratio. (**1-6**) Panoptic, obj.100x, bar = 20µm.

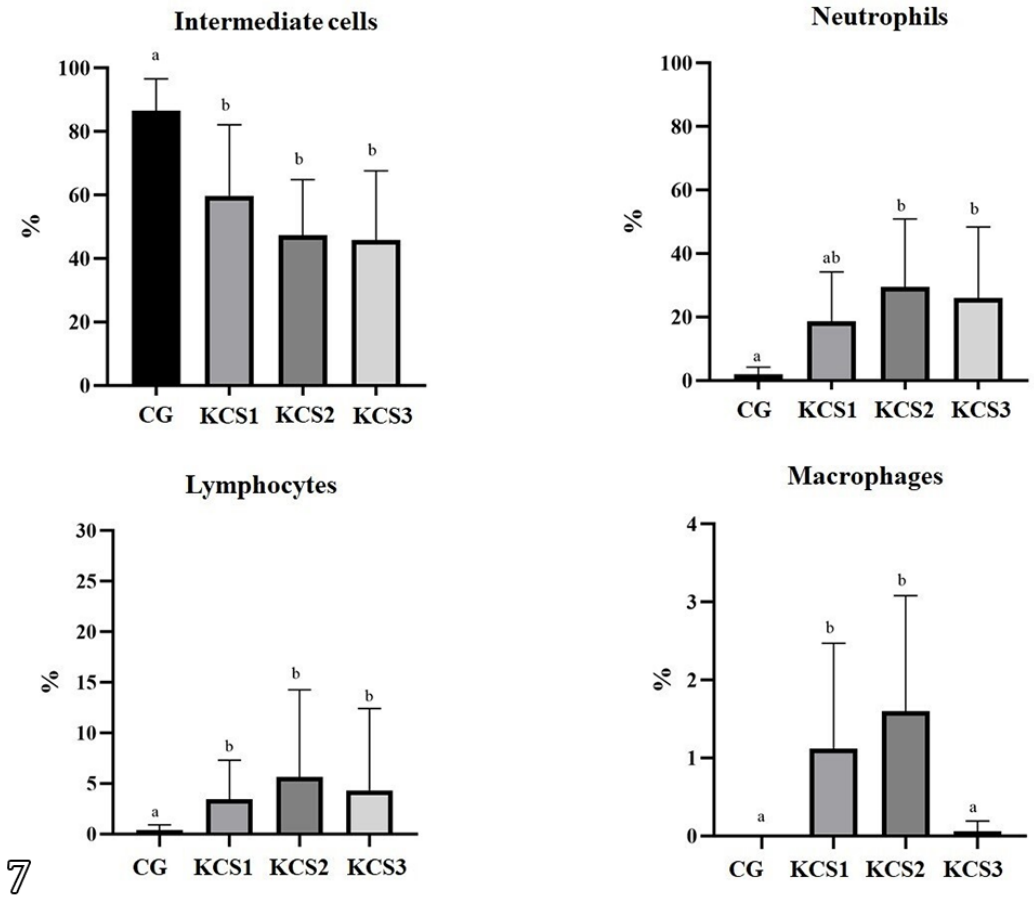

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Fig.7. Means and standard deviations of the frequency of intermediate epithelial cells, neutrophils, lymphocytes and macrophages obtained by cytomorphological examination of the conjunctiva of Shih Tzu dogs of groups CG, KCS1, KCS2 and KCS3. The difference between the mean values of columns with different overwritten is statistically significant (p>0.05) (a,b,c,d).

The frequency of lymphocytes had a negative correlation with TBUT (r=−0.43) and with the frequency of CECi (r=−0.60), as well as a positive correlation with conjunctival secretion (r=0.43). Neutrophils, lymphocytes, and macrophages were positively correlated among inflammatory cells. Furthermore, lymphocytes were positively correlated with plasma cells.

The cytomorphological evaluation in CG showed a predominance of intact neutrophils. Importantly, two cytological samples from CG had low cellularity compared to the others. KCS1 had a predominance of lymphocytes and neutrophils, the latter mostly degenerated. Degenerated neutrophils were also frequent in eye samples from the KCS2 and KCS3 groups. All evaluated groups presented free chromatin and epithelial scroll. The KCS2 and KCS3 groups showed emperipolesis, and some KCS samples had red blood cells. Cocci-like bacteria in conjunctival cytology samples were found in 50% (n=5) of those from CG, 62.5% (n=5) from KCS1, 71.4% (n=5) from KCS2, and 70% (n=7) from KCS3. Bacilli were observed in 10% (n=1) of the samples from the KCS1 and KCS3 groups (Fig.8-13).

The histomorphological analysis showed the presence of conjunctival edema, hyperemia, and metaplasia in all groups at different frequencies (Table 3). The variable conjunctival edema had a frequency above 80% in all groups. In contrast, conjunctival hyperemia occurred in half of the samples from CG, with an increase in the KCS1 group and presence in all samples from KCS2 and KCS3. The intact conjunctival epithelium revealed variations of two to five layers of cells interspersed with goblet cells. Flattening of epithelial cells, erosions on the epithelial surface, and cell debris were identified in KCS1, KCS2, and KCS3. Squamous metaplasia was identified in a sample from CG, 75% of those from KCS1, and all of the KCS2 and KCS3 groups.

All groups showed different amounts and frequencies of inflammatory infiltrate, goblet cells, squamous metaplasia, edema, and hyperemia. The predominant inflammatory infiltrate was lymphoplasmacytic, with a smaller proportion of neutrophils and macrophages.

The frequency of lymphocytes in the conjunctival samples was higher in the eyes from the KCS3 group than that of CG (Fig.14). No difference was observed between groups of eyes with KCS regarding the frequency of lymphocytes. The frequency of plasma cells was higher in groups with KCS than CG. Among KCS groups, the KCS3 group had a higher frequency than the KCS1 group. The frequency of neutrophils in the histomorphological evaluation showed no difference between groups, with a higher frequency of macrophages in the conjunctiva of eyes from KCS3 and CKCS2 than in the eyes from CG.

The frequency of plasma cells and macrophages in the histomorphological examination showed a positive correlation (r=0.63), as well as with pigmentation (r=0.56 and r=0.6, respectively) secretion (r=0.43 and r=045, respectively) and neovascularization (r= 0.61 and r=0.66, respectively), and a negative correlation with STT-1 (r=−0.52 and r= −0.61, respectively). The frequency of plasma cells was also negatively correlated (r= −0.49) with the frequency of CECi observed in the cytology.

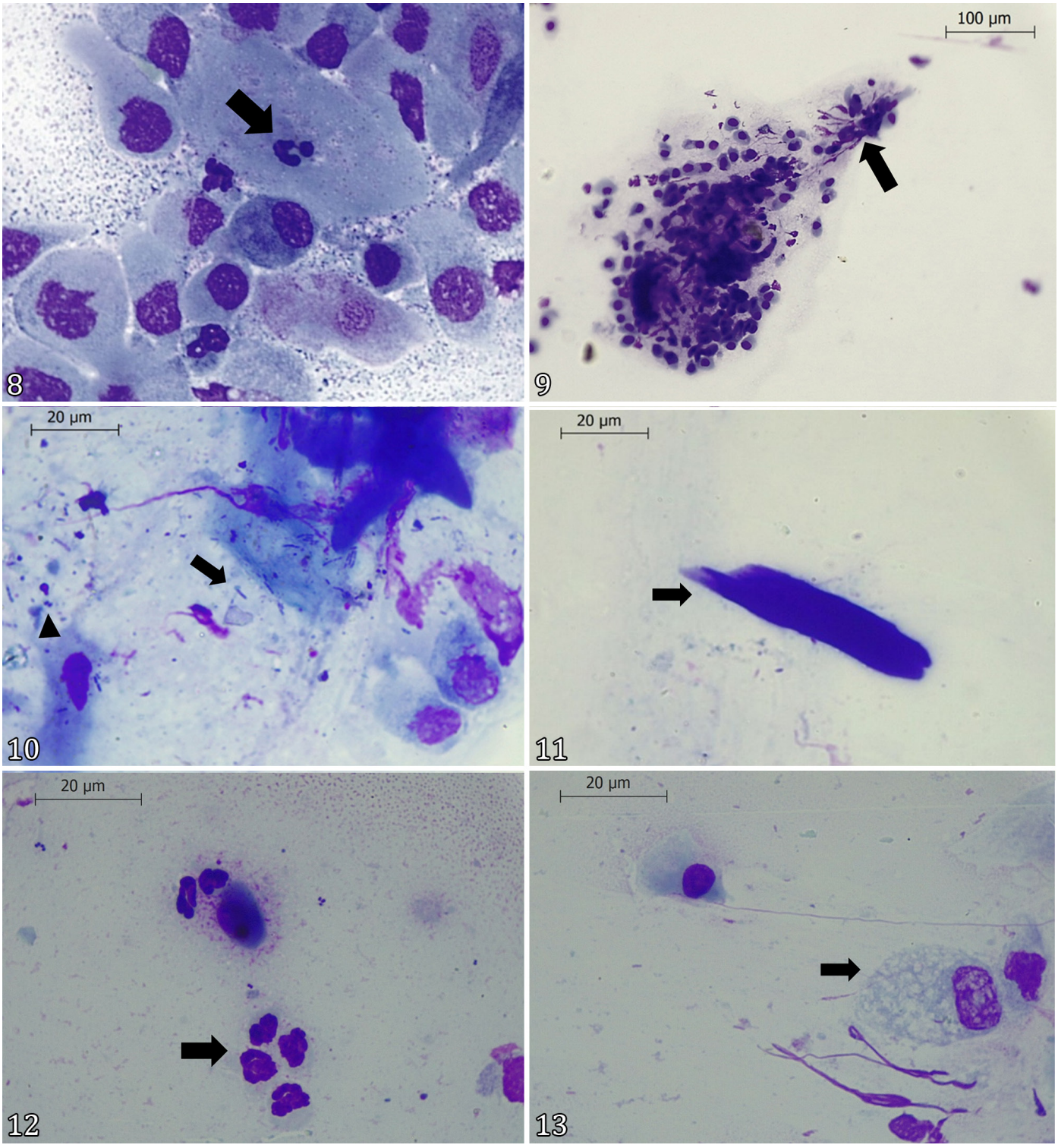

Fig.8-13. Photomicrographs of cytological samples of the conjunctiva of normal Shih-Tzu dogs with mild KCS (KCS1), moderate KCS (KCS2) and severe KCS (KCS3) exhibiting alterations related to the inflammatory process. (**8**) Emperipolesis in a keratinized conjunctival epithelial cell (arrow) in a KCS3 dog. (**9**) Free chromatin (arrow) in KCS1 dog. (**10**) Bacteria in the form of cocci (arrowhead) and bacilli (arrow) in KCS3 dog. (**11**) Lanceocyte (arrow) in KCS1 dog. (**12**) Degenerate neutrophils (arrow) in KCS2 dog. (**13**) Activated macrophage (arrow) in CCS3 dog. (**8** and **10**-**13**) Panoptic, obj.100x, bar = 20µm. (**9**) Panoptic, obj.20x, bar = 100µm.

Furthermore, the frequency of macrophages in the conjunctival stroma was negatively correlated with TBUT (r=−0.47). On the other hand, lymphocytes showed a positive correlation with pigmentation (r=0.54) and neovascularization (r=0.48), as well as with the frequency of neutrophils (r=0.52) and macrophages (r=0.48), on histopathological examination.

The count of conjunctival goblet cells on histomorphological examination showed higher numbers in CG and KCS1 compared to the KCS2 and KCS3 groups (Fig.14). Clinical and cytomorphological aspects of eyes from CG, KCS1, KCS2, and KCS3 are shown in Figure 15-30.

Considering the values of STT-1 and TBUT, 34 out of the 35 eyes of Shih Tzu dogs presented qualitative disturbance of the tear film, and 25 of them presented quantitative and qualitative tear deficiencies simultaneously. Only one evaluated eye had normal STT and TBUT values, observed in CG. In addition, one of the evaluated animals presented an eye with quantitative KCS and the other with a normal STT-1 value.

DISCUSSION

Quantitative KCS in dogs is classified according to the STT-1 value as mild, moderate, and severe (Giuliano 2013). The disease has been widely studied in veterinary medicine (Marek & Podhorska 1995, Carter & Colitz 2002, Cullen et al. 2005, Naranjo et al. 2005, Sanchez et al. 2007, Williams 2008, Lima et al. 2014, Liu et al. 2014, Radziejewski et al. 2018, Silva et al. 2018, Williams & Tighe 2018, Haeussler Jr. 2019, Vatnikov et al. 2020). However, few studies considered its different degrees for evaluations (Balicki

Table 3. Frequency (%) of inflammatory conjunctival edema, hyperemia, and metaplasia in the histopathological samples of the conjunctiva of eyes of Shih Tzu dogs from the control group (CG) and with mild (KCS1), moderate (KCS2), and severe (KCS3) keratoconjunctivitis sicca (KCS)

Groups	Edema	Hyperemia	Metaplasia
CG	80%	50%	10%
KCS1	100%	87.5%	75%
KCS2	83.33%	100%	100%
KCS3	100%	100%	100%

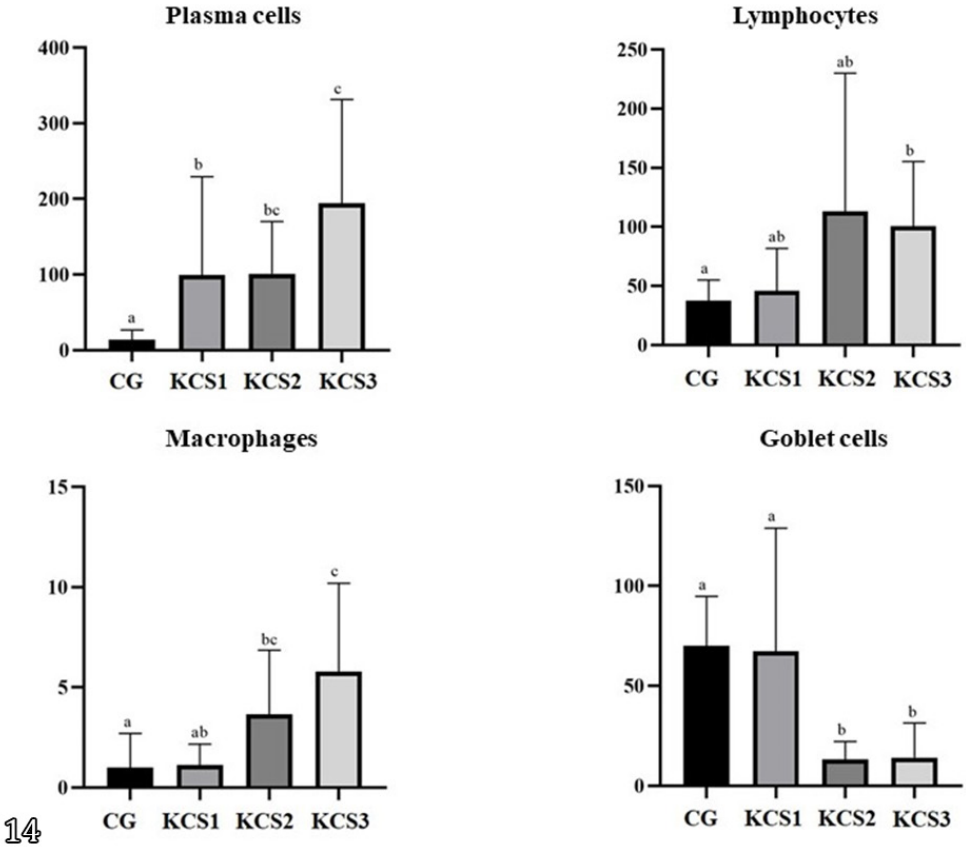

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Fig.14. Graphs of the means of the values and standard deviations of conjunctival goblet cells and inflammatory infiltrate in the conjunctival histopathological samples of the eyes of Shih Tzu dogs in the control group (CG) and with keratoconjunctivitis (KCS) mild (KCS1), moderate (KCS2) and severe (KCS3). The difference between the mean values of columns with different overwritten is statistically significant (p>0.05) (a,b,c,d).

et al. 2011, Williams & Griffiths 2017, Madruga et al. 2018), and no study contemplating clinical and cyto-histomorphological investigation at the different KCS stages was found.

The variation in the clinical presentation of KCS in specific dog breeds has been little explored (Herrera et al. 2007, Sanchez et al. 2007, Kitamura et al. 2019). The Shih Tzu breed was described as predisposed to the development of quantitative KCS (Kaswan & Salisbury 1990, Sanchez et al. 2007, Giuliano 2013, Arnold et al. 2014, Oliveira et al. 2014, Pereira et al. 2019a) and identified as one of the breeds most affected by the disease in surveys carried out in the United States (Kaswan & Salisbury 1990), United Kingdom (Sanchez

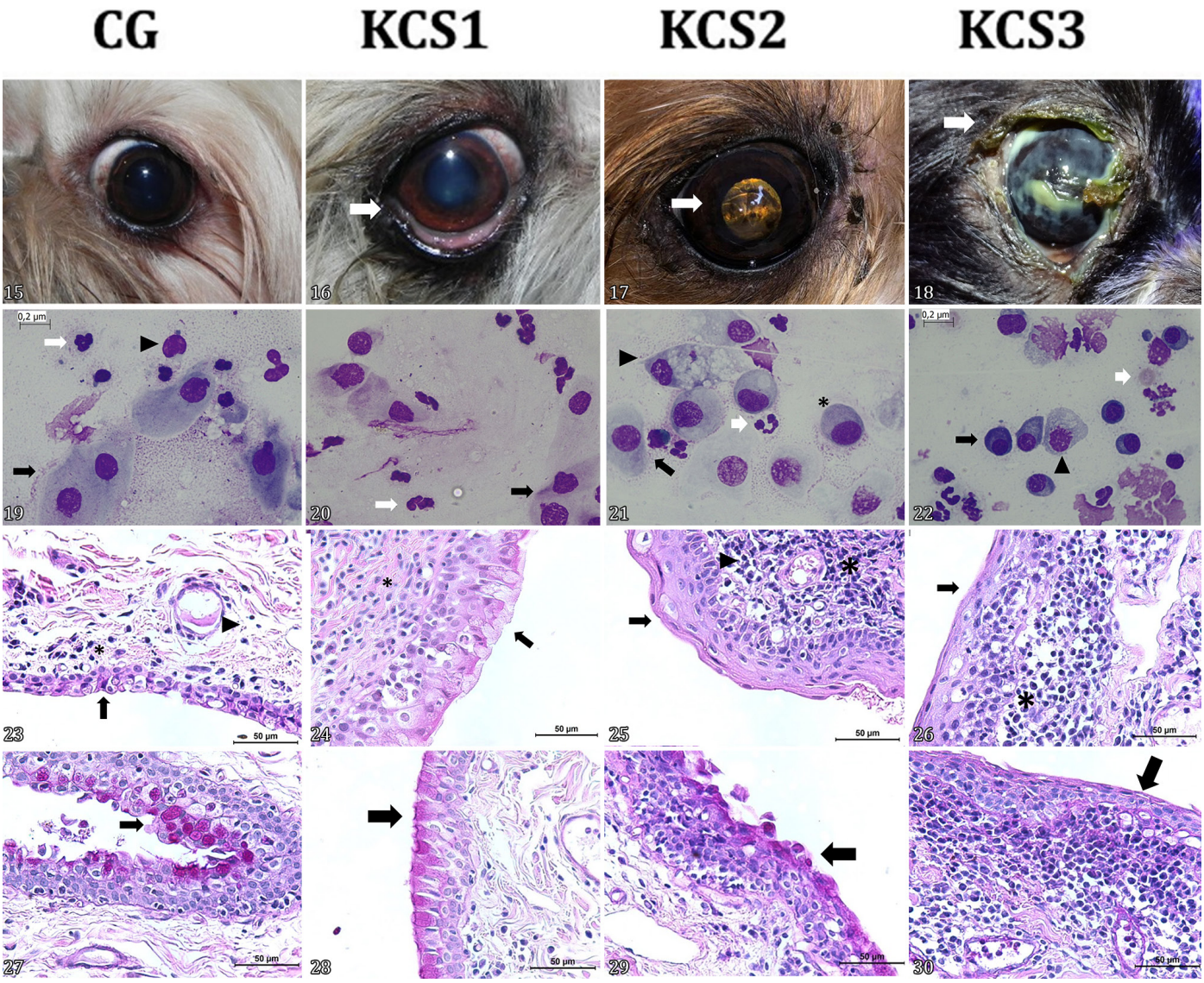

Fig.15-30. Ocular surface and photomicrographs of cytological and histological samples of the conjunctiva normal Shih-Tzu dogs with mild KCS (KCS1), moderate KCS (KCS2) and severe KCS (KCS3). (**15**) Eye surface without quantitative KCS. (**16**) Conjunctival secretion (arrow) and moderate eyelid hyperemia (asterisk). (**17**) Pigmentation in the four quadrants of the cornea (arrow). (**18**) Marked conjunctival secretion (arrow) and pigmentation in the four quadrants of the cornea (asterisk). (**19**) Intermediate cells (black arrow), neutrophils (white arrow) and lymphocytes (arrowhead). (**20**) Intermediate cells (black arrow) and neutrophils (white arrow). (**21**) Intermediate cell (black arrow), basal (asterisk), neutrophil (white arrow) and macrophage (arrowhead). (**22**) Degenerate neutrophil (white arrow), plasmocyte (black arrow) and intermediate cell (arrowhead). (**23**) Typical stratified epithelium (black arrow), lymphoplasmacytic inflammatory infiltrate (asterisk) and edema (arrowhead). (**24**) Epithelium stratified with goblet cells (black arrow). Stromal lymphoplasmacytic inflammatory infiltrate (asterisk). (**25**) Squamous metaplasia (black arrow), edema (arrowhead) and lymphoplasmocytic inflammatory infiltrate (asterisk). (**26**) Stratified epithelium presenting squamous metaplasia (red arrow) and stroma presenting edema and lymphoplasmacytic inflammatory infiltrate (asterisk). (**27** and **28**) Intact conjunctival epithelium and goblet cells (black arrow). (**29**) Conjunctival squamous metaplasia, lymphoplasmacytic inflammatory infiltrate and scarce goblet cells (black arrow). (**30**) Conjunctival squamous metaplasia, marked lymphoplasmacytic inflammatory infiltrate and absence of goblet cells (black arrow). (**19-22**) Panoptic, obj.100x, bar = 20µm; (**23-26**) HE, obj.40x, bar = 40µm; (**27-30**) periodic acid-Schiff, obj.40x, bar = 40µm.

et al. 2007), Taiwan (Liu et al. 2014), and Brazil (Oliveira et al. 2014). However, ophthalmologic evaluations between the different degrees of KCS in Shih Tzu dogs have not been found.

The mean values of STT-1 found in CG were similar to those described for Shih Tzu dogs (Lima et al. 2011, Sebbag et al. 2022) and lower than those observed by Kobashigawa et al. (2015). The mean TBUT values for dogs from CG were similar to those described for Shih Tzu dogs (Kobashigawa et al. 2015) and other brachycephalic breeds (Arnold et al. 2014), lower than those found for Beagles (Moore et al. 1987) and different from those described by Sebbag et al. (2022) in normal Shih Tzu dogs. Furthermore, TBUT values in Shih Tzu dogs for each stage were similar to those described by Madruga et al. (2018) in a study that evaluated dogs of different breeds with mild, moderate, and severe KCS. Moreover, dogs with KCS, regardless of the stage, have TBUT values ranging from 5.93 to 11 s (Oria et al. 2013, Chen & Poweel 2015, Dos Santos et al. 2019).

The TFBT reduction observed in dogs with moderate and severe KCS and the positive correlation between STT-1 and TBUT demonstrates the influence of decreasing the aqueous portion of the tear on tear film stability. In this sense, early tear film evaporation in these animals may be associated with mucosal layer dysfunction, implying secondary qualitative KCS (Moore 1990). Many mucin properties are changed in KCS (Davidson & Kuonen 2004), in addition to reducing GC density (Moore et al. 1987) and changing mucin production, expression, and glycosylation pathways (Bron et al. 1985, Hicks et al. 1998). Despite the positive correlation, it is worth highlighting the importance of evaluating tear film stability using TBUT, as STT-1 is not used to evaluate the mucous and lipid layers (Williams & Griffiths 2017, Silva et al. 2018, Pereira et al. 2019b).

Palpebral and bulbar conjunctival hyperemia and conjunctival secretion in the eyes of the evaluated Shih Tzu dogs presented the highest scores at the severe stages of the disease, in addition to a negative correlation with STT-1 and TBUT, corroborating studies in dogs with KCS (Balicki et al. 2011, Williams & Griffiths 2017, Silva et al. 2018, Zulim et al. 2018, Amalfitano et al. 2019). Hyperemia reflects conjunctival inflammation secondary to the ocular surface dryness (Amalfitano et al. 2019). Conjunctival secretion in animals with KCS occurs due to reduced flow of the aqueous portion of the tear, accumulating desquamation cells, mucus, bacteria, inflammatory cells, and debris on the ocular surface (Giuliano 2013). The difference between the palpebral conjunctival hyperemia in the eyes from the CG and KCS1 groups indicates that the inflammatory process occurs at the initial phase of the disease. Thus, the palpebral conjunctival hyperemia for Shih Tzu dogs seems to be a parameter that discriminates eyes without quantitative KCS and eyes with quantitative CCS at an early stage. Similarly, the conjunctival secretion was discriminating between eyes from the CG and KCS1 groups.

Similar to what Balicki et al. (2011) observed, there was severe corneal neovascularization and pigmentation in the eyes of dogs with severe KCS. These variables also showed a negative correlation with STT-1 and TBUT. Dryness and the inflammatory process of the ocular surface lead to an increase in angiogenic factors in the cornea, resulting in neovascularization, which, in turn, intensifies the local inflammatory infiltrate in the cornea and the carryover of melanin granules, resulting in pigmentation (Williams 2008, Silva et al. 2018, Zulim et al. 2018, Amalfitano et al. 2019).

The present study showed an increased frequency of conjunctival and corneal impregnation by the Rose Bengal dye as the disease progresses. The mechanism that leads to Rose Bengal impregnation has been discussed, and currently, the consensus is that the dye binds to tissues not covered by the mucin layer (Pflugfelder et al. 1997, Williams & Griffiths 2017).

Rose Bengal dye impregnation was also observed in the cornea and conjunctiva of CG dogs. This data, associated with the low density of GC in healthy Shih Tzu dogs, as described by Lima et al. (2011), provides evidence that this breed has a deficiency of the tear film mucous layer, even with normal STT-1 values. Anatomical characteristics of dogs of brachycephalic breeds, such as Shih Tzu, contribute to the development of ocular diseases such as lagophthalmos, medial canthal entropion, nasal trichiasis, and keratopathies secondary to exposure of the ocular surface (Gilger et al. 2007, Sebbag & Sanchez 2023). Conjunctival inflammation associated with these anatomical changes in Shih Tzu dogs is believed to be the result of reduced conjunctival goblet cell density and a deficiency of the tear film mucosal layer, as described by Kunert et al. (2002).

Regarding conjunctival cytology, the higher frequency of intermediate epithelial cells indicates the health of the ocular surface, as a positive correlation was observed between STT-1 and TBUT, and a negative correlation was found between corneal-conjunctival lesions and the frequency of inflammatory cells in conjunctival samples. Studies have also shown the predominance of CECi in the conjunctival cytology of KCS non-carrier animals (Borges et al. 2012, Morais et al. 2019).

Inflammatory cells were found in cytological samples of the conjunctiva of Shih Tzu dogs from CG. However, Borges et al. (2012) did not identify these cells in samples from KCS non-carrier dogs of different breeds. The frequency of neutrophils and lymphocytes in the conjunctival cytology of dogs from CG was similar to that described by Bolzan et al. (2005), who evaluated bulbar conjunctiva samples from dogs of different breeds without KCS, obtained through imprint with filter paper.

The frequency of neutrophils and lymphocytes in the cytopathological examination was higher in cases of lower STT-1 and TBUT, and the frequency of neutrophils was positively correlated with most clinical signs of KCS. In this sense, the predominance of neutrophils in conjunctival cytological samples from dogs with KCS has also been observed in other studies (Silva et al. 2018, Zulim et al. 2018, Vatnikov et al. 2020). The disparity between the profile of inflammatory cells predominant in the cytological and histological samples of the conjunctiva can be explained by the higher density of neutrophils and the mucopurulent conjunctival secretion collected together with the cytological sample. Moreover, in this study, the higher the conjunctival secretion score, the higher the frequency of neutrophils in cytology.

The disparity between the results of cytopathological and histopathological analyses was also demonstrated when evaluating GC. The sparse number of GC obtained from the cytopathological analysis shows that exfoliative cytology of the conjunctiva does not comprise an adequate technique for counting these cells, and the histomorphology evaluation is appropriate.

Overall, there was congruence between the histomorphological and clinical evaluations according to the KCS stage. The more the disease progresses, the more severe the inflammatory infiltrate on microscopy, as well as hyperemia, edema, and metaplasia, and the lower the count of conjunctival goblet cells. Clinically, the KCS2 and KCS3 groups developed more severe palpebral and bulbar conjunctival hyperemia conditions, ocular secretion, Rose Bengal dye impregnation, and lower TBUT values. Furthermore, the more accentuated the conjunctival histological changes, the more severe the corneal clinical manifestations, such as neovascularization and pigmentation.

The predominance of lymphoplasmacytic infiltrate in the stroma on the histological evaluation of the conjunctiva consisted of a finding similar to that observed in other studies using dogs with KCS (Izci et al. 2015, Silva et al. 2018, Zulim et al. 2018). Despite this, the inflammatory infiltrate was also described in CG, corroborating with Lima et al. (2011), who evaluated the conjunctival tissue of quantitative KCS non-carrier Shih Tzu dogs. In addition, anatomical changes in brachycephalic dogs contribute to an evaporative disturbance and dryness of the ocular surface (Sebbag & Sanchez 2023).

No data were found regarding the evaluation of goblet cells at the different KCS stages in Shih Tzu dogs. On the other hand, a reduction in conjunctival goblet cell density was observed in the moderate and severe stages of the disease compared to CG, with no discrimination regarding this parameter at the initial phase. The instability and, indirectly, the deficiency of the tear film mucous layer at the moderate and severe KCS stages in Shih Tzu dogs can be confirmed considering these findings, the Rose Bengal impregnation, and the low TBUT values.

It is inferred that there was a relationship between the worsening of the inflammatory infiltrate and squamous metaplasia in the groups with more severe cases of KCS. This relationship has been described in humans with dry eye syndrome, in which the alteration of the ocular surface homeostasis mechanisms, associated with the chronic inflammatory process of the disease, favors the squamous metaplasia of the conjunctival epithelium (Almeida et al. 2009, Morais et al. 2019).

One study identified lower corneal sensitivity in brachycephalic dogs and, consequently, lower response to stimulation and tear production tests compared to non-brachycephalic dogs (Bolzanni et al. 2020). In addition, another study pointed out a lower blink rate in these dogs than in non-brachycephalic dogs (Williams & Denny 2020). Considering this information, the findings of this study regarding qualitative and quantitative KCS, and those of other authors regarding the ocular health of brachycephalic dogs and, exclusively, the Shih Tzu breed (Lima et al. 2011, Kobashigawa et al. 2015), the importance of an early ophthalmic evaluation in dogs of this breed stands out. In this context, the evaluation aims to identify changes in the tear film's quality, quantity, and distribution and, consequently, establish early therapy, minimizing the KCS effects, risks of loss of visual acuity, and blindness in these animals.

CONCLUSIONS

Keratoconjunctivitis sicca (KCS) carrier Shih Tzu dogs show worsening clinical signs as the disease progresses. Compared to the initial stages of KCS in Shih Tzu dogs, moderate and severe stages have more noticeable ocular surface alterations besides evidence of mucin deficiency, tear film instability, and consequent qualitative dysfunction of the tear.

The cytomorphological examination is limited to evaluating the inflammatory infiltrate and quantifying conjunctival goblet cells compared to the histomorphological examination. However, intermediate epithelial cells are a promising cytological marker of eye health in Shih Tzu dogs.

Quantitative KCS non-carried Shih Tzu dogs have qualitative and tear film distribution disorders, which makes routine ophthalmologic evaluations necessary in these animals, aiming to identify early changes and mitigate the deleterious effects on the ocular surface.

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