Original Article

Evaluation of tear quantity and quality treated with gintonin eye drops in Labrador retrievers and English springer spaniels

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Abstract

The aims of this study were to determine the relationship between tear osmolarity (TO), Schirmer tear test-1 (STT-1) and tear film break-up time (TBUT) values in canines and to determine the impact of eye drops containing gintonin - a ginseng-derived lysophosphatidic acid (LPA) receptor ligand - on these indices. Fourteen canines (four English springer spaniels and ten Labrador retrievers) were randomized into two groups: the control group received only placebo eye drops (carboxymethylcellulose), while the experimental group received carboxymethylcellulose eye drops containing 5 μg of gintonin. Treatment was applied twice per day for a period of four weeks and STT-1, TBUT and TO were evaluated both prior to and following the treatment period; the latter using I-PEN® VET (I-MED Animal Health, QC, Canada). Pearson's correlation coefficients were calculated to investigate potential associations between baseline TO, STT-1 and TBUT values. Within-group statistical significance of value changes induced by treatment was determined using Student's t-test. Positive correlations exist between TO and STT-1 values, as well as between TO and TBUT values (P = 0.001 and P = 0.032, respectively). Gintonin treatment significantly increased only TBUT (21.0 ± 11.2 s versus 7.5 ± 2.8 s) and TO (317.1 ± 18.1 mOsm/L versus 302.2 ± 19.3 mOsm/L) values (P < 0.01 and P < 0.05, respectively). The results suggest that gintonin improves canine tear film quality, as indicated by increased TBUT and TO values.

Keywords: Gintonin, Schirmer tear test-1, Tear film break-up time, Tear osmolarity, Tear film quality

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Received July 17, 2020.
Accepted December 16, 2020.
doi: 10.14456/tjvm.2021.3

Introduction

Historically, veterinary ophthalmologists have made the diagnosis of keratoconjunctivitis sicca (KCS, known colloquially as dry-eye) on the basis of tear volume. A tear film lateral flow of less than 10 mm per minute, as measured by the Schirmer tear test-1 (STT-1), is generally considered diagnostic of dry-eye (Williams, 2005). The tear film is structurally complex, consisting of three layers: an outer lipid layer, a middle aqueous layer and an inner mucin layer (Ledbetter and Gilger, 2013). Disruption of this architecture may render the eye susceptible to drying. Poor tear film quality is also thought to produce dry-eye, even when tear production volume is normal (Moore, 1990). Therefore, several indices for the assessment of tear film quality have been developed. Tear osmolarity (TO) and tear film break-up time (TBUT) represent two such indices (Messmer *et al*., 2016). The TBUT test quantifies how long the tear film remains intact on the cornea, reflecting mucin layer function. In canines, sufficient TBUT is important for corneal protection. In humans, TO is a popular diagnostic index for dry-eye. Even in isolation, it has adequate diagnostic value, exhibiting a sensitivity of 73% and a specificity of 92% for any measurement over 312 mOsm/L (Lemp *et al*., 2011).

Gintonin – a specialized lysophosphatidic acid (LPA) receptor ligand - is a ginseng constituent with several known pharmacological effects (Kimura *et al*., 2006). For example, it promotes healing of the corneal epithelium when used in eye drops (Kim *et al*., 2017). Mechanistically, LPA ligates a specific G-protein-coupled receptor, prompting diverse downstream cell signaling pathways that induce cell proliferation and migration, and regulate cytokine and matrix metalloproteinase (MMP) expression (van Meeteren and Moolenaar, 2007; Pua *et al*., 2009). Because MMP-9 promotes corneal extracellular matrix degradation and epithelial loss (Corrales *et al*., 2006) and increased MMP-9 concentrations are associated with low TBUT and increased TO values, among other indices (Messmer *et al*., 2016), gintonin (similarly to cyclosporine) can be used to beneficially regulate MMP-9 levels. Although several studies have demonstrated gintonin's pharmacological effects (Corrales *et al*., 2006; Kim *et al*., 2017; Kimura *et al*., 2006), no study has yet evaluated its quantitative and qualitative impact on tear film.

The aim of the present study was twofold: (1) to determine the relationship between STT-1, TBUT and TO values of canine tear film, and (2) to determine the impact of gintonin-containing eye drops on these indices.

Materials and Methods

Fourteen canines exhibiting normal eye health were enrolled from a kennel club that manages two breeds: English springer spaniels and Labrador retrievers. The canine subjects thus consisted of four English springer spaniels (three female, one male; median age 23 months) and ten Labrador retrievers (five female, five male; median age 71.9 months). All experiments involving animals were approved by the Institutional Animal Care and Use Committee of Konkuk University (Approval No. KU18197). All canines underwent complete ophthalmic examination, including slit-lamp biomicroscopy, indirect ophthalmoscopy and applanation tonometry. Tear film quantity and quality were assessed via the STT-1, TBUT and TO. An STT-1 measurement of < 15 mm/min and a TBUT measurement of < 20 secs were considered abnormal (Giuliano, 2013; Moore, 1990). The I-PEN® VET (I-MED Animal Health, QC, Canada) was used to measure TO levels. The device was positioned on the conjunctival surface of the lower eyelid with a single-use test card in contact with the ocular surface (Fig. 1). Because no reference threshold value exists that defines abnormal canine TO, we considered normal canine TO to be 337.4 ± 16.2 mOsm/L, as provided by Sebbag *et al*. (2017) (Sebbag *et al*., 2017).

Figure 1 Measurement of tear osmolarity (TO). The device was positioned on the conjunctival surface of the lower eyelid, with a single-use test card in contact with the ocular surface.

Study subjects were randomized into two equal groups. The control group received only eye drops containing 5 mg/ml carboxymethylcellulose (Nunen®, Hanmi Pharm. Co. Ltd., Seoul, South Korea), while the experimental group received carboxymethylcellulose eye drops containing 5 µg gintonin. Treatment was administered twice a day for four weeks, prior to the re-evaluation of tear film indices.

Gintonin was isolated from Panax ginseng using a previously described method (Kim *et al*., 2017). Reagents used during isolation were obtained as follows: RPMI 1640 medium, Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), horse serum (HS), penicillin, and streptomycin were obtained from Invitrogen (USA); lysophosphatidic acid (LPA; 1-oleoyl-2-hydroxy-snglycero-3-phosphate; 857130P) was obtained from Avanti Polar Lipids (Alabaster, USA); and all other reagents - including the cell permeant chelator BAPTA-AM and N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) - were obtained from Sigma-Aldrich (USA) (Choi *et al*., 2013). Isolated gintonin was dissolved in saline and diluted with a medium before use.

Statistical analyses were performed using SPSS version 24.0 (IBM, Armonk, NY, USA) for Windows. Pearson's correlation coefficients were calculated to investigate potential associations between baseline TO, STT-1 and TBUT values. Within-group statistical significance of value changes induced after four weeks of treatment was determined using Student's t-test. A *P*-value of < 0.05 was considered statistically significant.

Results

Mean (± SD) TO, STT-1 and TBUT baseline values were as follows: 305.5 ± 18.7 mOsm/L (abnormal), 20.0 ± 4.6 mm/min (normal), and 11.4 ± 6.6 s (abnormal), respectively. A significant positive correlation existed at baseline between STT-1 and TO values (P = 0.001, r = 0.62, Fig. 2) and between TBUT and TO values (P = 0.032, r = 0.4, Fig. 3). After treatment, control group STT-1 and TBUT values were marginally increased, and TO was marginally decreased but these changes did not achieve statistical significance (Table 1). After treatment, the experimental group STT-1 value marginally increased (but did not achieve statistical significance), while TBUT and TO values increased significantly (Table 1).

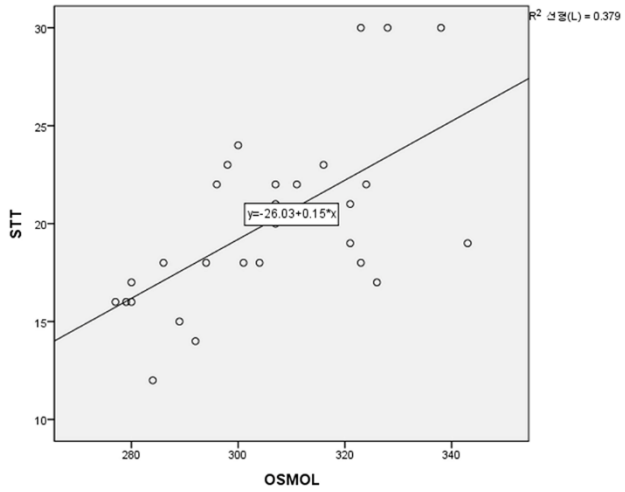

Figure 2 Correlation of Schirmer tear test-1 (STT-1) and tear osmolarity (TO) values at baseline: TO increased proportionally with STT-1 (P < 0.01, r = 0.62).

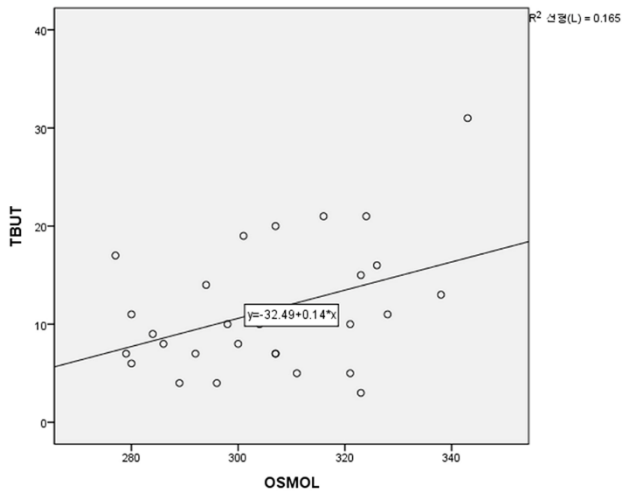

Figure 3 Correlation of tear film break-up time (TBUT) and tear osmolarity (TO) values at baseline: TO increased proportionally with TBUT (P = 0.032, r = 0.41).

Table 1 Comparisons of Schirmer tear test-1 (STT-1), tear film break-up time (TBUT) and tear osmolarity (TO) values before and after treatment using carboxymethyl cellulose eye drops with or without gintonin.

Group	Index	Before treatment	After treatment	*P*-value
Control (without gintonin)	STT-1 (mm/min)	20.0 ± 4.4	21.4 ± 3.8	0.388
	TBUT (s)	15.3 ± 7.1	21.5 ± 9.1	0.054
	TO (mOsm/L)	308.6 ± 18.1	304.2 ± 22.5	0.578
Experimental (with gintonin)	STT-1 (mm/min)	20.1 ± 4.9	21.3 ± 4.3	0.496
	TBUT (s)	7.5 ± 2.8	21.00± 11.2	0.001
	TO (mOsm/L)	302.2 ± 19.3	317.1 ± 18.1	0.045

Discussion

Dry-eye is common in canines, with a prevalence of up to 35% (Kaswan *et al*., 1998), and its importance has increased over time. This condition does not indicate a simple tear film volume inadequacy (Moore, 1990): when the quality of the tear film is sub-standard, this is referred to as qualitative tear deficiency dry-eye. Therefore, the present study considered indices indicative of both tear film volume and quality. Regarding the STT-1 - the most-commonly used clinical diagnostic test for dry-eye - the obtained baseline mean (± SD) value of 20.0 ± 4.6 mm/min suggests that a diagnosis of dry-eye (requiring an STT-1 value of < 10 mm in the presence of clinical symptoms (Williams, 2005)) is not warranted. Thus, tear film volume as measured by the STT-1 was within the normal reference range for both groups. It was not significantly altered by treatment in either group.

The multiple factors that influence qualitative tear deficiency may be divided into two categories. The first concerns disturbances of the tarsal or meibomian glands (Giuliano, 2013). Abnormal meibomian glands can produce highly polar lipids that disrupt the nonpolar surface of the tear film. Such abnormal lipids may also be directly toxic to surface cells (Giuliano, 2013). Furthermore, inflammation of the mucocutaneous junction (i.e. blepharitis or meibomianitis), also results in abnormal lipid secretion. Surface pathology presumably results from the combined insults of absent meibomian secretions, exposure to the exogenous environment, trichiasis, and - in some cases - spastic blepharitis (Giuliano, 2013). The second category concerns insufficient preocular mucin production. Chronic, diffuse conjunctival inflammatory cell infiltrates may decrease or eliminate goblet cells (Giuliano, 2013). Although the cause of infiltrates is often indeterminate, both infectious and autoimmune mechanisms have been hypothesized (Giuliano, 2013). Canines included in the present study were undergoing training and were thus in residence at the kennel club. At the time of conducting the present study (Spring), the city where the kennel club is located was experiencing poor air quality (including fine dust, yellow dust and air pollution), producing high levels of human eye pathology. We speculate that training stress and poor air quality led to chronic low-grade conjunctival inflammation in canines examined during the present study, thereby impairing goblet cell functionality.

The innermost mucin tear film layer plays an important role in maintaining tear stability and its function is reflected by TBUT. In the present study, the mean (± SD) baseline TBUT value was 11.4 ± 6.6 s, which is lower than normal (20 s (Nam and Maeng, 2019)) but higher than the diagnostic threshold for mucin deficiency (5 s (Giuliano, 2013)). Therefore, these canines may have been at increased risk for tear deficiency dry-eye (perhaps again due to training stress and poor air quality), rather than yet exhibiting frank pathology.

In the present study, the mean (± SD) TO baseline value was 305.5 ± 18.7 mOsm/L, lower than the reported normal reference values (339.0 ± 23.0 mOsm/L (Williams, 2017) and 337.4 ± 16.2 mOsm/L (Sebbag *et al*., 2017)). However, low TO is not a clinical sign of dry-eye, although it may occur during the onset stage and thus may progress to dry-eye in later life (Sebbag *et al*., 2017). Based on the positive statistical association between TO and TBUT, lower values of one may be a causative factor in lower values of the other. Given the dearth of previous research comparing canine TO and TBUT, contextualization of this finding and speculation regarding the causes of lower TO values is difficult. However, training stress and poor air quality may again be implicated. Furthermore, a study has reported that dolichocephalic dogs have lower TO than brachycephalic dogs (Williams, 2017).

Feline conjunctivitis studies considering tear quantity, TO and TBUT have demonstrated that TBUT was the most sensitive indicator of conjunctivitis (Davis and Townsend, 2011). In the present study, carboxymethylcellulose with gintonin (relative to carboxymethylcellulose-only) significantly increased TBUT values from 302 to 317 (P = 0.045) mOsm/L. This may reflect a therapeutic increase in the number of conjunctive goblet cells, mediated by gintonin's cell-regenerative effects. Consistent with our canine findings, a prior study demonstrated that medications containing gintonin, such as Solcoseryl® (Solco Basle Ltd., Birsfelden, Switzerland), promote healing of the corneal epithelium (Kim *et al*., 2017). Use of Solcoseryl® has also resulted in increased TBUT values (Nam and Maeng, 2019).

Osmolarity increases are due to either decreased tear production or increased tear evaporation, which

increases ocular surface electrolyte concentrations. In humans, there is thus a negative correlation between tear quantity and osmolarity (Bunya *et al*., 2013), while the present canine study has instead demonstrated a positive correlation. While TO is appropriate as a diagnostic indicator in humans, veterinary science-specific evidence is lacking. Consistent with human findings, a canine study demonstrated that TO increased with decreased tear quantity, providing values of 339 ± 23 and 350 ± 27 mOsm/L, respectively, for healthy versus dry-eye-afflicted dogs (Williams, 2017). However, another canine study demonstrated that tear quantity is proportional to TO, providing TO values of 337.4 ± 16.2 and 306.2 ± 18.0 mOsm/L, respectively, for healthy versus dry-eye-afflicted dogs (Sebbag *et al*., 2017). Such conflicting results suggest that further canine TO studies are warranted.

Results of the present study suggest that the impact of gintonin treatment on TO is comparable to that achieved by five months of cyclosporine, which increased TO from 306.2 to 330.5 mOsm/L (Sebbag *et al*., 2017). Cyclosporine administration decreases MMP-9 levels (Pflugfelder *et al*., 2017). It has also been reported that conjunctival goblet cell density increases with the use of cyclosporine eye drops, improving tear film quality and decreasing epithelial apoptosis (Kunert, 2002; Pflugfelder *et al*., 2017).

We hypothesize that gintonin eye drops have a similar effect. Control of tear film MMP-9 levels by gintonin is attributed to its unique proteinaceous components and high LPA concentration. In the absence of direct evidence, such effects are thought to be consistent across mammalian species, given the conserved roles of LPA and MMPs in the corneal inflammatory response and regenerative capacity. In support of this argument, we have previously confirmed that gintonin also modulates rabbit corneal LPA levels (Kim *et al*., 2017). Further studies are needed to elucidate the specific mechanisms by which gintonin improves TBUT and TO indices.

In conclusion, carboxymethylcellulose supplemented with gintonin significantly increased canine TBUT and TO values, changes which can be considered reflective of improved tear film quality. Thus, gintonin shows promise as a therapeutic agent in canine dry-eye.

Acknowledgements

We would like to thank Dr. Dong-Bum Ji of the Ji dogn Bum Animal Hospital, Busan Korea, who supported use of the I-PEN® VET and provided study-related consultation.

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