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Reference values for selected dry eye tests in normal Beagle dogs: a pilot study

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
ABSTRACT

Background: Dry eye disease (DED) cannot be diagnosed by a single test because it is a multifactorial disorder of the ocular surface. Although studies on various dry eye tests (DETs) in dogs have been reported, standard criteria have not been established except for the Schirmer tear test 1 (STT-1).

Objectives: To establish reference values for DETs in dogs with normal STT-1 values (≥ 15 mm/min) and to analyze the correlation between DETs.

Methods: The STT-1 was performed in 10 healthy Beagle dogs (20 eyes). After 20 min, interferometry (IF) for estimating the lipid layer thickness (LLT) of the tear film, tear meniscus height (TMH), non-invasive tear breakup time (NIBUT), and meibomian gland loss rate of the upper eyelid (MGLRU) and lower eyelid (MGLRL), were measured using an ocular surface analyzer.

Results: Mean values for STT-1, TMH, and NIBUT were as follows: 21.7 ± 3.4 mm/min, 0.41 ± 0.21 mm, and 19.1 ± 9.5 sec, respectively. The most frequent LLT range, measured by IF, was 30–80 nm (11 eyes), followed by 80 nm (5 eyes) and 80–140 nm (4 eyes). MGLRU ≤ 25% was measured in 11 eyes and 26%–50% in 9 eyes; MGLRL ≤ 25% in 8 eyes and 26%–50% in 12 eyes. Besides positive correlation between TMH and NIBUT ($P = 0.038$), there were no significant associations between DETs.

Conclusions: Data obtained in this study provided normative references that could be useful for diagnosing DED and for further research into correlation between DETs in dogs with DED.

Keywords: Dog; non-invasive tear breakup time; ocular surface analyzer; dry eye tests; tear meniscus height

INTRODUCTION

Dry eye disease (DED), or any disorders that have abnormalities in precorneal tear film, has been routinely classified as quantitative or qualitative keratoconjunctivitis sicca (KCS) in veterinary ophthalmology [1]. While quantitative KCS lacks a middle aqueous layer, termed as an aqueous deficient dry eye, qualitative KCS lacks an inner mucin layer that binds the aqueous layer to the lipophilic corneal epithelium or outer lipid layer, limiting aqueous

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Conflict of interest

The authors declare no conflicts of interest.

Author Contributions

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evaporation, termed as evaporative dry eye [1]. The coexistence of these 2 categories can result in varying signs and symptoms [2].

In 2017, DED in humans was newly defined as a multifactorial disorder of the ocular surface, that induces loss of homeostasis with the hyperosmolarity and tear film instability, inflammation of the ocular surface, and neurosensory abnormalities [3]. The various etiologies of DED make it challenging to diagnose and evaluate the treatment effect using only a single test. Therefore, combinations of dry eye tests (DETs) are needed to accurately diagnose DED [4].

In veterinary ophthalmology, many studies have evaluated tear film instability and tear components. Schirmer tear test-1 (STT-1) and phenol red thread test (PRTT) are performed to diagnose quantitative KCS [1,5,6]. Tear film break-up time (TFBUT), tear osmolarity, ferning test, and interferometry (IF) are used for qualitative KCS in dogs [5-9]. IF analyzes the images of the interference colors caused by the refractive index difference between the front surface of the lipid layer and the aqueous-lipid layer interface when the corneal surface is illuminated with a white light [6,10].

Except for STT-1 with a normal range of ≥ 15 mm/min in dogs, the reference ranges for DETs to evaluate canine KCS have not yet been established [11]. In other studies on DETs, the results in dogs and human differed. For example, tear osmolarities of normal dogs were 337.4 ± 16.2 or 339.2 ± 22.7 mOsm/L [7,8] and were higher than normal values in humans (300.8 mOsm/L) [12]. Normal canine TFBUT, 21.5 ± 7.4 sec [5], was also longer than the human value of 10 sec [13].

In humans, clinical symptoms of dry eye, including stinging, burning, foreign body sensation, dryness, epiphora, and blurry vision, are assessed using the ocular surface disease index (OSDI) [14], which is not possible in animals. Since a significant relationship was found between OSDI and ophthalmic tests, such as the tear meniscus height (TMH), non-invasive tear breakup time (NIBUT), lipid layer thickness (LLT), and meibomian gland loss rate, in human studies [15-17], we thought it was necessary to study the reference values of these tests and the association between them in dogs.

The volume of the tear meniscus, which consists of the bulbar conjunctiva and eyelid margin and acts as a reservoir for tears, is assessed by TMH, tear meniscus curvature, and tear meniscus cross-sectional area in practice [18]. In this study, TMH was measured for quantitative assessment of the tear film volume, measuring the tear volume at a cross-sectional area between the central lower eyelid and the corneal or bulbar conjunctival surface [18].

The purpose of this study was to establish reference values for selected DETs using an ocular surface analyzer (OSA) for assessing tear film stability in normal Beagle dogs with normal STT-1, and to analyze the correlation between DETs.

The OSA (I.C.P OSA; SBM Sistemi, Italy) can quickly analyze which layer among the aqueous, mucin, and lipid layers has abnormalities, and help define the type of DED and select appropriate treatment [19].

MATERIALS AND METHODS

Experimental animals

Ten healthy Beagle dogs (20 eyes) were included in this study. The study involved 3 intact male and 7 intact female Beagle dogs and the age and body weight (± SD) of dogs were 3.9 ± 1.5 (range, from 2 to 6) years and 13.2 ± 2.3 (range, from 8 to 16) kg, respectively. All dogs were confirmed to be clinically healthy through physical and ophthalmic examinations, such as hand-held direct ophthalmoscope (Cat. No. 11720; Welch Allyn, USA) and slit-lamp biomicroscopy (SL-D7; Topcon Corp, Japan), and all dogs had no abnormal clinical signs of ophthalmic diseases, such as mucoid to purulent ocular discharge, chemosis, hyperemia, corneal pigmentation, corneal neovascularization, epiphora, or blepharitis. This study was approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University (acceptance No.: SNU-180927-3). All procedures were performed according to the IACUC guidelines.

STT-1

Tear production was measured using the STT strips (Schirmer Tear Test; Merck Animal Health, USA) without topical anesthesia in the order of the right eye (OD), then the left eye (OS). The STT strip was folded at the notch portion of the strip before unpacking the plastic package to avoid the imbibition of oils from the examiner's fingers touching the strip. The strip was then positioned into the conjunctival sac of the lower eyelid at the 1/2 to 1/3 point from the lateral canthus for 1 min. The length from the notch to the end of the wetted strip margin was recorded in millimeters.

To avoid stimulating reflex tearing and influencing the tear film characteristics caused by the STT-1, the other diagnostic tests, including the IF for estimating LLT of tear film, TMH, NIBUT, and meibography for estimating the meibomian gland loss rate of the upper eyelid (MGLRU) and lower eyelid (MGLRL), were performed using an OSA, 20 min after STT-1 measurement.

IF

By evaluating these interferometric patterns according to the IF grading system (**Table 1**) [6,10], the LLT of the precorneal tear film could be determined. The dog's eyes were naturally opened, and the corneal surface was photographed using an OSA immediately after inducing

Table 1. Interferometry grading system according to tear film LLT in this study

Grade	IF pattern	Descriptions	LLT (nm)
0	Not present	Invisible lipid layer	
1	Open meshwork	Gray, very transparent, little reflection, knitted pattern [6] Indistinct gray, marble-like appearance. Frequently visible only by post blink movement [10]	15 [6]
2	Closed meshwork	Gray, more dense and reflective, tightly knitted pattern [6] Well-defined gray, marble-like pattern with tight meshwork [10]	30 [6]
3	Flow	Reflective, with horizontal vertical waves [6] Constantly changing wave-like pattern [10]	30-80 [6]
4	Amorphous	White/bluish with predominantly horizontal waves [6] Blue-whitish appearance with no discernable features [10]	80 [6]
5	Normal fringes	Yellow, brown, blue, and purple on a gray transparent background [6] Colored interference fringes. Gradual color changes [10]	80-140 [6]
6	Abnormal fringes	More intense colors with predominantly horizontal waves [6] Discrete areas of highly variable colored fringes. Rapid color changing on small areas [10]	140-370 [6]

The number in square bracket means the reference of IF grading system.
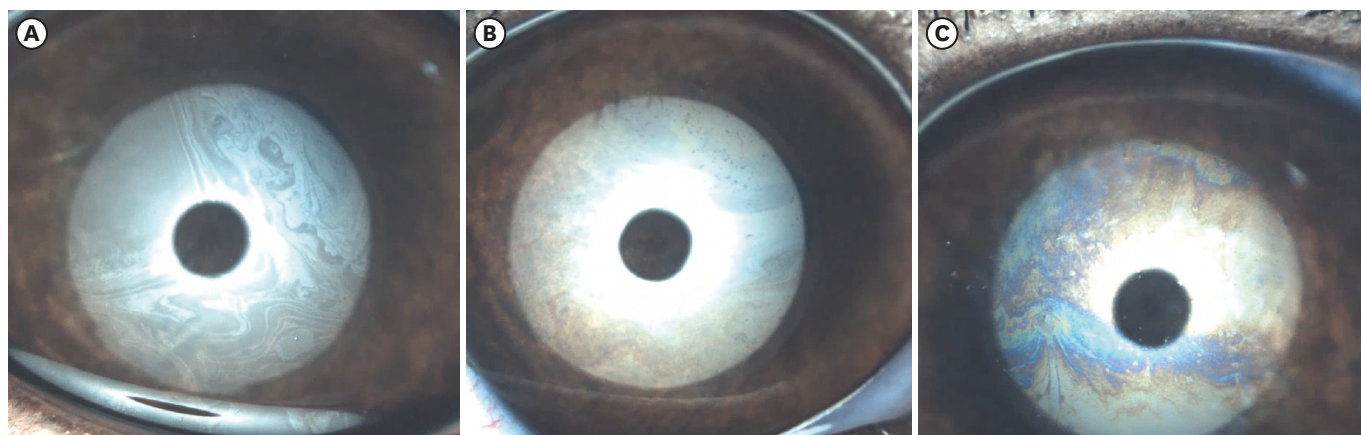LLT, lipid layer thickness; IF, interferometry.

Fig. 1. Interferometry grading patterns observed by an ocular surface analyzer in this study. (A), Flow pattern (right eye of case 2). (B), Amorphous pattern (right eye of case 4). (C) Normal color fringes pattern (left eye of case 5).

palpebral reflex. These interferometric patterns were classified into 7 steps from 0 to 6 as follows: grade 0, no lipid layer visible; grade 1, open meshwork; grade 2, closed meshwork; grade 3, flow pattern; grade 4, amorphous pattern; grade 5, normal fringes; and grade 6, abnormal fringes (**Fig. 1**).

TMH

For TMH measurement, the ocular surface including the lower eyelid was photographed with an ocular surface analyzer. The upper and lower tear meniscus borders were manually selected by the examiner, and then TMH, a distance between upper and lower meniscus border, was automatically measured by the software integrated into the equipment (**Fig. 2**).

NIBUT

As in the IF measurement, immediately after the complete blinking of the eye, the examiner kept the dog's eye open with the thumb and index finger. With this state maintained, the corneal surface was illuminated by the light source to generate black lattice lines on the outer surface of the cornea, and the time interval from the first opening of the eyelid to the time of
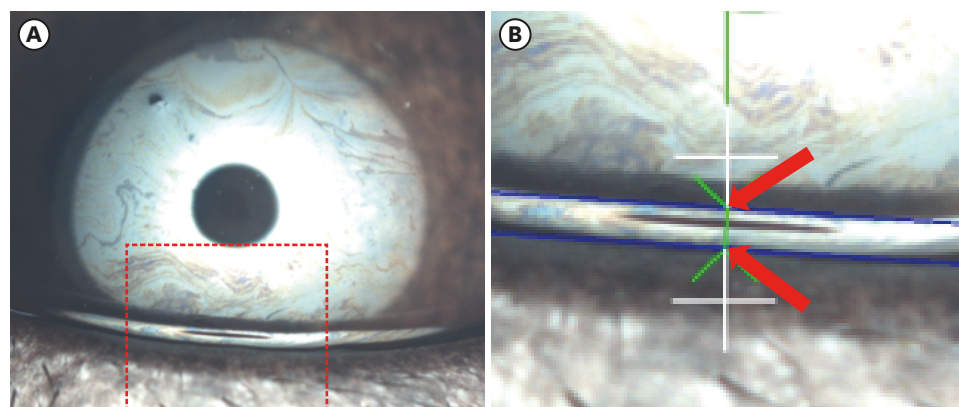
Fig. 2. Measurement of TMH in the left eye of case 5. (A), Original ocular surface image photographed by an OSA. (B), A magnified image of the red dotted rectangular area in (A). After manually selecting the upper and lower tear meniscus borders (red arrows), the TMH, a distance between upper and lower meniscus border, was automatically measured by the software integrated in the OSA equipment.
TMH, tear meniscus height; OSA, ocular surface analyzer.

the first perturbation (deformation or distortion) or break-up of the reflected grid images on the corneal surface [18] was recorded in seconds.

Meibography

To measure the meibomian gland loss rate (MGLR), the everted tarsal plate areas of the upper and lower eyelids were photographed by a non-contact, non-invasive infrared camera. Meibomian gland area was automatically highlighted with green lines by the software integrated in the OSA and the meibomian gland loss rate was calculated as the percentage of meibomian gland loss in relation to the entire tarsal plate area (**Fig. 3**). The same procedure was repeated 3 times, and the results were averaged and graded from 0 to 4 as follows: grade 0, area of loss 0%; grade 1, area of loss ≤ 25%; grade 2, area of loss 26%–50%; grade 3, area of loss 51%–75%; and grade 4, area of loss > 75% [20].

All ophthalmic examinations were performed by the same examiner (YK) and proceeded in awake animals with minimal physical restraint and with no medications, such as sedatives, or systemic or topical anesthetics, in the same examination room under the same calm conditions (ambient temperature 20°C–25°C, humidity 35%–40%).

Statistical analyses

Statistical analyses were performed using SPSS 25 (IBM Corp., USA). A statistical normality of the data was assessed with the Shapiro-Wilk test. To obtain statistical results for 20 eyes of 10 Beagle dogs, it was necessary to confirm that there was no significant difference between OD (10 eyes) and OS (10 eyes) in the results of STT-1, IF, TMH, NIBUT, MGLRU, and MGLRL.
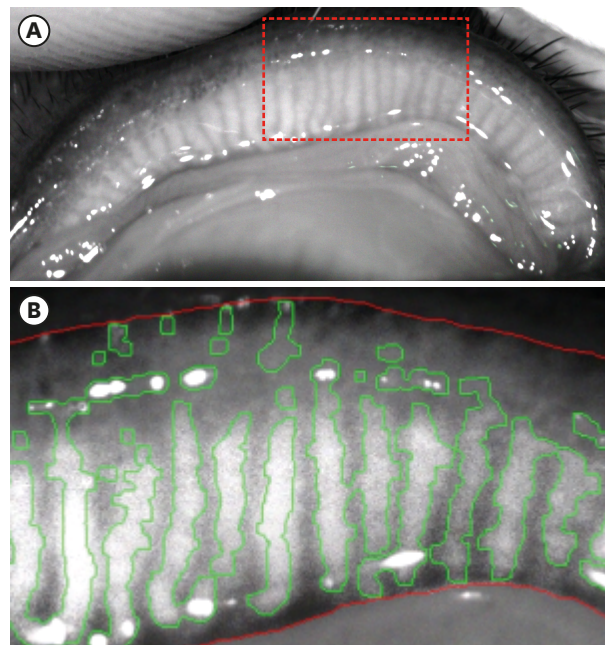

Fig. 3. Meibographic image of the upper eyelids in case 10. (A) Original picture of the everted tarsal plate areas taken by an infrared light camera built into the OSA in the left upper eyelid. (B) A magnified image of the red dotted rectangular area in (A). Meibomian gland area was automatically highlighted with green lines by the software integrated in the OSA and the meibomian gland loss rate was calculated as the percentage of meibomian gland loss in relation to the entire tarsal plate area. The same procedure was repeated 3 times, and the results were averaged and graded from 0 to 4 as follows: grade 0, area of loss 0%; grade 1, area of loss ≤ 25%; grade 2, area of loss 26%–50%; grade 3, area of loss 51%–75%; and grade 4, area of loss > 75% [20].OSA, ocular surface analyzer.

Therefore, the STT-1, TMH, and NIBUT were compared between OD and OS using the Mann-Whitney U, nonparametric test, because the distributional assumption for parametric testing was not satisfied. The IF, MGLRU, and MGLRL were compared between OD and OS using the Chi-squared test as well as Fisher's exact test. As a result, since there was no significant difference between OD and OS, statistical analyses were performed on 20 eyes. The data were expressed as the mean ± SD and calculated for all 20 eyes. Since the variables were a combination of continuous (STT-1, TMH, and NIBUT) and categorical (IF, MGLRU, and MGLRL), the correlations among the tests were calculated using the Spearman's rank correlation analysis. Statistically significance was set at $p < 0.05$.

RESULTS

Distribution of test results

In 20 eyes of 10 normal Beagle dogs, the test values (mean ± SD) of the STT-1, TMH, and NIBUT were 21.7 ± 3.4 mm/min, 0.41 ±0.21 mm, and 19.1 ± 9.5 sec, respectively (**Table 2**). The most frequently measured IF value was grade 3 (11 eyes, 55%), followed by grade 4 (5 eyes, 25%) and grade 5 (4 eyes, 20%). These results mean that of the LLT measured by IF grading system, 30–80 nm (11 eyes) was the most frequent, followed by 80 nm (5 eyes) and 80–140 nm (4 eyes) (**Fig. 4**). The estimated values of the MGLRU and MGLRL are shown in **Fig. 5**. These results mean that an MGLRU of ≤ 25% was measured in 11 eyes and 26%–50% in 9 eyes, and an MGLRL of ≤ 25% was measured in 8 eyes and 26%–50% in 12 eyes. There were no cases in which the meibomian gland loss rate exceeded 50% in the upper and lower eyelids in normal Beagle dogs.
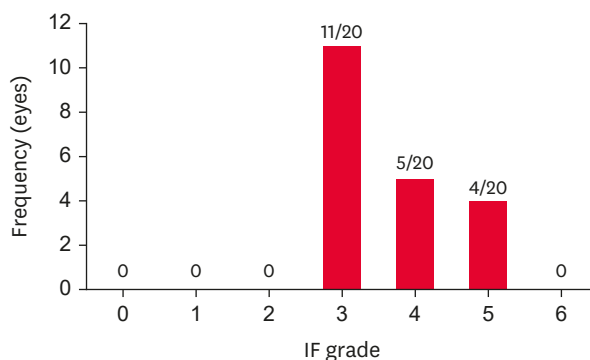

Fig. 4. Bar graph showing the distribution of IF grade suggesting tear film lipid layer thickness. The most frequently measured interferometry value was grade 3 (11 eyes, 55%) and the rest were measured at frequencies of 5 eyes for grade 4 (25%), and 4 eyes for grade 5 (20%).
IF, interferometry.

Table 2. Mean values of STT-1, TMH, and NIBUT results in OD, OS, and OU

Variables	Mean ± SD			Minimum	Maximum
	OD (n = 10)	OS (n = 10)	OU (n = 20)		
STT-1	22.0 ± 4.0	21.3 ± 2.8	21.7 ± 3.4	15	28
TMH	0.40 ± 0.25	0.43 ± 0.16	0.41 ± 0.21	0.15	0.91
NIBUT	19.8 ± 11.0	18.4 ± 8.2	19.1 ± 9.5	8.2	37

No significant differences were determined between OD and OS in the STT-1, TMH, and NIBUT ($p = 0.567$, $p = 0.449$, and $p = 0.880$, respectively) using the Mann-Whitney U test. Statistical significance was set at $p < 0.05$.
STT-1, Schirmer tear test-1; TMH, tear meniscus height; NIBUT, non-invasive breakup time; OD, right eye; OS, left eye; OU, both eyes.
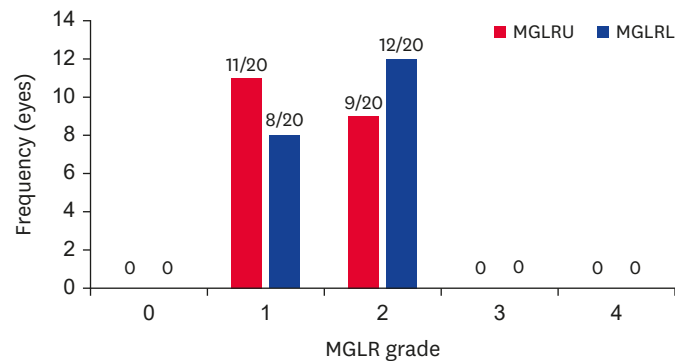
Fig. 5. Bar graph showing the distribution of MGLRU and MGLRL. The MGLRU showed a grade 1 result in 11 eyes (55%), and grade 2 in 9 eyes (45%) and MGLRL showed a grade 1 result in 8 eyes (40%) and grade 2 in 12 eyes (60%). MGLR, meibomian gland loss rate; MGLRU, meibomian gland loss rate of the upper eyelid; MGLRL, meibomian gland loss rate of the lower eyelid.
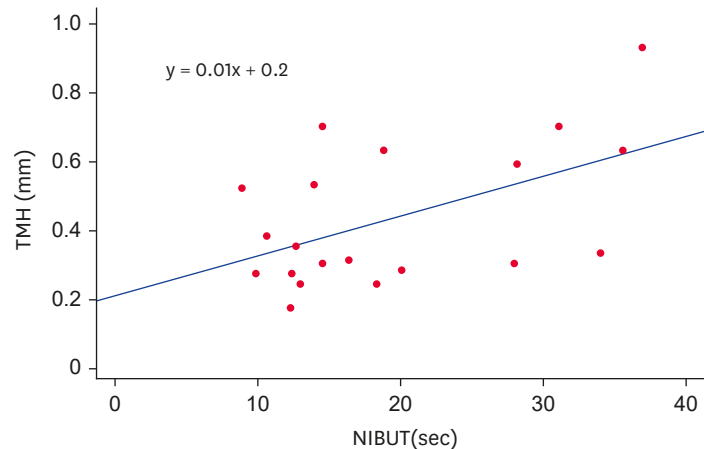

Fig. 6. Correlation between the NIBUT and TMH. The significant positive correlation was noted by Spearman's rank correlation analysis ($r = 0.467$, $p = 0.038$) in 20 eyes of 10 Beagle dogs. The r value from 0.40 to 0.59 means the moderate strength of the correlation between 2 variables.
NIBUT, non-invasive breakup time; TMH, tear meniscus height.

Correlation analysis among the tests

The STT-1 values did not significantly correlate with IF ($r = -0.168$, $p = 0.478$), TMH ($r = -0.182$, $p = 0.443$), NIBUT ($r = 0.205$, $p = 0.385$), MGLRU ($r = -0.123$, $p = 0.605$), or MGLRL ($r = -0.054$, $p = 0.822$). In addition, the TMH had a significantly positive correlation with NIBUT ($r = 0.467$, $p = 0.038$) (**Fig. 6**), and no significant associations were found among the other tests.

DISCUSSION

In humans, a variety of studies have shown that mean values of LLT of tear film in normal populations are quite variable, such as 79.55 ± 37.31 nm [16], 68.3 ± 13.7 nm [21], and 42 nm [22] in different studies. In another study, more than 74% of patients with severe dry eye syndrome had an LLT of less than 60 nm, and more than 72% of patients without dry eye had an LLT ≥ 76 nm [23]. In the present study, the IF grade 3, which means an LLT of 30–80 nm [6], was the most frequent in dogs without DED (**Fig. 4**). Since the lipid layer, which is the outermost layer of the ocular tear film, is known to prevent the evaporation of the aqueous layer [24], it is expected that the thinner the oil layer, the more the aqueous layer evaporates

and the STT-1 value decreases. Furthermore, the LLT is positively correlated with the Schirmer tear test with topical anesthesia in subjects with symptoms of dry eye in humans [25]. However, the correlation between the STT-1 and LLT was not confirmed ($r = -0.168$, $p = 0.443$) in the present study.

The following assumptions are possible for why there is no correlation between STT-1 and LLT. First, while Isreb et al. [25] reported a positive correlation between STT-1 and LLT in people with DED, our study conducted these tests in normal Beagle dogs. In fact, Choi et al. [26] reported a weak correlation (Pearson correlation coefficient, $r = 0.025$) between STT-1 and LLT in 75 human subjects without DED. Therefore, further studies in dogs with DED are warranted. Second, although most dogs with epiphora have anatomical abnormalities of the eyelids that lead to the overflow of tears, a previous study showed that epiphora can be caused by compensatory reflex hypersecretion, in which the lacrimal system is oversecretes tears to compensate for water loss by excessive ocular surface exposure secondary to the lack of a lipid layer in evaporative dry eye [27]. Hence, in the assessment of causes of epiphora in dogs, LLT values should be included as a causal factor.

In humans, Mainstone et al. [28] determined that the mean range of TMH was 0.244 ± 0.089 mm in dry eyes and 0.46 ± 0.173 mm in normal eyes and suggested that the diagnostic sensitivity and specificity of the DET was 93.3% and 66.7%, respectively, when the cut-off level of dry eye diagnosis was below 0.35 mm in humans. In the present study, the TMH mean was 0.41 ± 0.21 mm and was consistent with the previous study in humans [28]. Therefore, it is also necessary to determine whether TMH is associated with quantitative KCS in dogs as it is in human patients with DED. In addition, if the TMH results are not related with the quantitative KCS in dogs, further research is needed into the factors that can affect TMH results, such as corneal irritation by STT-1 strips, light stimulation due to other tests, and time interval between tests measurement. Previously, Sebbag et al. [29] concluded that a sufficient time interval of ≥ 10 min should be allowed for the replenishment of tear film. Based on this result, in order to avoid stimulating reflex tearing and to minimize these interference effects, we set a time interval of 20 min between the STT-1 and other selected tests in the present study.

The most common test for evaluating the tear film instability in both human and veterinary ophthalmology is the TFBUT using a fluorescein dye [11,18]. However, the disadvantage of the TFBUT is that the time can be affected by using the different amounts of fluorescein dye. Moreover, fluorescein dye is known to destabilize the tear film regardless of whether the conventional test strip or solution is used. Benzalkonium chloride, which was used as a preservative in eyedrops, reduces the tear film stability. Consequently, if eyedrops containing benzalkonium chloride are used for TFUBT using fluorescein dye, TFBUT may be shortened. These factors can affect the clinical evaluation of TFBUT [13,30,31].

Therefore, the TFBUT can be complemented by the non-contact NIBUT, which observes reflected grid images of the placid disk from the corneal surface with ophthalmic topography systems [32] and is becoming a more popular diagnostic test in clinical practice and research in humans [18]. Pauk et al. [33] reported that the NIBUT was significantly more sensitive than the TFBUT in evaluating DED when the cut-off level for dry eye diagnosis was 12 sec for NIBUT and 8 sec for TFBUT.

Two studies have evaluated the relationship between TFBUT and NIBUT in humans. In one study, the NIBUT was longer than the TFBUT by 1.85 ± 1.48 sec and the 2 variables were

significantly correlated with each other [34]. In contrast, the TFBUT and NIBUT were found to have similar results [35]. In veterinary ophthalmology, the mean range of TFBUT for 16 normal Beagle dogs was reported as 21.53 ± 7.42 sec (range: 10–35 sec) [36]. In addition, another study reported that the mean TFBUTs were 19.96 ± 5.01 sec for OD and 19.38 ± 4.80 sec for OS for 24 normal Beagle dogs [37]. The NIBUT values in our study were similar to previously reported TFBUT results [36,37], suggesting that NIBUT could be applied to evaluate tear film stability in dogs.

There were several factors that made NIBUT examination difficult in the present study: a sudden elevation of the third eyelid, unexpected movements of the eye, mucoid discharge on the surface of the cornea, and non-cooperation of animals (head shaking, barking, and panting) during the NIBUT examination (**Fig. 7**). The factors mentioned above, together with irregularity of the corneal surface secondary to keratitis, can make TFBUT examination difficult. As the test was repeated, the amount of mucoid discharge increased because of the light and drying of the ocular surface caused by longer ocular surface exposure time. Because of this, the NIBUT is relatively difficult to measure in dogs.

In this study, NIBUT was significantly correlated with TMH (p = 0.038) (**Fig. 6**). In a human study, Hong et al. [38] also reported that the NIBUT values had a significantly positive correlation with the STT-1, TMH, and TFBUT. Therefore, when it is difficult to measure the NIBUT, it may be helpful to evaluate the tear film instability by replacing the NIBUT with TMH or TFBUT. Although the STT-1, IF, MGLRU, and MGLRL were not correlated with the NIBUT in this study, further studies are needed as there is a possibility that different statistical significance may be obtained in studies of a larger number of dogs with DED.

In our study, there were no grades 0, 3, or 4 in the MGLR of the upper and lower eyelids (**Fig. 5**). These results mean that there were no cases in which the meibomian gland was lost more than 50% in normal Beagle dogs. The frequency of grade 2 MGLRL was higher than that of grade 2 MGLRU. These results are similar to those reported by Pult et al. [39] in humans. In that study, MGLRL was higher (mean: 32.3%; upper eyelid: 26.9%) (P < 0.001). MGLRL was significantly related to dry eye population by regression analysis and significantly increased in patients with dry eye [17]. However, it was more difficult to sufficiently expose the meibomian gland area of the lower eyelid than the upper eyelid in this study in dogs. In human studies, a positive correlation between MGLRU and MGLRL has been confirmed
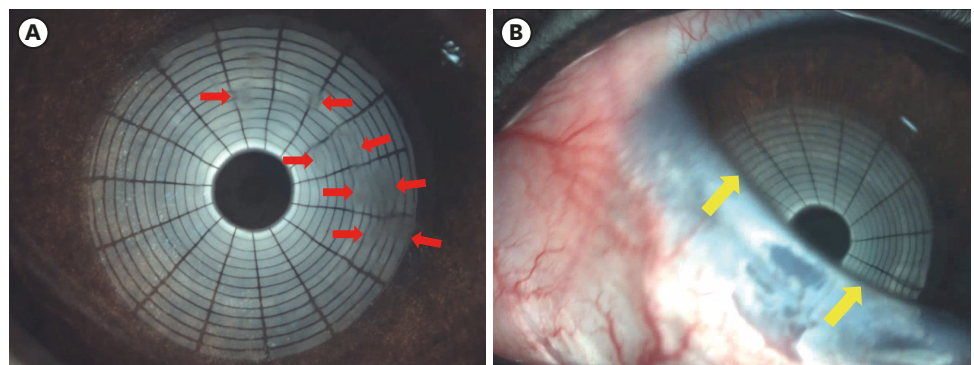

Fig. 7. NIBUT measurement using an ocular surface analyzer. (A) Red arrows indicate the areas where the grid was deformed due to the mucus on the corneal surface. This artifact made NIBUT measurement difficult. (B) During the NIBUT test, the measurement failed due to sudden elevation of the third eyelid (yellow arrows).
NIBUT, non-invasive breakup time.

[39]. Therefore, it may be necessary to study whether MGLRL has a stronger correlation than MGLRU in dogs with DED. If it is found that MGLRL and MGLRU are strongly correlated, and that MGLRL is also associated with other DETs or KCS through further studies with a larger population of normal dogs and using dogs with KCS, it may be recommended to evaluate KCS using MGLRU, which is easier to perform than MGLRL in dogs.

This study had the following limitations: there may have been factors that contributed to the tear film instability, including ambient temperature, humidity, fluorescein dye, and air circulation [18]. However, the influence of these factors was not evaluated in this study. We completed all examinations in 1 day to minimize the impact of these factors.

In addition, Williams et al [8] suggested that the normal ranges of tear osmolarity in humans and dogs were different, resulting from higher tear evaporation due to lower eye blink rate in dogs (3–5 times/min) [40] than in humans (10–30 times/min) and different exposure of the ocular surface in different skull shapes (higher exposure in brachycephalic than mesaticephalic or dolichocephalic breeds). However, there was no comparison of results according to each species and breed characteristics since this study was only performed in normal Beagle dogs. Further studies are needed to determine the reference values of various DETs in different species and breeds.

In conclusion, this study could provide normative diagnostic reference values for selected DETs, such as LLT, TMH, NIBUT, MGLRU, and MGLRL in normal dogs and help the clinicians evaluate the quantitative and qualitative KCS in dogs. Besides TMH having a positive correlation with NIBUT, there were no significant correlations among selected DETs in this study using normal Beagle dogs. Based on the results, additional research should assess the changes in the measured values of the selected DETs and the correlation between the DETs in different breeds of dogs with tear film instability, including abnormal STT-1.

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