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Association of serum 25-hydroxyvitamin D concentrations with Schirmer tear test 1 and tear film breakup time in dogs

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ABSTRACT

Background: The association between vitamin D and canine keratoconjunctivitis sicca (KCS) has not been investigated in dogs.
Objectives: To investigate the association of serum 25-hydroxyvitamin D [25(OH)D] concentrations with Schirmer tear test 1 (STT-1) and tear film breakup time (TFBUT) in dogs.
Methods: Sixty-one clinically healthy, client-owned dogs were enrolled. STT-1 and TFBUT were measured in 122 (61dogs) and 82 (41 dogs out of total 61 dogs) eyes, respectively. Serum 25(OH)D concentrations were evaluated by quantitative chemiluminescent immunoassay. The dogs were classified into 6 groups according to the evaluations (STT-1: group 1, normal [≥ 15 mm/min] in both eyes; group 2, normal in one eye and abnormal [< 15 mm/min] in the fellow eye; group 3, abnormal in both eyes; TFBUT: group 4, normal [≥ 20 sec] in both eyes; group 5, normal in one eye and abnormal [< 20 sec] in the fellow eye; group 6, abnormal in both eyes).
Results: STT-1 was positively correlated with TFBUT ($p < 0.001$). Among the STT-1 groups, the mean serum 25(OH)D concentration in group 1 was significantly higher than in groups 2 and 3 with positive correlation ($p < 0.001$). However, there were no significant differences among the TFBUT groups 4, 5, and 6.
Conclusions: In dogs, it was found that serum 25(OH)D concentrations had a greater effect on quantitative KCS than qualitative KCS. Therefore, it is considered that measurement of serum 25(OH)D concentration could be included in the diagnostic tests in canine quantitative KCS patients.

Keywords: Canine; dry eye; dry eye test; keratoconjunctivitis sicca; vitamin D

INTRODUCTION

Vitamin D deficiency is known to be associated with poor skeletal maturation, cancers, cardiovascular disease, hypertension, diabetes mellitus, periodontal disease, osteoarthritis, and rheumatoid arthritis in humans [1]. Additionally, it was suggested that vitamin D deficiency was related to tear hyperosmolarity, impairment in tear film function, and development of dry eye syndrome in humans [2,3]. Meanwhile, other studies suggested that vitamin D concentration was not correlated with dry eye disease (DED) [4,5].

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Conflicts of Interest
The authors declare no conflicts of interest.

In dogs, several studies had suggested that decreased levels of serum 25-hydroxyvitamin D [25(OH)D] concentrations are related to various diseases, including cancer [6-8], inflammatory bowel disease [9], renal disease [7,10], congestive heart failure [11], and hyperparathyroidism [7]. However, the association between serum vitamin D concentrations and keratoconjunctivitis sicca (KCS) has not been investigated in dogs yet.

Among three layers of the tear film composed of aqueous, mucin, and lipid layer, the aqueous production deficiency (quantitative KCS) is diagnosed when Schirmer tear test 1 (STT-1) is < 15 mm/min with ocular discharge, blepharospasm, conjunctival hyperemia, corneal neovascularization, pigmentation, corneal ulcer, and reduced vision [12]. Qualitative KCS with abnormalities of the mucin and/or lipid layer is evaluated using tear film breakup time (TFBUT), conjunctival biopsy to determine goblet cell numbers, meibum expression, and meibometry [12,13]; the most commonly performed diagnostic test in dogs is TFBUT [12,13]. Dogs with clinical signs such as blepharitis, meibomianitis, and conjunctivitis are diagnosed with qualitative KCS. Especially, mucin deficiency KCS is diagnosed if the TFBUT values are less than 20 sec [12].

Vitamin D (calciferol) is known as a fat-soluble secosteroid that contributes to the maintenance of the calcium and phosphate homeostasis and is involved in various biological functions, including cellular health and gene regulation [14]. Vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol) are the most important subtypes of vitamin D; they are first hydroxylated in the liver to 25(OH)D (calcifediol) and is later converted to 1,25-dihydroxyvitamin D [$1,25(OH)_2D$, calcitriol] after the second hydroxylation in the kidney. Serum $1,25(OH)_2D$ is an activated form of vitamin D that binds to vitamin D receptors, which are expressed in a wide variety of target cells that control calcium concentration, allow differentiation of immune cells, and reduce inflammation [1,15,16]. However, $1,25(OH)_2D$ does not represent the actual vitamin D status since it has a short half-life (4 h) and its levels can be normalized or increased by the action of the parathyroid hormone even when the vitamin D concentration is low [14,17,18]. Therefore, serum 25(OH)D is used instead as an indicator of the vitamin D status as it has a relatively long half-life (2–3 weeks) [1,18,19].

The purpose of this study was to measure the representative dry eye test (DET), STT 1 for quantitative KCS and TFBUT for qualitative KCS in dogs and to investigate the correlation between these two test values and serum 25(OH)D concentration in dogs.

MATERIALS AND METHODS

Animals studied

The study population consisted of 61 client-owned dogs from 14 different breeds that presented at Dana Animal Hospital Eye Center. The mean age (± standard deviation, SD) of the dogs at presentation was 9.48 ± 4.13 years (range, 1–17 years). The breeds were Shih Tzu (n = 16), Maltese (n = 15), toy Poodle (n = 6), Bichon Frise (n = 4), Pomeranian (n = 4), Crossbreed (n = 3), Yorkshire Terrier (n = 3), Chihuahua (n = 2), Pekingese (n = 2), miniature Schnauzer (n = 2), American Cocker Spaniel (n = 1), French Bulldog (n = 1), Spitz (n = 1), and West Highland White Terrier (n = 1). The study included 1 intact male, 20 castrated males, 15 intact females, and 25 spayed females (**Table 1**). This study was approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University (SNU-221014-1) and conducted after written informed consent was obtained from each owner.

Table 1. Age, body weight, sex according to the groups in this study

Group	No. of dogs	Age (yr)	Body weight (kg)	Sex
1	33	8.6 ± 4.3	5.2 ± 2.4	1 M, 13 CM, 9 F, 10 SF
2	5	9.2 ± 6.1	5.7 ± 2.5	2 CM, 1 F, 2 SF
3	23	10.8 ± 3.1	5.3 ± 1.8	5 CM, 5 F, 13 SF
4	8	4.4 ± 2.8	5.6 ± 3.0	4 CM, 1 F, 3 SF
5	4	9.3 ± 3.5	5.5 ± 1.6	2 CM, 1 F, 1 SF
6	29	10.9 ± 3.5	4.8 ± 1.7	6 CM, 8 F, 15 SF

Mean values of age and body weight was expressed as mean ± SD.
M, intact male; CM, castrated male; F, intact female; SF, spayed female.

Dogs with distichiasis, entropion, ulcerative keratitis, buphthalmos due to chronic glaucoma, cataract, anterior uveitis, eyelid or third eyelid deformities, facial nerve paralysis, history of previous ophthalmic surgery, illnesses including diabetes mellitus and other hormonal diseases, and dogs who have recently underwent treatment using systemic drugs or eye drops that could influence lacrimal function or received lacrimostimulant eyedrops, such as cyclosporine and tacrolimus, were excluded from this study.

All dogs underwent complete ophthalmic examinations, including STT-1, slit-lamp biomicroscopy (SL-D7, Topcon Corp, Japan), intraocular pressure measurement using rebound tonometer (Tonovet; Icare Finland Oy, Finland), fluorescein dye staining test followed by TFBUT, and routine neuro-ophthalmic assessment tests of menace response, dazzle reflex, pupillary light reflex, and palpebral reflex during the first admission.

STT-1

Tear production in 61 dogs (122 eyes) was measured using STT strips (Schirmer tear test, Merck Animal Health, USA) without topical anesthesia (STT-1) first in the right eye (OD) and then in the left eye (OS). The STT strip was folded at the notched portion of the strip before unpacking the plastic package to avoid the imbibition of oils from the examiner's fingers. 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 moistened strip margin was recorded in millimeters.

TFBUT

TFBUT was performed ten minutes after the STT-1 examination in 41 dogs (82 eyes) out of a total of 61 dogs. A fluorescein dye strip (Fluorescein sodium; Optitech Eyecare, India) was moistened with one drop of sterile saline solution without preservatives and placed on the lower conjunctival fornix. Complete blinking of the dog's eyelids (2–3 times) was achieved by stimulating the lateral palpebral reflex. Next, the examiner kept the dog's eyes open with the thumb and index finger. The time interval between the last eyelid opening and appearance of a dark streak due to tear film breakup was recorded as the TFBUT (sec) using broad beam illumination with a cobalt blue filter integrated in slit-lamp biomicroscopy. The same procedure was repeated three times, and the results were averaged for each eye to enhance the repeatability.

All the ophthalmic examinations, including STT-1 and TFBUT, that were conducted as part of the study were performed by one examiner (YK) in a single examination room under the same calm conditions (ambient temperature, 20°C–25°C; humidity, 35%–40%). To minimize the effects of the variables that could affect the measured values of STT-1 and TFBUT, all the strips used in the present study were obtained from the same manufacturer,

and one drop of sterile saline without preservatives was consistently applied to dilute the fluorescein dye strips.

Measurement of serum 25(OH)D concentration

After performing ophthalmic examinations, whole blood samples were drawn from 61 dogs, collected in a serum separator tube (BD Vacutainer SST II Advance Tube), and centrifuged within 30 min. The obtained serum (minimum 1 mL) was protected from light and stored at 2°C–8°C in an airtight plastic tube before measurement. All the serum samples were sent to a single commercial laboratory (Neodin BioVet, Korea) within 2 days for measurement of the serum 25(OH)D concentration and were evaluated by quantitative chemiluminescent immunoassay.

Group classification according to STT-1 and TFBUT

The dogs were classified into 6 groups depending on their STT-1 and TFBUT values to study the association between these values and the serum 25(OH)D concentration in a clinical setting. In this study, STT-1 values ≥ 15 mm/min and TFBUT ≥ 20 sec were regarded as normal. The dogs were classified int the following groups: group 1 (normal STT-1 in both eyes), group 2 (normal STT-1 in one eye and abnormal in fellow eye), group 3 (abnormal STT-1 in both eyes), group 4 (normal TFBUT in both eye), group 5 (normal TFBUT in one eye and abnormal in fellow eye), and group 6 (abnormal TFBUT in both eyes).

Statistical analyses

All data were analyzed using SPSS (version 21 for Windows, SPSS Inc., USA). Student's *t*-test was used to investigate the association of STT-1 and TFBUT with the serum 25(OH) D concentration in each group. Pearson's correlation analysis was used to analyze the relationships between STT-1 and TFBUT, between STT-1 and serum 25(OH)D concentration, between TFBUT and serum 25(OH)D concentration, between age, body weight, and serum 25(OH)D concentration. All measurements were expressed as mean ± SD. Differences with $p < 0.05$ were considered statistically significant.

RESULTS

The mean (± SD) values for age, body weight, and breed distribution according to groups are summarized in **Table 1**. There were no significant age differences among groups 1, 2, and 3; however, among in groups 4, 5, and 6, groups 5 and 6 had a significantly higher age ($p = 0.021$, $p < 0.001$, respectively) than in group 4. Meanwhile, there were no significant body weight differences among all groups, and no correlation was found between age, body weight, and serum 25(OH)D concentration.

Correlation between STT-1 and TFBUT

The mean ± SD STT-1 of the 71 eyes with an STT-1 measurement of ≥ 15 mm/min in a total of 122 eyes was 18.97 ± 2.44 mm/min, whereas that in 51 eyes with an STT-1 measurement < 15 mm/min was 8.92 ± 4.12 mm/min. The mean ± SD TFBUT of 20 eyes with a TFBUT of ≥ 20 sec was 24.58 ± 4.00 sec, whereas that in 62 eyes with a TFBUT of < 20 sec was 9.74 ± 4.34 sec. Results revealed that there was a significant positive correlation between STT-1 and TFBUT (n = 82; Pearson's correlation, $r = 0.497$, $p < 0.001$) (**Fig. 1**). The correlation between STT-1 and TFBUT was not significant when STT-1 was normal (≥ 15 mm/min) (Pearson's correlation, $r = 0.023$, $p = 0.873$). On the other hand, when STT-1 was abnormal (< 15 mm/min), a significant positive correlation was observed between them (Pearson's correlation, $r = 0.446$, $p = 0.009$)

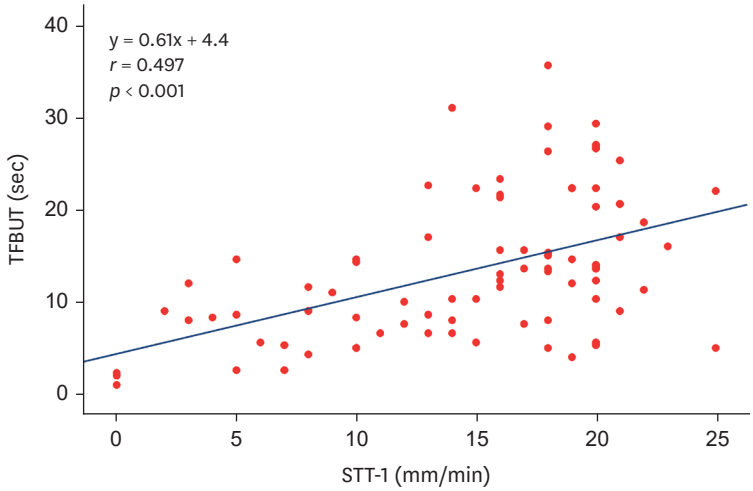

Fig. 1. Correlation between STT-1 (mm/min) and TFBUT (sec) in dogs. STT-1 was significantly correlated with TFBUT (n = 82 eyes; Pearson's correlation analysis, $r = 0.497$, $p < 0.001$).
STT-1, Schirmer tear test 1; TFBUT, tear film breakup time.

Association of serum 25(OH)D concentrations with STT-1 and TFBUT

In the present study, the STT 1 values showed a significantly positive correlation with the serum 25(OH)D concentration (n = 122 eyes; Pearson's correlation analysis, $r = 0.353$, $p < 0.001$) (**Fig. 2**). On the other hand, no significant correlation was found between TFBUT and serum 25(OH)D concentration (n = 82 eyes; Pearson's correlation analysis, $r = 0.043$, $p = 0.663$) (**Fig. 3**). In addition, there was a stronger correlation between serum 25(OH)D concentration and STT 1 when both STT 1 and TFBUT were abnormal (STT 1 < 15 mm/min, TFBUT < 20 sec; Pearson's correlation, $r = 0.501$, $p = 0.004$).

Comparison of mean serum 25(OH)D concentrations between the groups according to the range of STT-1 and TFBUT values

Among the STT-1 values, the mean ± SD serum 25(OH)D concentration in group 1 (31.65 ± 10.20 ng/mL) was significantly higher than in group 2 (21.66 ± 10.55 ng/mL; $p = 0.049$)

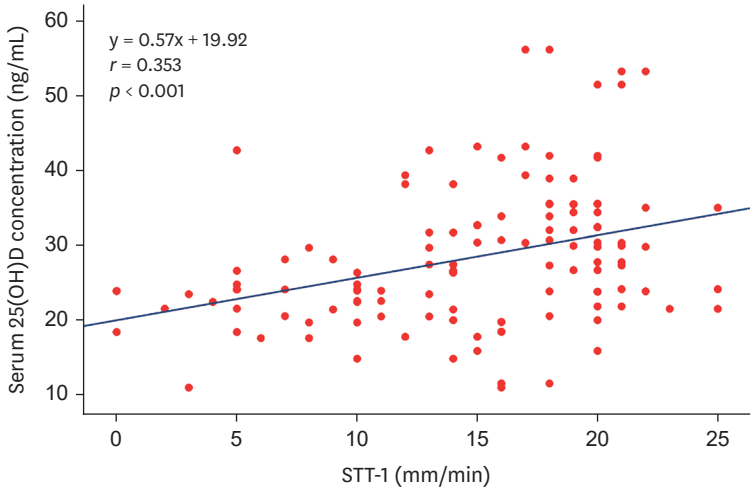

Fig. 2. Correlation between STT-1 (mm/min) and serum 25(OH)D concentration (ng/mL). STT-1 was significantly correlated with serum 25(OH)D concentration (n = 122 eyes; Pearson's correlation analysis, $r = 0.353$, $p < 0.001$).
STT-1, Schirmer tear test 1; 25(OH)D, 25-hydroxyvitamin D.

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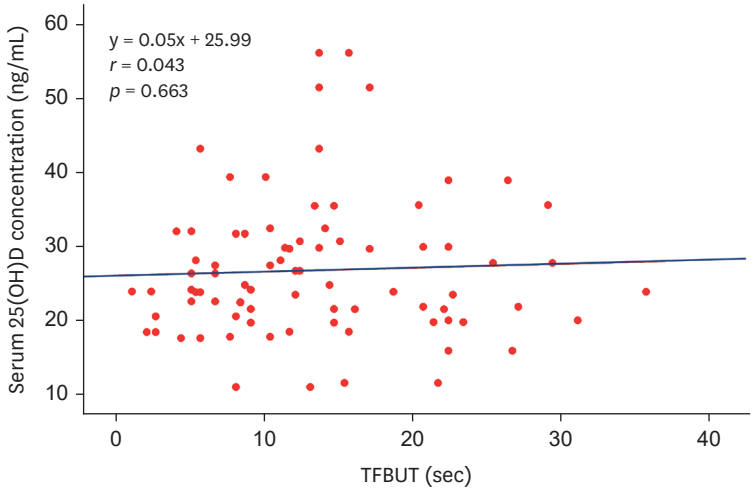

Fig. 3. Correlation between TFBUT (sec) and serum 25(OH)D concentration (ng/mL). TFBUT was not significantly correlated with serum 25(OH)D concentration (n = 82 eyes; Pearson's correlation analysis, $r = 0.043$, $p = 0.663$).
TFBUT, tear film breakup time; 25(OH)D, 25-hydroxyvitamin D.

and group 3 (24.88 ± 6.27 ng/mL; $p = 0.007$); however, no significant difference was found between groups 2 and 3 ($p = 0.37$) (**Fig. 4**). Among the TFBUT groups, there were no significant differences in the mean ± SD serum 25(OH)D concentrations between groups 4 (26.13 ± 8.19 ng/mL), 5 (20.03 ± 5.79 ng/mL), and 6 (27.75 ± 9.98 ng/mL) (**Fig. 5**).

DISCUSSION

The relationships between vitamin D deficiency and DED remain unclear, although vitamin D supplementation is effective in treating DED in humans. Jee et al. [4] and Jeon et al. [5] suggested that the serum 25(OH)D concentration was not significantly related to DED. In

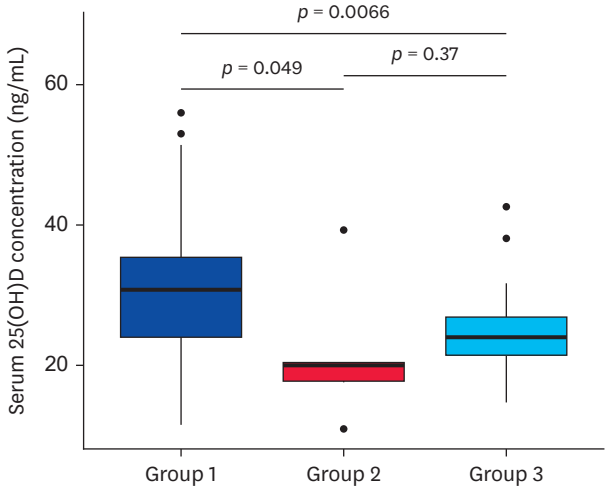

Fig. 4. Boxplot analysis to compare serum 25(OH)D concentrations among group 1 (normal STT-1 in both eyes), group 2 (normal STT-1 in only one eye), and group 3 (abnormal STT-1 in both eyes). The mean serum 25(OH)D concentration (±SD) in group 1 (31.65 ± 10.20 ng/mL) was significantly higher than in group 2 (21.66 ± 10.55 ng/mL, $p = 0.049$) and group 3 (24.88 ± 6.27 ng/mL, $p = 0.0066$). However, no significant difference was found between group 2 and group 3. Results with $p < 0.05$ were considered statistically significant.
25(OH)D, 25-hydroxyvitamin D; STT, Schirmer tear test 1.

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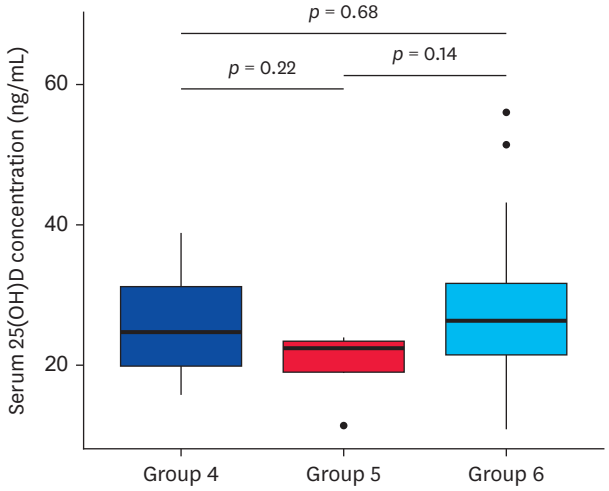

Fig. 5. Boxplot analysis to compare serum 25(OH)D concentrations among group 4 (normal TFBUT in both eyes), group 5 (normal TFBUT in only one eye), and group 6 (abnormal TFBUT in both eyes). There were no significant differences in the mean (±SD) serum 25(OH)D concentrations among group 4 (26.13 ± 8.19 ng/mL), group 5 (20.03 ± 5.79 ng/mL), and group 6 (27.75 ± 9.98 ng/mL). Results with $p < 0.05$ were considered statistically significant. 25(OH)D, 25-hydroxyvitamin D; TFBUT, tear film breakup time.

contrast, it was reported that the serum vitamin D concentration was significantly lower in patients with DED [20]. Yildirim et al. [3] found that the STT-1 and TFBUT in patients with vitamin D deficiency were lower than those in healthy subjejcts and recommended that the patients with vitamin D deficiency should be evaluated for DED.

In the present study, STT-1 and TFBUT were positively correlated; this finding was strengthened when STT-1 was in the abnormal range. However, although STT-1 had a positive correlation with the serum 25(OH)D concentration, TFBUT was not significantly correlated with serum 25(OH)D concentration in this study. Based on these results, it was expected that the serum 25(OH)D concentration would have a greater association with the quantitative KCS than with qualitative KCS in dogs. Additionally, the serum 25(OH)D concentration was significantly higher in group 1 than in groups 2 and 3, which suggests that even if the quantitative KCS was present in only one eye, the serum 25(OH)D concentration was likely to be low, similar to dogs with quantitative KCS in both eyes. Therefore, a decreased serum 25(OH)D concentration should be included in the differential diagnoses of systemic causes of KCS in dogs, along with diabetes mellitus, hyperadrenocorticism, and hypothyroidism [13].

Caution should be taken when interpreting the results of the serum 25(OH)D concentration as the normal range in dogs has not yet been fully established. Dawson-Hughes et al. [21] suggested that in humans, vitamin D concentrations < 20 ng/mL, 21–29 ng/mL, and > 30 ng/mL indicate deficiency, relative deficiency; sufficiency. Meanwhile, studies that defined the sufficient serum 25(OH)D concentration in healthy dogs remain controversial [6-11]. Selting et al. [6] reported that the serum 25(OH)D concentration was measured very broadly (9.5–249 ng/mL) in healthy dogs, and that the lowest concentration of 25(OH)D that inhibited the synthesis of parathyroid hormone (between 100 and 120 ng/mL) was suggested as an adequate serum 25(OH)D concentration; in this study, no group reached a serum 25(OH) D concentration of > 100 ng/mL. However, these results were supported by the findings published by Sharp et al. [22], who suggested that the serum 25(OH)D concentration in dogs could be classified as sufficient (> 100 ng/mL), insufficient (25–100 ng/mL), and deficient (< 25 ng/mL), and that most dogs (86%) did not have sufficient serum 25(OH)D concentrations.

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In addition, Agarwal et al. [23] reported that lipemia in humans was negatively correlated with serum vitamin D concentrations in the range of 15%–20%. Notably, intact male dogs had higher serum 25(OH)D concentrations (83.3 ng/mL; range, 23.9–249.2) than neutered male dogs (60.4 ng/mL; range, 16.9–135.0) and intact females (67.7 ng/mL; range, 32.3–143.0) [23].

Since canine KCS has the highest prevalence of immune-mediated KCS, drugs, such as cyclosporine, tacrolimus, and pimecrolimus, are frequently prescribed to treat KCS [12]. Lee et al. [24] reported that the intraperitoneal injections of cyclosporine and tacrolimus administered for 1 week increased the serum $1,25(OH)_2D$ concentration in mice. In humans, serum vitamin D concentrations in patients with vernal keratoconjunctivitis increased after 1% topical cyclosporine treatment [25]. In contrast, Reichel et al. [26] reported that cyclosporine treatment for 2 years did not cause significant alterations in vitamin D concentration. Therefore, dogs which had previously been administered eye drops, such as cyclosporine or tacrolimus, were excluded from this study.

Vitamin D supplementation is known to enhance tear secretion and tear film stability and reduce ocular surface inflammation, tear hyperosmolarity, and clinical symptoms of DED in humans [27-29]. Therefore, further research would be needed to investigate the effects of vitamin D supplementation on canine quantitative and qualitative KCS through a comparative analysis of STT-1, TFBUT, clinical symptoms, and the results of other dry eye tests (such as fluorescein staining score of the cornea, tear meniscus height, lipid layer thickness of tear film, and meibomian gland dropout) before and after vitamin D supplementation in dogs.

This study had the following limitations: first, we did not include additional differential diagnosis of other systemic occult diseases associated with changes in the serum 25(OH)D concentration as differential diagnoses. Therefore, it was not possible to determine whether the serum 25(OH)D concentration was affected by other systemic diseases unrelated to KCS. Further investigations of underlying diseases are warranted in dogs with low serum 25(OH)D concentration. second, only STT-1 and TFBUT were used as tests for diagnosing quantitative and qualitative KCS, respectively. Other parameters, such as clinical symptoms and other ophthalmic tests, were not included. However, since KCS is a multifactorial disease of the ocular surface caused by various causes, it is recommended that the combination of various DETs and clinical signs should be considered for the analysis of KCS [30,31]. Therefore, additional studies would be needed to analyze the correlation between serum 25(OH)D concentration and other DETs.

In conclusion, this study demonstrated that the serum 25(OH)D concentrations were positively correlated with STT-1 for quantitative KCS, but not with TFBUT for qualitative KCS in dogs. Therefore, measuring of serum 25(OH)D concentrations could be considered in canine quantitative KCS since serum 25(OH)D concentrations were lower in dogs with quantitative KCS. Further studies are warranted on the potential of therapeutic vitamin D supplementation and whether the serum 25(OH)D concentrations can affect the outcomes of treatment in dogs with quantitative KCS.

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