• Basic Research •

Effect of natural extract eye drops in dry eye disease rats

Soo Youn Choi[1,2], *Youngsub Eom*[1,3], *Jee Yong Kim*[4], *Dong Hoon Jang*[4], *Jong Suk Song*[1], *Hyo Myung Kim*[1]

[1]Department of Ophthalmology, Korea University College of Medicine, Seoul 02841, Republic of Korea
[2]BGN World Tower Eye Clinic, Seoul 05551, Republic of Korea
[3]Department of Ophthalmology, Korea University Ansan Hospital, Korea University College of Medicine, Gyeonggi-do 15355, Republic of Korea
[4]Medical O Co., Ltd., Seoul 06025, Republic of Korea
Correspondence to: Youngsub Eom. Department of Ophthalmology, Korea University Ansan Hospital, Korea University College of Medicine, 123, Jeokgeum-ro, Danwon-gu, Ansan-si, Gyeonggi-do 15355, Republic of Korea. hippotate@hanmail.net
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Abstract

● **AIM:** To investigate the therapeutic effect of natural extract eye drops containing bee venom, musk, and deer antlers in dry eye disease (DED) animal models.

● **METHODS:** Scopolamine-injected DED rats and lacrimal gland-excised rats were allocated into control, saline, and natural extract groups respectively and a normal group (lacrimal gland excision was not performed) in lacrimal gland-excised rats. After eye drop instillation 4 times a day for 5d, corneal fluorescein staining (CFS) scores, tear MUC5AC levels, and tear lactic dehydrogenase (LDH) levels were measured.

● **RESULTS:** In scopolamine-injected rats, the natural extract-treated group had significantly lower CFS scores (1.7±0.5, 4.7±1.4, 3.8±1.9, *P*=0.006) and tear LDH levels (0.10±0.01, 0.19±0.01, 0.16±0.08 OD, *P*=0.014) but higher tear MUC5AC levels (12.9±3.7, 7.9±2.0, 9.7±3.6 ng/mL, *P*=0.041) compared with the control and saline-treated groups. There were no significant differences between the control and saline-treated groups. In lacrimal gland-excised rats, the natural extract-treated group also had lower CFS scores (4.3±1.2, 11.5±2.3, 9.0±1.9, *P*<0.001, *P*=0.001) and tear LDH levels (0.30±0.08, 0.48±0.12, 0.39±0.05 OD, *P*<0.05) but higher tear volume (4.3±0.9, 1.9±0.7, 2.8±1.1 mm, *P*=0.005, *P*=0.124) and tear MUC5AC levels (8.2±2.0, 2.9±1.2, 5.4±2.2 ng/mL, *P*<0.001, *P*=0.047) compared with the control and saline-treated groups. There were no significant differences in the CFS scores, tear MUC5AC level, and tear LDH level between the normal and natural extract-treated groups.

● **CONCLUSION:** The natural extract consisting of bee venom, musk, and deer antlers may have effectiveness in DED treatment by restoring the damaged ocular surface, increasing tear volume, and recovering the tear mucin layer in DED rats.

● **KEYWORDS:** bee venom; musk; deer antler; natural extract; dry eye disease; rat

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INTRODUCTION

Dry eye disease (DED) is a multifactorial disease that causes a variety of symptoms such as ocular surface damage and foreign body sensation, burning sensation, and blurred vision, due to tear film instability, along with elevated tear osmolarity and ocular surface inflammation[1-2]. Multiple treatments, including artificial tear drops, steroid drops, therapeutic contact lenses, and punctual plug insertion, have been introduced for DED in the clinical field[3-4]. However, these treatments have a number of limitations. Artificial tears only show a temporary effect, while steroid drops may cause cataracts or glaucoma when used long-term. In addition, therapeutic contact lenses may lead to infection, and punctual plug insertion may result in lacrimation. Accordingly, a large number of studies have been conducted to find a new therapeutic agent for DED capable of suppressing ocular surface inflammation, which is a key mechanism causing DED.

Multiple efforts have been made to develop new medicines based on natural extracts with a long history of use in the East[5]. One study revealed that eye drops made from retinispora extracts promoted the expression level of antioxidant proteins[6]. In DED patients treated with antioxidant and anti-inflammatory multi-component functional foods, the expression level of inflammation-related proteins was decreased, indicating that the foods were effective in DED treatment[7]. In addition, Choi *et al*[8] reported that wearing

glasses containing medicinal plant extracts with antioxidant effects ameliorated DED. Previous animal studies have shown that application of an hyaluronic acid complex containing essential omega-3 fatty acid or mineral oil into eyes reduced corneal surface irregularities and epithelial barrier damage, as well as decreased oxidative stress and inflammatory cytokine expression in the ocular surface[9-10]. Another report showed that administration of ethyl alcohol complex extracted from medicinal plants to dry eyes improved clinical features of DED by decreasing oxidative stress and inflammatory makers in the cornea[11]. Accumulating evidence has shown that extracts composed of bee venom, musk, and deer antlers exhibited anti-inflammation and antioxidation effects[12-15].

We hypothesized that application of natural extracts containing bee venom, musk, and deer antlers, which exhibit anti-inflammatory and antioxidant effects, might alleviate inflammation in DED, which involves inflammatory responses at the ocular surface. In this study, we examined the therapeutic efficacy of the extracts by evaluating the restoration of the damaged ocular surface and the normalization of the tear mucin layer on the ocular surface in DED model rats.

MATERIALS AND METHODS

Ethical Approval All procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Approval for this study was obtained from the Korea University Medicine-Institutional Animal Care and Use Committee, Seoul, South Korea (KOREA-2017-0005).

Natural Extract Samples Freeze-dried natural extract powders from 100 mg bee venom (Goldleben Inc., Cheongju, Korea), 1 g musk (Uri-Herb Medicine Inc., Seoul, Korea), and 15 g deer antlers (Hans Medicine Inc., Chungcheongnam-do, Korea) were mixed and put into a distilling extractor (DM-100) with 1350 mL deionized distilled water for extraction at 107°C for 3h. Distilled extracts of 900 mL were obtained after filtering the mixture 3 times. The extract was placed in a microtube, and 2 μL of extract eye drops was instilled in the eye of natural extract-treated group four times a day using a pipette.

Animals A total of 33 male Sprague-Dawley rats aged 5-6wk weighing 200-250 g were used in this study. Among them, 9 rats were established as the scopolamine-injected DED model and the remaining 24 animals as the lacrimal gland-excised DED model. To establish scopolamine-injected DED animal models, 9 rats were subcutaneously administered with 0.1 mL (15 mg/mL) scopolamine (S0929-5G; Sigma-Aldrich GmbH, Steinheim, Germany) 3 times a day (10 a.m., 1 p.m. and 4 p.m.) for 5d. The animals were maintained in a constant temperature and humidity chamber (Orient Bio Inc., Sungnam, Korea) controlled at 23±1°C and relative humidity 30%±5% from 2d before to 2d after the subcutaneous scopolamine injection. The eye drop treatment was conducted 3d after the injection.

To establish the lacrimal gland-excised DED animal model, the lacrimal gland was excised from one eye of 18 out of 24 rats. After general anesthesia, surgical site cleansing was performed using povidone-iodine. An about 2 mm skin incision was made using scissors, and blunt dissection was performed under the subcutaneous fascia. The extra orbital lacrimal gland was removed, and the skin wound was closed with a single 7-0 nylon suture. Antibiotic ophthalmic ointment (Tarivid eye ointment; Santen Pharmaceutical Co, Osaka, Japan) was applied to the surgical site[16]. The eye drop treatment was conducted 6 days following the excision. For the remaining 6 rats, surgical procedures were not performed.

Methods

Evaluation of the effect of the natural extract eye drop in scopolamine-injected rats The 9 scopolamine-injected rats were distributed into 3 groups of 3 rats each: a non-treated control group, saline-treated group, and natural extract-treated group. Eye drops were applied to the both eyes 4 times a day for 5d. Corneal fluorescein staining (CFS) scores, tear MUC5AC, and lactate dehydrogenase (LDH) levels were measured in both eyes in the three groups after eye drop treatment[17]. For CFS, fluorescein sodium-impregnated paper strips (Haag-Streit, Bern, Switzerland) were applied to the upper bulbar surface after retracting the upper eyelid after wetting the end of the strip with 5 μL saline. After eye drop application, the eye was gently closed 5 times, the flowing tears and dyes were wiped, and CFS scores were assessed under a cobalt blue light using a slit lamp microscope according to the National Eye Institute scoring scheme[18].

Tear samples were collected according to the methodology of previous studies[17]. Tear MUC5AC and LDH levels were measured using a MUC5AC ELISA kit (MyBiosource, San Diego, CA, USA) and an LDH ELISA kit (Promega, Madison, WI, USA), respectively[17].

Evaluation of the effect of the natural extract eye drop in lacrimal gland-excised rats The 24 rats were assigned into 4 groups of 6 rats each, including one non-excised normal group and 3 lacrimal gland-excised groups divided into the non-treated control, saline-treated, and natural extract-treated group. Eye drops were applied to the affected eye 4 times a day for 5d. In the non-excised normal group, these treatments were not conducted. Lacrimal gland excision was carried out in one eye and the evaluation on the eye drop was also performed in the excised eye. In all groups, CFS scores were measured using fluorescein staining and tear volume was measured using a Phenol Red Thread test after eye drop treatments. For the Phenol Red Thread test, phenol red-impregnated cotton threads (Zone-Quick; Showa Yakuhin Kako Co. Ltd., Tokyo, Japan) were placed to the lateral canthus of the lower eyelid for 30s with the end of the threads folded by 2 mm and removed;

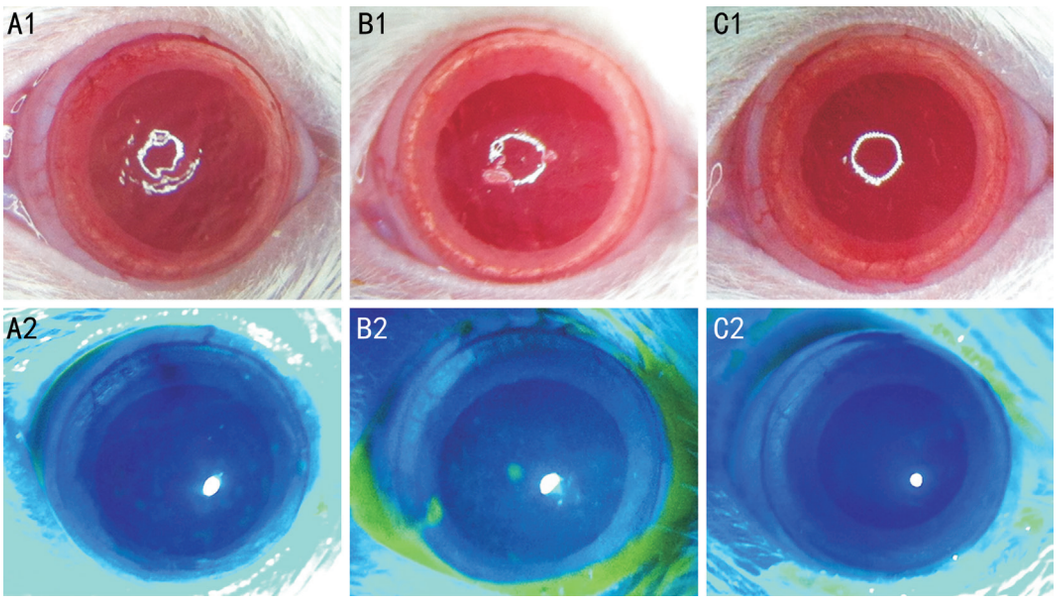

Figure 1 Representative anterior segment photographs of rat eyes with and without fluorescein staining in a scopolamine-induced dry eye rat model The normal saline and natural extract group received eye drop instillation four times a day for 5d; the control group received no treatment. A1, A2: Control group; B1, B2: Normal saline group; C1, C2: Natural extract group.

the length of the part that turned red was measured. Tear MUC5AC and LDH levels were measured using ELISA kits as described above.

Statistical Analysis In a previous study that investigated the effects of medicinal plant extracts in a dry eye mouse model, the mean corneal fluorescein staining score (±SD) was 12.55±1.77 in the dry eye group, 11.73±2.05 in the BSS-treated group, and 8.15±2.21 in the extract-treated group[11]. According to the results of that previous study, 6 measurements were needed in each group to achieve a power of 90% and a 5% significance to identify a difference in means with one-way analysis of variance (ANOVA). Thus, this study included 6 eyes of 3 rats in each group of scopolamine-injected rats and 6 rats in each group of lacrimal gland-excised rats. The normal distribution of each variable was verified by the Kolmogorov-Smirnov test. For statistical verification, one-way ANOVA was applied to normally distributed variables for comparison of 3 groups or more and the Kruskal-Wallis test to non-normally distributed ones. In case of significant difference following one-way ANOVA, the post-hoc Tukey test was performed to compare two groups. In addition, in case of significant difference following the Kruskal-Wallis test, two groups were compared based on the significant level adjusted by the Bonferroni correction[19]. For statistical analysis, Social Sciences Statistics Standard 20 (IBM Corp., Armonk, NY, USA) was used, and *P* values of less than 0.05 were regarded as statistically significant.

RESULTS

Effect of the Natural Extract Eye Drop in Scopolamine-Injected Rats

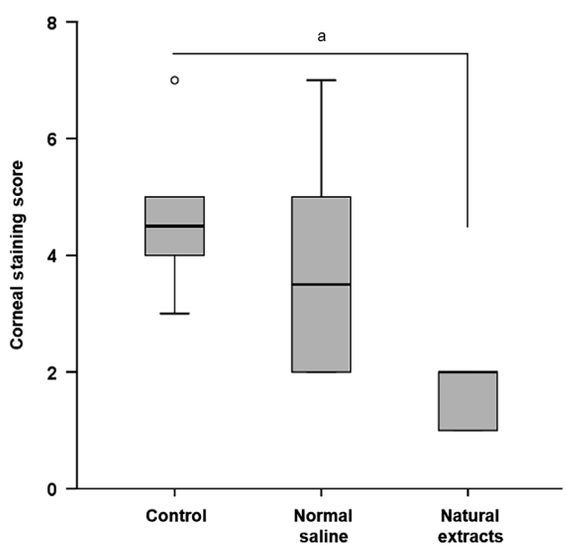

Figure 2 Comparison of corneal staining score among the control, normal saline, and natural extract groups [a]*P*<0.05 compared with the natural extract group.

Corneal fluorescein staining scores The mean CFS scores were 4.7±1.4, 3.8±1.9, and 1.7±0.5 in the non-treated control, saline-treated, and natural extract-treated group, respectively (*P*=0.006). The CFS score of the natural extract-treated group was significantly lower than that of the control group (*P*=0.006), but not significantly different from that of the saline-treated group (*P*=0.080). In contrast, there was no significant difference in the CFS score between the saline-treated group and the control group (Kruskal-Wallis test, Figures 1 and 2).

Tear MUC5AC level The mean tear MUC5AC levels were 7.9±2.0, 9.7±3.6, and 12.9±3.7 ng/mL in the control,

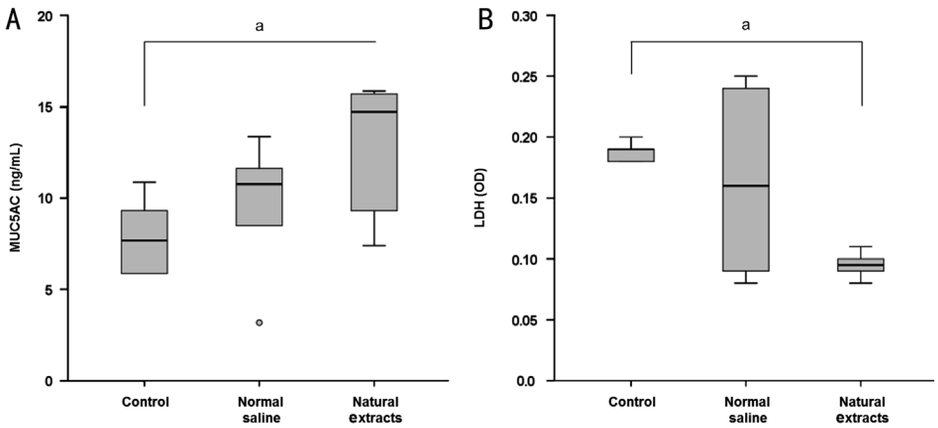

Figure 3 Comparison of MUC5AC levels (A) and LDH activity (B) in tear samples among the control, normal saline, and natural extract groups [a]P<0.05 compared with the natural extract group.

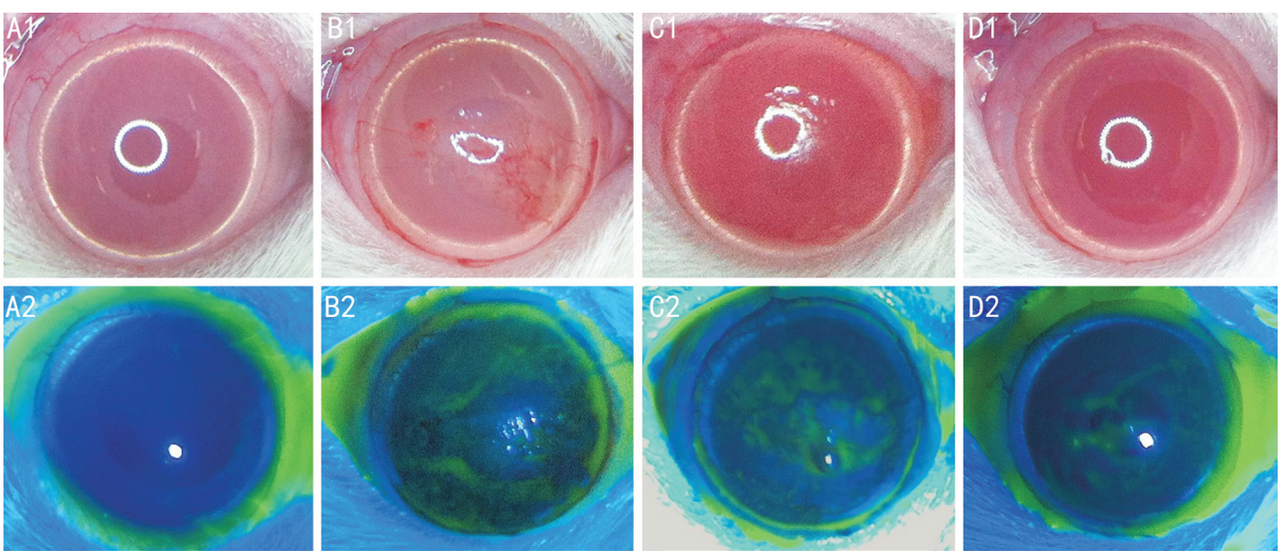

Figure 4 Representative anterior segment photographs of rat eyes with and without fluorescein staining in a dry eye rat model developed by removing the lacrimal gland The normal saline and natural extract group received eye drop instillation four times a day for 5d, while the control group did not receive treatment. The normal group in which the lacrimal gland was not removed also did not receive eye drops. A1, A2: Normal group; B1, B2: Control group; C1, C2: Normal saline group; D1, D2: Natural extract group.

saline-treated, and natural extract-treated group, respectively (P=0.041). The tear MUC5AC level of the natural extract-treated group was significantly higher compared with that of the control group (P=0.035). The tear MUC5AC level of the saline-treated group was not significantly different from that of the control group (P=0.588; one-way ANOVA; Figure 3A).

Tear LDH level The tear LDH levels were 0.10±0.01, 0.16±0.08, and 0.19±0.01 optical density (OD) in the natural extract-treated, saline-treated, and control group, respectively (P=0.014). We only observed a significant difference in the tear LDH level between the natural extract-treated group and the control group (P=0.013; one-way ANOVA; Figure 3B).

Effect of the Natural Extract Eye Drop in the Lacrimal Gland-Excised Rats

Corneal fluorescein staining score The mean CFS scores were 1.5±1.8, 11.5±2.3, 4.3±1.2, and 9.0±1.9 in the normal, control, natural extract-treated, and saline-treated group, respectively (P<0.001). The CFS score of the natural extract-treated group was significantly reduced compared with that of the control and saline-treated groups (P<0.001, P=0.001, respectively), but not significantly different from that of the normal group (P=0.062). The CFS score of the saline-treated group was significantly increased compared to that of the normal group (P<0.001), but not significantly different from that of the control group (P=0.114; one-way ANOVA; Figures 4 and 5A).

Tear volume The mean tear volume was 8.4±1.4 mm, 1.9±0.7 mm, 4.3±0.9 mm, and 2.8±1.1 mm in the normal, control, natural extract-treated, and saline-treated groups, respectively (P<0.001). The tear volume of the natural extract-treated group was significantly increased compared to that of the control group (P=0.005), but significantly lower than that of the normal group (P<0.001). There was no significant difference in tear volume between the saline-treated group and the control group (P=0.451; one-way ANOVA; Figure 5B).

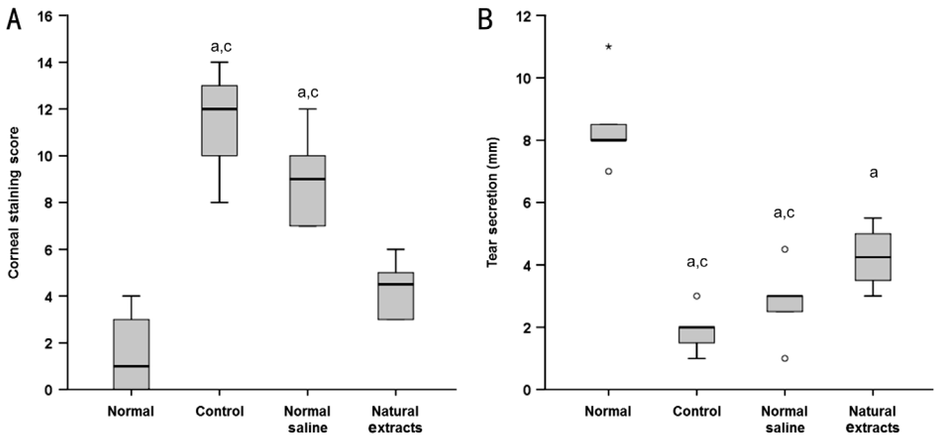

Figure 5 Comparison of corneal staining score (A) and tear secretion (B) among the normal, control, normal saline, and natural extract groups [a]*P*<0.05 compared with the normal group; [c]*P*<0.05 compared with the natural extract group.

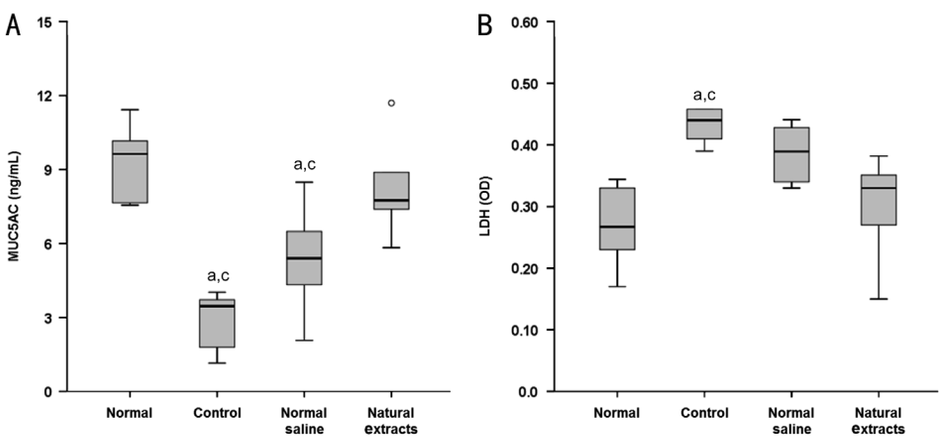

Figure 6 Comparison of MUC5AC levels (A) and LDH activity (B) in tear samples among the normal, control, normal saline, and natural extract groups [a]*P*<0.05 compared with the normal group; [c]*P*<0.05 compared with the natural extract group.

Tear MUC5AC level The mean tear MUC5AC level of the natural extract-treated group (8.2±2.0 ng/mL) was not significantly different from that of the normal group (9.3±1.5 ng/mL, *P*=0.685), but significantly higher than that of the control (2.9±1.2 ng/mL, *P*<0.001) and saline-treated groups (5.4±2.2 ng/mL, *P*=0.047). There was no difference in tear MUC5AC levels between the saline-treated group and the control group (*P*=0.109; one-way ANOVA; Figure 6A).

Tear LDH level The mean tear LDH levels were 0.27±0.07 and 0.30±0.08 OD in the normal and natural extract-treated group, respectively, which were significantly lower than that of the control group (0.48±0.12 OD, *P*=0.002, *P*=0.033, respectively). The mean tear LDH levels of the saline-treated group (0.39±0.05 OD) was not significantly different from that of the control group (*P*>0.999; Kruskal-Wallis test; Figure 6B).

DISCUSSION

DED is one of the most common ophthalmic disorders and results in epithelial cell damage on the ocular surface, goblet cell loss, and abnormal mucin secretion owing to increased tear osmolarity and activated ocular surface inflammation, exhibiting diversity clinical features by tear film instability[20]. Current treatments for DED include a conservative treatment that preserves some tears using artificial tear drops or punctual plugs, as well as an active treatment that reduces ocular surface inflammation using cyclosporine, steroid, and autologous serum eye drops and augments ocular surface stability by increasing mucin secretion in conjunctival goblet cells using a 3% diquafosol sodium eye drop application[4,21].

Natural extracts containing bee venom, musk, and deer antlers have been reported to have anti-inflammatory effects and have been used in traditional Korean medicine. Therefore, in this study, we examined whether natural extracts from bee venom, musk, and deer antlers, used as traditional therapeutic agents in the East, are effective in DED treatment by evaluating their therapeutic efficacy in DED rats. The results showed that following instillation of distilled eye drops including a mixture of the three natural extracts, CFS scores and tear LDH levels were significantly decreased and the tear MUC5AC levels were significantly increased in both scopolamine-injected and lacrimal gland-excised rats compared with non-treated controls. These findings confirm that the extracts cured

the ocular surface injured by DED and improved the ocular surface conditions.

Systemic scopolamine administration with desiccating stress has been commonly used for establishing an aqueous-deficient dry eye animal model, probably from lacrimal gland inflammation and/or ocular surface neuropathy[22]. Stevenson *et al*[16] introduced the extraorbital lacrimal gland excision for establishing an animal model of severe aqueous tear-deficient dry eye in which tear secretion is reduced due to lack of lacrimal glands. A lacrimal gland excision model showed more severe decrease of tear secretion and severe corneal epitheliopathy compared with a scopolamine-induced dry eye model[16]. Similar to previous results, lacrimal gland-excised rats showed more severe corneal fluorescein staining score and higher LDH level but lower MUC5AC level compared with scopolamine-injected rats in this study. We tried to determine whether natural extracts had a therapeutic effect in both dry eye animal models because each might have its own meaning in the pathogenesis of DED[16].

The current results suggest that the therapeutic efficacy of the natural extracts may be attributed to their anti-inflammatory and antioxidant effects[12-15]. Bee venom is a secretion produced in the poison sac of bees that contains melittin, apamin, adolpin, and mast cell-degranulating peptide. Melittin and apamin, the main components of bee venom, exhibit anti-inflammatory effects by suppressing lipopolysaccharide (LPS)-induced nitric oxide, the nuclear factor kappa B (NF-κB) signal pathway, and transcription of proinflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α[23-24]. Phospholipase A2, the main enzyme component of bee venom, plays a crucial role in axonal outgrowth in the periphery, synaptic transmission, and nervous system survival, thus contributing to protection of tissues in arthritis, asthma, and Parkinson's disease[14].

Musk is obtained by drying secretions produced in the musk gland of male musk deer and contains a variety of ingredients including glucocorticoids, lipids, and peptides[25]. A previous study demonstrated that musk shows an anti-inflammatory effect on arthritis and decreases the histamine and serotine in affected paw of animals[15]. Another study reported that musk strengthens the heart by anti-inflammation and β-adrenergic stimulation and shows androgenic hormone, antihistamine, and convulsion control effects[26-27].

Deer antlers are obtained by drying non-keratinized immature deer horns found in members of the deer family and contain water-soluble proteins, polypeptides, and free amino acids. Deer antlers have been used to treat arthritis and showed multiple efficacies such as immunity enhancement, pain relief, and anti-inflammation[12,28]. Deer antler also had a protective effect on cisplatin-induced renal toxicity through its antioxidant and anti-inflammatory effects[29].

MUC5AC is a major mucin secreted from conjunctival goblet cells and has a role in evenly spreading out the tear film on the ocular surface. MUC5AC is decreased in tears of patients with Sjögren's syndrome or severe DED[30-31]. In our study, tear MUC5AC levels were increased in DED animals after instillation of the natural extract eye drop compared with the control group, indicating that the natural extracts have a restorative effect on the tear mucin layer in DED.

LDH is a cytosolic enzyme that converts lactate into pyruvate and is found in most living cells. Detection of LDH in the extracellular space indicates cell membrane damage and is considered as a biomarker of cytotoxicity. Thus, we evaluated the extent of ocular surface damage by investigating tear LDH levels[32-33]. In the present study, the tear LDH levels were reduced following eye drop application of the natural extracts to DED rats compared with the control group, indirectly suggesting that the natural extracts may work to restore the damaged ocular surface.

This study has a number of limitations. First, this study used a small number of animals to draw conclusions. However, the sample size was calculated from the results of a previous study. Second, although we verified the therapeutic efficacy for DED using the natural extract eye drop, we did not identify the active ingredients responsible for the therapeutic effect. Accordingly, further studies should be conducted to identify the active ingredients effective in DED treatment from the natural extract eye drop with the aim of developing a therapeutic agent using the active ingredients. Third, safety for usage of the natural extract eye drop and toxicity of ocular cells were not assessed in this study. Thus, whether the eye drop explored in our study can be immediately applied to the clinic has not been determined. Moreover, ophthalmic safety and appropriate concentration of this eye drop concerning ocular cell toxicity and therapeutic effect should be assessed before the eye drop can be introduced as a medicine. Finally, although our study demonstrated the efficacy of the natural extract eye drop in DED animals, further studies are needed to elucidate whether this eye drop application is also clinically effective in DED patients.

In conclusion, this study revealed that eye drop application of natural extracts of mixed bee venom, musk, and deer antler could increase the amount of tears and restore the tear mucin layer and the damaged ocular surface. Also, these extracts might be developed as a compelling therapeutic agent with anti-inflammatory and antioxidant effects on DED with identification of active ingredients of the natural extracts and assessment of appropriate concentrations required for therapeutic effects and ocular safety.

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Conflicts of Interest: Choi SY, None; **Eom Y,** None; **Kim JY,** None; **Jang DH,** None; **Song JS,** None; **Kim HM,** None.

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