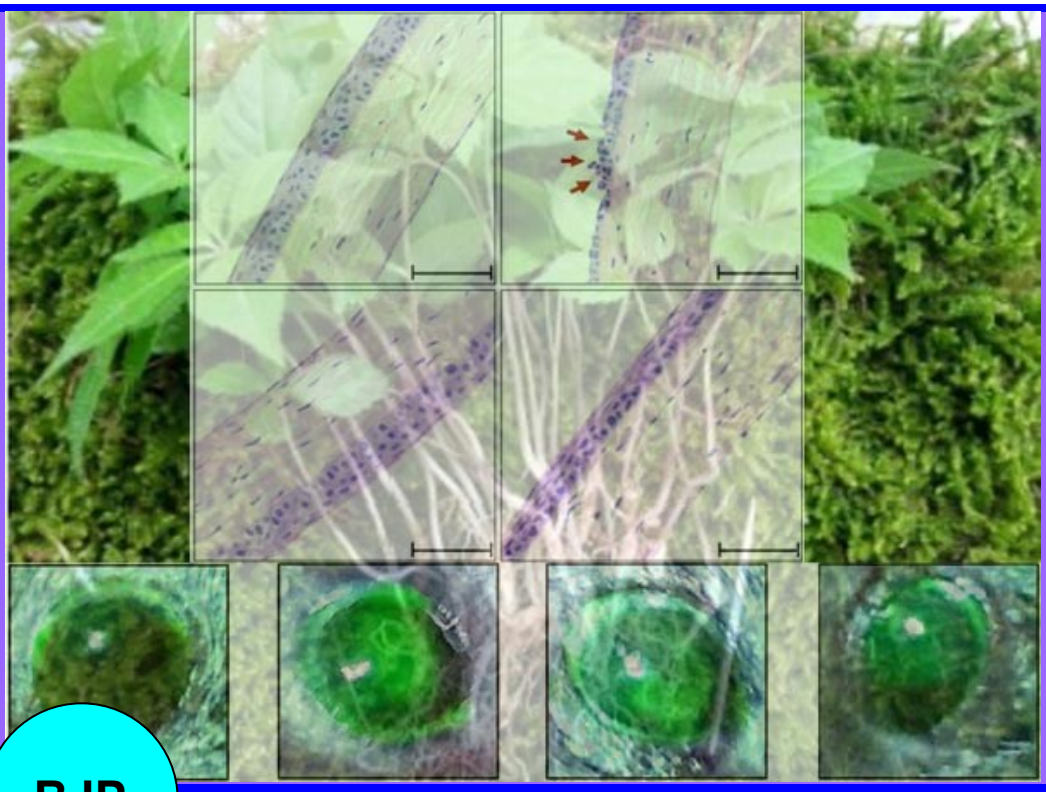

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Amelioration of dry eye syndrome by oral administration of cultivated wild ginseng extract

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Abstract

Cultivated wild ginseng extract has been reported to have chemopreventive, chemotherapeutic, hepatoprotective, cardioprotective and anti-inflammatory effects. We investigated the preventive effect of ginseng extract (oral administration, 0.5 or 1.0 mg/kg twice daily for 2 weeks) on dry eye syndrome using benzalkonium chloride-induced dry eye mouse model. Corneal epithelial cells and tear volume were measured using fluorescein corneal staining, followed by histologic assessment, TUNEL assay and Western blot analysis for TNF-α expression. Tear volume was restored to control level with lower fluorescein corneal staining score. Structural damage by benzalkonium chloride of corneal epithelial cells was significantly inhibited in the histologic assessment. Both TUNEL assay and TNF-α expression level showed that apoptotic-positive cells were significantly decreased. These findings indicate that ginseng extract could be a promising supplement in dry eye syndrome treatment.

Introduction

Dry eye syndrome (keratoconjunctivitis sicca) is a multifactorial disease of ocular surface accompanying visual disturbance, hyperemia, photophobia and ocular discomfort (Lemp et al., 2007). Its pathogenesis is triggered by dysfunction of the lacrimal functional unit leading towards an alteration in tear quantity and composition, which results in damage of the conjunctival and corneal epithelium with accompanying inflammatory events (Messmer et al., 2010; Javadi and Feizi, 2011; Tsubota et al., 2017). Accordingly, drugs contain-ing anti-inflammatory agents, especially steroid eye drop, has been the most common and effective treatment of dry eye syndrome. However, non-steroidal anti-inflammatory drugs (NSAIDs) are increasingly being demanded as alternative to steroidal drug, in order to avoid its potential adverse effects, such as glaucoma (Colligris et al., 2014; Phulke et al., 2017).

Ginsenosides, which are the active components of the herb ginseng, have been regarded as main active ingredients for the pharmaceutical activities of ginseng roots (Yun, 2003). Previous reports showed that ginsenoside possess remarkable pharmaceutical activities in cancer (Kang et al., 2009), anti-inflammatory (Wang et al., 2012), anti-allergic (Bae et al., 2002) and anti-aging effect (Kang et al., 2009). Ginsenoside compound K (20-O-beta-D-glucopyranosyl-20(S)-protopanaxadiol) is one of the most biologically and pharmaceutically active protopanaxadiol (PPD)-type ginsenoside (Shin et al., 2017). It is noteworthy that compound K has anti-inflammatory effect through suppression of inflammation related-genes (Wang et al., 2016), indicating that compound K could be promising drug candidate for dry eye syndrome.

In the present study, the protective effect of cultivated wild ginseng extract using benzalkonium chloride-

induced dry eye mouse was examined.

Materials and Methods

Preparation and analysis of ginseng extract

Cultivated wild ginseng was fully dried at 121°C for 15 min, followed by grinding a total of 60 g. The ground ginseng was extracted 5 times with 300 mL of >85% ethanol. After centrifugation at 10,000 rpm for 10 min, the supernatant was harvested and evaporated at 50°C using rotary evaporator. The extracted saponin (10 g) was re-suspended in water (1,000 mL) to form a bulk active protopanaxadiol. The suspension material was prepared in an active form (compound K) component in order to decompose the sugar side chains of the saponin by a pectinase enzyme solution (170 mL) using a peristaltic pump (50 mL/min) and exposed overnight at 4°C. Compound K was collected by centrifugation at 10,000 rpm for 10 min, supernatant were re-suspended in alcohol (200 mL) and filtrated. The filtrated material was evaporated and used in this study. Cultivated wild ginseng extracts were obtained and analyzed as previously described (Ok et al., 2016).

Animal experiment

Male C57BL/6 ICR mice (7 weeks old) were obtained from the Hyochang Science, Korea. In order to minimize environmental changes, the mice were housed for one week. The mice were divided into control group, positive group, and two treatment groups (four mice in each group). The control group was treated with 100 µL saline orally at 10:00 AM for 2 weeks, followed by saline intraocularly (5 µL) in the eyes at 2:00 PM for 2 weeks (control group). The positive control group was administered orally 200 µL solvent once per day at 10:00 AM, then 5 µL benzalkonium chloride eye drops at 2:00 PM for 2 weeks. The two treatment groups received ginseng extract orally (either 100 µL of 0.5 mg/kg or 1 mg/kg, respectively) at 10:00 AM, followed by receiving 5 µL benzalkonium chloride eye drops at 2:00 PM for 2 weeks.

Ocular surfaces evaluation with fluorescein staining

The corneal epithelial surface was assessed with fluorescein staining (Zhang et al., 2014). One microliter of 0.1% sodium fluorescein was dropped into the right eye. After 1 min, the corneal epithelial surface was observed with a cobalt blue filter under a slit-lamp microscope (DM6C ophthalmoscope, Zumax Medical Co. Ltd., Germany). Fluorescein corneal staining scores (ranging between 1 and 4) from all groups were determined by four independent observers and then averaged. The cornea was divided into four quadrants, which were scored respectively. Staining scores were determined as follows: 1; staining absent, 2; sparse punctuate staining less than 10 spots, 3; approximately one-third confluency of punctuate staining, 4; nearly half confluency of punctuate staining. The scores from four quadrants were summed to determine grade (total, 16 points). The analysis was performed in a double-blind manner.

Histopathology

The hematoxylin and eosin staining were used to measure benzalkonium chloride-induced damage of corneal epithelial cells. The eyeball was fixed in 10% formalin solution. Then the cornea was isolated, following by dehydration and paraffin embedding. The cornea (4 µm) was cut from the paraffin blocks and were stained with 0.1% hematoxylin and 1% eosin. Each section was observed under a light microscope (DP-70, Olympus, Japan).

TUNEL assay

TUNEL assays were carried out to observe apoptotic

Box 1: Phenol Red Thread Test

Principle

When a cotton thread impregnated with phenol red touches the tear (slightly alkaline), the thread turns from yellow to red. Then the length of red color in mm is recorded.

Uses

This test is used to detect the dry eye syndrome

Requirements

Ketamine; Phenol red thread (Tiankin Jingming New Technological Development Co., Ltd., China); Pilocarpine; Xylazine

Procedure

Step 1: Each mouse was anesthetized by ketamine (60 mg/kg body weight) and xylazine (6 mg/kg)

Step 2: Pilocarpine (2.5 mg/kg) was injected intraperitoneally to stimulate the tear secretion

Step 3: The phenol red thread was placed under the lateral one fifth of the inferior palpebral margin

Step 4: The thread absorbed the tear that contact it for 5 min (0–5 min and 5–10 min)

Calculation

The tear volume was gauged by the length of the phenol red thread left in contact with the eye

Notes

1. Less time is required for each eye
2. Thread is difficult to handle initially due to its light and flexible nature

References

Arakaki et al., 2014; Senchyna and Wax, 2008

cells in corneal epithelial layer. The formalin embedded eyeball sections were deparaffinized and rehydrated sections (5 µm). Then obtained sections were placed in 3% hydrogen peroxide for 10 min at room temperature, and treated with 20 µg/mL proteinase K for 10 min at 37°C. After washing three times in phosphate buffered saline, apoptotic cells were stained using commercial kit (*In situ* cell death detection kit, Roche, Germany) according to the supplier's instructions.

Western blot analysis

After TUNEL staining observation, cryosections were used for TNF-α expression using Western blotting analysis. Cells were lysed and extracted in RIPA buffer (50 mM Tris-Cl [pH 7.6], 150 mM NaCl, 1%Triton X-100, 1%NP-40, 1%sodium deoxycholate, 0.1%sodium dodecyl sulfate, 1 mM EDTA). The supernatants were harvested by 14,000 rpm centrifugation at 4°C for 15 min. Total proteins (30 µg) were separated using 15% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE). Nonspecific binding was blocked by TBST (25 mM Tris–HCl, 50 mM NaCl, 0.05% Tween-20) containing 5% dry milk for 1 hour at room temperature, followed by incubation 4°C for 16 hours with 1 : 400 and 1 : 10,000 diluted primary antibodies of anti-TNF-α and anti-β-actin, respectively. After 1 hour of incubation at room temperature with appropriate secondary antibody, the signal was detected by the ChemiDoc-It2 Imaging system (UVP 97-0650-05) using enhanced chemiluminescent solutions (ECL Substrate, Thermo Scientific, USA). The relative densities were determined using ImageJ software (version 1.51j8; public domain program created by Wayne Rasband, National Institutes of Health, USA).

Statistical analysis

The data is expressed as the mean ± standard deviation. Statistical analyses were performed and analyzed by one-way analysis of variance (n=4). p value below 0.01 was considered significant.

Results

The contents of cultivated wild ginseng extract (active saponin) by HPLC had a result of a compound K of 52%, Rd of 34.4%, F2 of 2.69%, and Rg3 of 2% with respect to the total saponin content.

Amount of tear in dry eye mouse model

Figure 1 shows that there was a significant decrease (approximately 25.7%, p<0.01) of tear volume in 0.2% benzalkonium chloride-induced mice (positive control group), which confirms that dry eye condition was successfully induced. No significant difference was observed in the ginseng extract (0.5 mg/kg)-treated group compared to the positive control group. Remarkably, decreased tear volume in ginseng extract (1 mg/kg)-treated group were restored to more than 95% of the control (p<0.001). There was statistically significant differences between the positive control group and the 1 mg/kg-treated group with respect to the protective effects of ginseng extract.

Clinical evaluations of dry eye

After 2 weeks of oral administration of ginseng extract, fluorescein staining was carried out for clinical evaluations on corneal surface of dry eye mouse (Figure

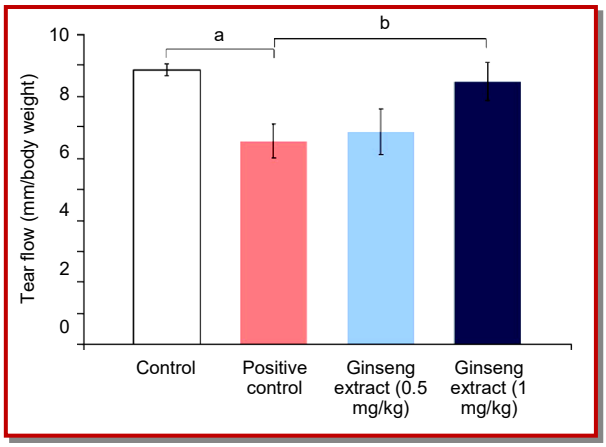

Figure 1: Effects of ginseng extract (0.5 and 1 mg/kg) administration on tear secretion in benzalkonium chloride-induced dry eye syndrome mouse model. Results are expressed as mean ± SD; (n=4, [a]p<0.01, [b]p<0.001)

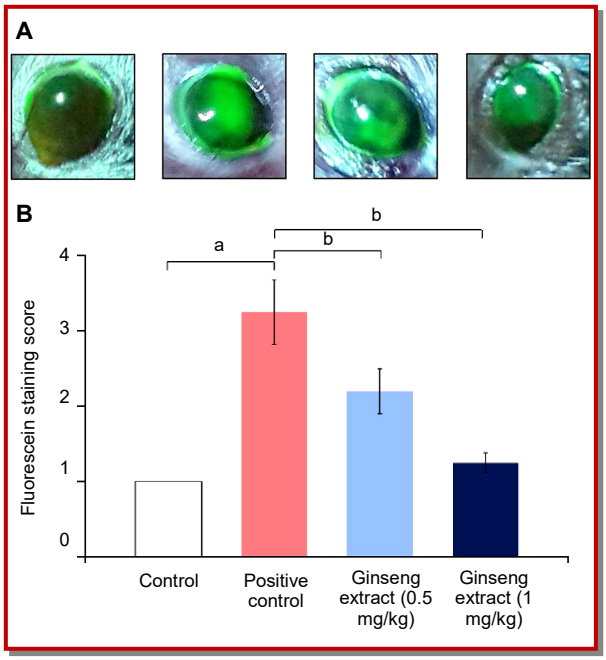

Figure 2: Clinical evaluation of dry eye including representative images of corneal fluorescein staining (A) and staining scores (B) after 14 days and in the normal control. Results are expressed as mean ± SD for 4 mice per group ([a]p<0.01, [b]p<0.001)

2A). There was a remarkable increase of fluorescein staining scores in positive control group (mean score: 3.4, $p<0.01$), compared to the normal group (mean score: 1.9, 1.1, respectively, Figure 2B). It is noteworthy that fluorescein staining scores were dramatically decreased in benzalkonium chloride-treated group, compared to the positive control group ($p<0.001$).

Structural damage of corneal epithelial cells

After 2 weeks of treatment, damaged cells with irregular and vacuolated shapes were observed in the corneas of positive control group (benzalkonium chloride 0.2% treated) as shown in Figure 3B compared to normal group (Figure 3A). It should be noted that benzalkonium chloride-induced damages of the superficial epithelium in the corneas were remarkably recovered to the morphology of the normal group, in both ginseng extract-treated groups (Figure 3C and 3D), which indicates the protective role of ginseng extract in dry eye.

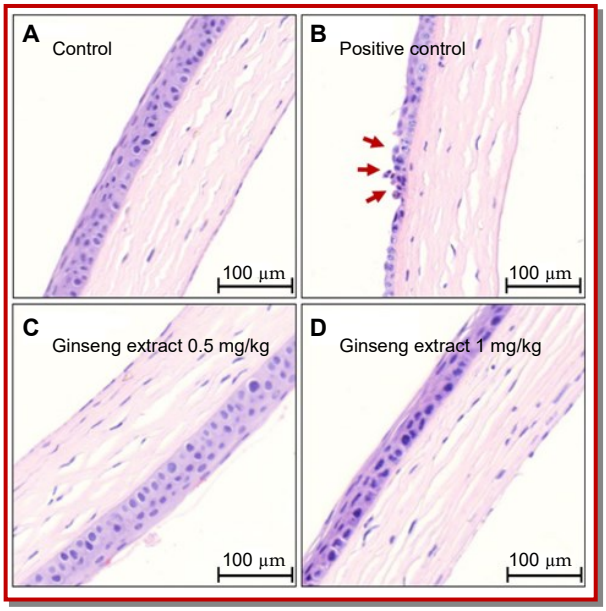

Figure 3: Effect of ginseng extract on corneal epithelial cells of benzalkonium chloride-induced mice. Red arrows indicates irregular and vacuolated corneal epithelial cells

Effect of ginseng extract on apoptosis in corneal epithelial cells

In order to investigate the preventive role of ginseng extract against benzalkonium chloride-induced apoptosis, TUNEL assay was performed (Figure 4A). Figure 4B shows that significant increase (3.6-fold, $p<0.01$) of TUNEL positive cells in positive control group in comparison to control group. There was considerable reduction of TUNEL positive cells in ginseng extract-treated group, compared to positive control group ($p<0.01$). Interestingly, the proportion of apoptotic cells were also decreased with the increase of ginseng extract concentration, which demonstrates anti-apoptotic activity of ginseng extract.

To further examine the anti-apoptotic effect of ginseng extract, expression level of TNF-α was assessed by Western blotting. Compared with control group, TNF-α expression was enhanced in positive control group (Figure 5A). Remarkably, TNF-α induction by benzalkonium chloride treatment was significantly suppressed in ginseng extract 0.5 mg/kg treated group by 52.6% (Figure 5B). Most importantly, it should be noted that expression of TNF-α was completely abolished.

Discussion

In present study, we examined the ginseng extract as nutritional supplement for amelioration of dry eye mouse syndrome model. Interestingly, ginseng extract restored the decreased amount of tear when compared to control group. Furthermore, two weeks treatment of ginseng extract remarkably recovered the damages in

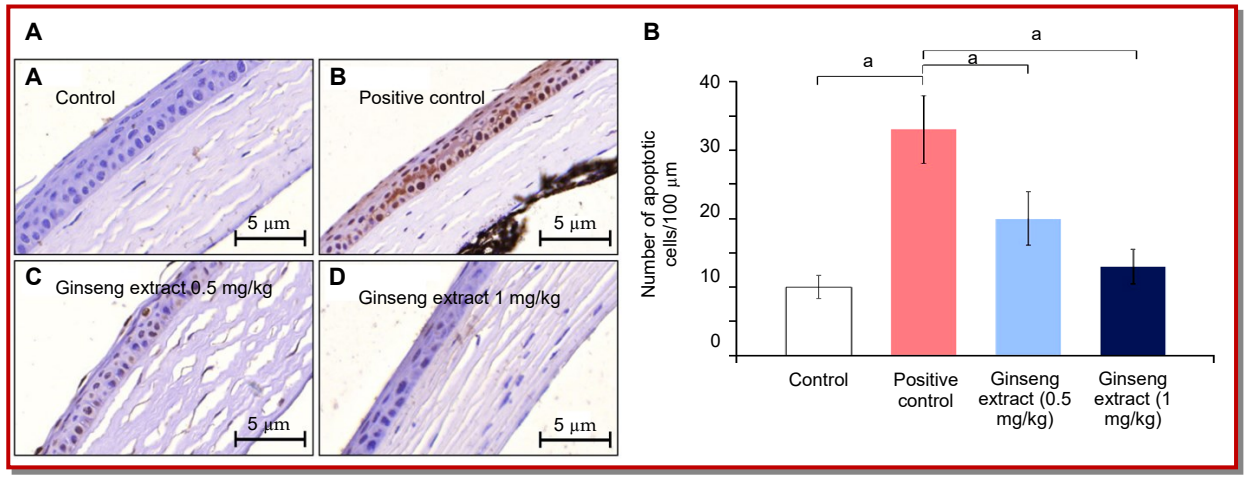

Figure 4: Evaluation of apoptosis via TUNEL assay on corneal sections. (A) Representative TUNEL staining images of corneal epithelial cells. (B) The number of apoptotic (TUNEL-positive) cells per 100 µm tissue. Results are expressed as means ± SD for 4 mice per group. (n = 4, [a]$p<0.01$)

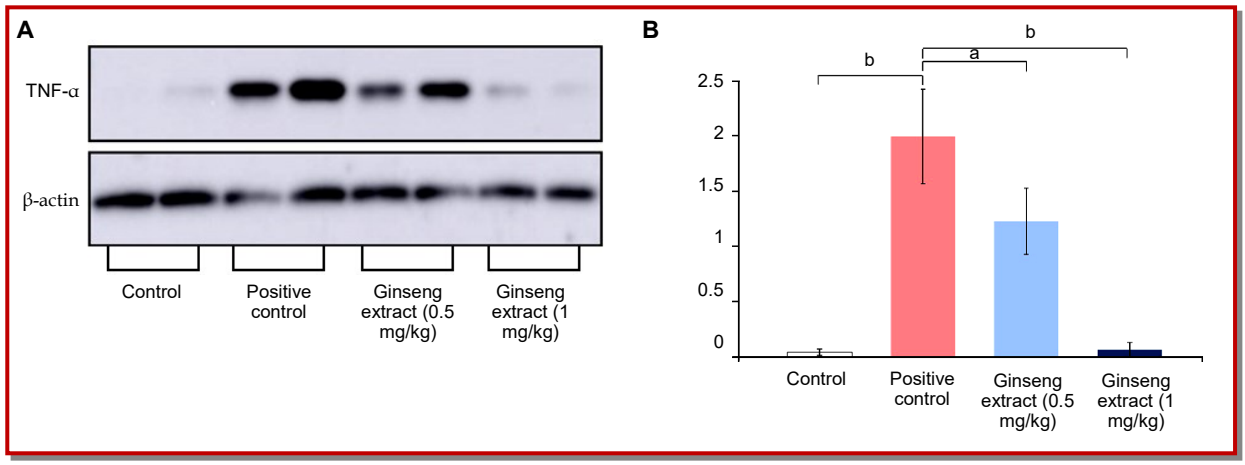

Figure 5: Effect of ginseng extract on TNF-α expression in cornea tissue of benzalkonium chloride-induced dry eye syndrome mouse. (A) Western-blot analysis for TNF-α. (B) The expression of TNF-α was gauaged by normalization based on β-actin intensity as shown in (A). The expression value of the control was set to 1.0. (n = 4, $^{a}p<0.05$, $^{b}p<0.01$)

the superficial epithelium of corneas to the normal morphology. In parallel, ginseng extract reduced the apoptotic cells with accompanying suppression of TNF-α expression, which is crucial in pro-inflammatory reactions. These results demonstrated that oral administration of ginseng extract significantly mitigated the benzalkonium chloride-induced damage of mouse corneal epithelial cells via suppression of inflammation, which indicating that ginseng extract could be a prospective supplement for dry eye syndrome.

The final concentrate is, hereinafter, referred to as gingseng extract because the PPD-based active ingredients such as compound K, Rd, F2, and Rg3 occupy 91% and the content of the compound K is highest as 52%. Ginsenoside metabolite compound K such as active saponin is a kind of dammarane-type triterpene ginseng saponins. There have been many studies demonstrating anti-inflammatory activity of compound K (Cuong et al., 2009; Joh et al., 2011). Wang et al., 2012 reported compound K could reduce mRNA expression of inflammatory-related genes including tumor necrosis factor (TNF), interleukin 1 (IL-1), interleukin 4 (IL-4), interferon g (INFG), and prostaglandin-endoperoxide synthase 2 (PTGS2). Furthermore, Wu et al., 2014 revealed that compound K inhibited the activation of mitogen-activated protein kinases (MAPKs) and Toll-like receptors 4 (TLR4)/lipopolysaccharide-induced nuclear factor kB, with accompanying reduced-expression of pro-inflammatory cytokines secreted by macrophages (Kim et al., 2017). There is another evidence that compound K remarkably inhibited superoxide generation, NADPH oxidase activities, and Ser345-p47phox phosphorylation in macrophages (Cuong et al., 2009). Recent studies using *in vivo* animal models shows that ginsenosides metabolites including compound K exert anti-inflammatory activities in several inflammatory diseases models as well (Kim et al., 2017). It is noteworthy that Korean red ginseng significantly improved the tear film stability and total ocular surface disease index score, as compared to placebo (Kim et al., 2010).

Consistent with this result, ginseng has been suggested as prospective drug candidate for glaucoma or cataract based on the anti-apoptotic/anti-oxidative activity of ginsenosides (Kim et al., 2010; Lee et al., 2010; Huynh et al., 2013)

Conclusion

Ginseng extract significantly inhibited inflammation in dry eye syndrome mouse model by suppression of TNF -α expression. It could be promising supplement for dry eye syndrome treatment.

Financial Support

Kyungsung University Research Grants in 2018

Ethical Issue

The animal experiment in present study was carried out in strict accordance to the World Health Organization guideline, and the Institutional Review Board of Kyungsung University for the evaluation of the safety and efficacy of herbal medicines (confirmation number: Research-17-013A).

Conflict of Interest

Authors declare no conflict of interest

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