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Blueberry Stem Extract Prevents Lacrimal Hyposecretion in Non-obese Diabetic Mice *via* Activation of AMPK

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Abstract. *Background/Aim: Tears secreted from the lacrimal gland are essential for preserving the ocular surface. Thus, dysfunction of the lacrimal gland in Sjögren's syndrome (SS) can lead to dry eye, resulting in a reduced quality of life. We previously reported that blueberry 'leaf' water extract prevents lacrimal hyposecretion in male non-obese diabetic (NOD) mice in a SS-like model. In this study, we investigated the effect of blueberry 'stem' water extract (BStEx) on lacrimal hyposecretion in NOD mice. Materials and Methods: Male NOD mice were fed 1% BStEx or control (AIN-93G) for 2, 4, or 6 weeks from 4 weeks of age. Pilocarpine-induced tear secretion was measured using a phenol red-impregnated thread. The lacrimal glands were histologically evaluated by HE staining. Inflammatory cytokine levels in the lacrimal glands were measured using ELISA. Immunostaining was performed to examine aquaporin 5 (AQP5) localization. The expression levels of autophagy-related proteins, AQP5, and phosphorylated AMPK were measured using western blotting. Results: After feeding BStEx to mice for 4 or 6 weeks, tear volume was observed to have increased in the BStEx group compared with that in the control group. There were no significant differences in inflammatory cell infiltration, autophagy-related protein expression, or the localization and expression of AQP5 in the lacrimal glands between the two groups. In contrast, AMPK phosphorylation increased in the BStEx group. Conclusion: BStEx prevented lacrimal hyposecretion in the SS-like model of male NOD mice, probably by opening tight junctions via the activation of AMPK in lacrimal acinar cells.*

Tears secreted from the lacrimal gland are essential for preserving the ocular surface because of their physiological functions of wetting the cornea and sclera, providing oxygen and nutrition, and protecting against bacteria and foreign substances. Tears are also necessary for clear vision because light must be correctly refracted through tears. Thus, dysfunction of the lacrimal gland can lead to a reduced number of tears and, in turn, dry eye, resulting in a reduced quality of life. There are several causes of lacrimal dysfunction, including Sjögren's syndrome (SS) (1), radiotherapy for head and neck tumors (2), and metabolic syndrome (3). In addition, the increased amount of time spent working with visual display terminals, such as computers and smartphones, has the potential to cause lacrimal dysfunction and dry eye (4, 5).

Primary SS is an autoimmune disease accompanied by the infiltration of inflammatory cells into exocrine glands, such as

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the lacrimal and salivary glands. This infiltration is considered to induce lacrimal and salivary hyposecretion by destroying the tissue of the lacrimal and salivary glands (6). However, to date, there has been no causal treatment for dry eye or dry mouth; thus, their therapy is limited to symptomatic treatment, including artificial tears and saliva, application of topical solutions of immunosuppressive agents to the ocular surface, and systemic use of muscarinic agonists (parasympathomimetic agents) (7). On the other hand, one of our research groups previously revealed that a non-inflammatory factor could cause lacrimal hyposecretion in SS-like model mice (8), indicating that therapies other than anti-inflammatory can be developed.

Non-obese diabetic (NOD) mice were originally developed as type-1 diabetic mice but were later developed as SS model mice owing to spontaneous inflammation in the lacrimal and salivary glands, followed by lacrimal and salivary hyposecretion. The onset of diabetic symptoms and inflammation rate in the glands depend on sex. It has been reported that 80% of females and less than 20% of male NOD mice up to 30 weeks of age exhibit diabetic symptoms (9). Inflammation is reported to be more common in male lacrimal glands and female salivary glands of NOD mice (10, 11). In addition, according to the breeder company of NOD mice (CLEA Japan, Inc., Tokyo, Japan), the initial onset age of diabetes is 18 weeks in both male and female NOD mice. Therefore, male NOD mice up to 18 weeks of age are considered a useful model of SS lacrimal hyposecretion without diabetic symptoms. Inflammatory cell infiltration into the lacrimal glands (dacryoadenitis) and lacrimal hyposecretion have been observed in male NOD mice (11-13). We previously confirmed that these two symptoms occur almost simultaneously starting at 6 weeks of age in male NOD mice (8).

Rabbiteye blueberries (*Vaccinium virgatum* Aiton) are a nutritionally important food cultivated in Miyazaki, Japan. Blueberry leaves and stems contain various polyphenols, which differ from those found in blueberry fruits. Blueberry stem hot water extract (BStEx) and blueberry leaf hot water extract (BLEx) are already used in food products and health foods in Japan. The major polyphenols in BStEx and BLEx are proanthocyanidins, which are presumably composed of a polymer of cyanidin and catechin with one interflavan B-type linkage, two interflavan A-type linkages, and phenylpropane cinchonain I units (14). Additionally, it has been reported that BStEx contains proanthocyanidins with a relatively lower degree of polymerization than BLEx (14). Furthermore, BStEx and BLEx contain other phenolic compounds such as catechins, epicatechins, chlorogenic acid, quinic acid, and caffeic acid (15, 16). Previous studies have shown that BStEx inhibits lipid synthesis in the liver (17), increases anti-adult T-cell leukemia (ATL) activity (18), and promotes viral proliferation (14). We reported the first evidence of the cytoprotective effect of BStEx against blue light-induced retinal cell damage in the field of eye health (16). In addition, we recently revealed that BLEx has a preventive effect on lacrimal hyposecretion (15).

Table I. *Composition of control (AIN93G) and blueberry stem extract (BStEx)-contained diets.*

Ingredients	Control diet (%)	BLEx diet (%)
Casein	20.0	20.0
L-cystine	0.3	0.3
Cornstarch	52.9	51.9
Sucrose	10.0	10.0
Soybean oil	7.0	7.0
Cellulose	5.0	5.0
Vitamin mix (AIN-93G VX)	3.5	3.5
Mineral mix (AIN-93 MX)	1.0	1.0
Choline bitartrate	0.25	0.25
Tert-butylhydroquinone	0.0014	0.0014
Blueberry stem water extract (BStEx)	–	1.0

In this study, we investigated the effects of BStEx on lacrimal hyposecretion in male NOD mice.

Materials and Methods

Materials. BStEx was provided by Biolabo Co. Ltd. The stems of rabbiteye blueberry (*Vaccinium virgatum* Aiton; Kunisato 35 Gou) grown in Miyazaki, Japan were provided by Nanaha Corporation. Hot water at 98˚C was added to the stems and boiled for 60 min for extraction, then filtered. The extracted solution was freeze-dried to obtain BStEx powder. AIN-93G formula diet and the diet mixed with 1% BStEx (the composition of the two types of diets is shown in Table I) were purchased from Oriental Yeast, Tokyo, Japan. Medetomidine (Dorbene), midazolam (Dormicum injection®), and Butorphanol (Vetorphale) were purchased from Kyoritsu Seiyaku (Tokyo, Japan), Astellas Pharma (Tokyo, Japan), and Meiji Seika Pharma (Tokyo, Japan) respectively. The thread impregnated with phenol red (Zone-Quick) was purchased from Ayumi Pharmaceutical (Tokyo, Japan). Pilocarpine hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-aquaporin 5 (AQP5) antibody for western blotting was purchased from Bioss Antibodies, Inc. (Woburn, MA, USA). Rabbit anti-AQP5 antibody (Aff Ra-TM14cFY) for immunostaining was a gift from T Matsuzaki (Gunma University, Maebashi, Gunma, Japan). The anti-α-tubulin antibody was purchased from GeneTex (Irvine, CA, USA). Anti-phospho-AMPK, anti-AMPK, anti-ATG5, anti-LC3A/B, and anti-rabbit IgG horse-radish peroxidase (HRP)-linked antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Envision™ +Dual Link System-HRP was purchased from Agilent Technologies Inc. (Santa Clara, CA, USA).

Mice. Male non-obese diabetes/ShiJcl mice were purchased from CLEA Japan. All mice were maintained under controlled conditions at 23±2˚C, 50±5% humidity, and light/dark cycle in an animal facility at the University of Miyazaki. Mice were divided into two groups and fed ad libitum with AIN-93G formula diet as control or

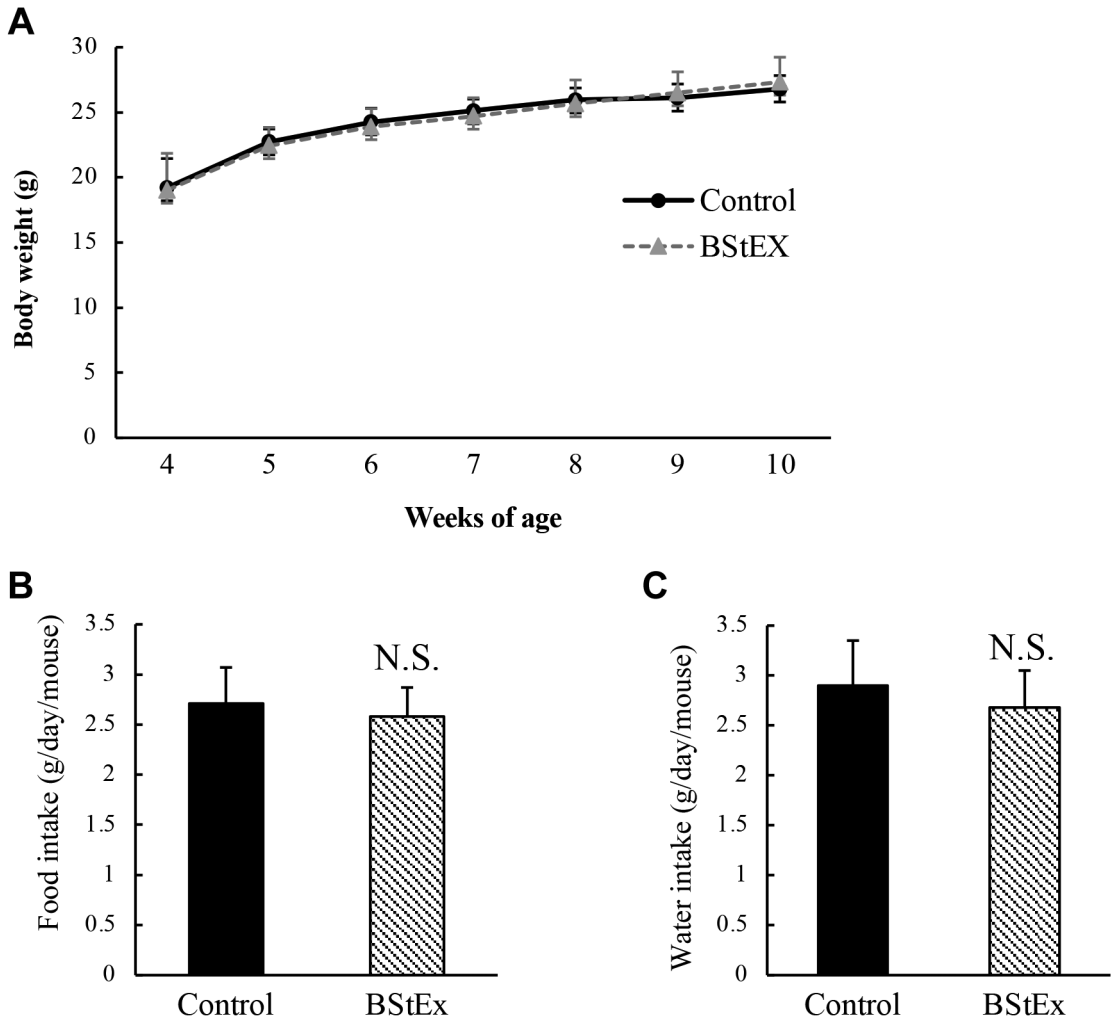

Figure 1. *Body weight changes and amount of food and water intake of non-obese diabetic (NOD) mice fed with control diet or 1% BStEx diet. There were no significant differences in (A) body weight, (B) amount of food intake, and (C) amount of water intake for 6 weeks from 4 weeks of age to 10 weeks of age between BStEx and control groups. Data are represented as mean±SD (n=6). BStEx group: NOD mice fed with an AIN-93G diet containing 1% blueberry stem hot water extract. Control group: NOD mice fed with a control AIN-93G diet.*

AIN-93G diet mixed with 1% BStEx for 2 to 6 weeks (from the age of 4 to 10 weeks).

All animal studies were approved by the University of Miyazaki's Committee on the Ethics of Animal Experiments (approval Number:2019-032) and carried out in accordance with the guidelines issued by the Committee.

Measurement of tear secretion volume. The tear volume of mice was determined using the cotton thread test using a phenol red-impregnated thread based on a previous report (8). Tear volumes from mice at 6, 8, and 10 weeks of age were measured.

Histological analysis. The histological analysis was performed based on our previous report (15). Images of HE-stained lacrimal gland tissue sections were photographed using a NanoZoomer 2.0-RS Digital slide scanner C10730-13 (Hamamatsu Photonics K.K., Shizuoka, Japan) and observed using NDP.View2 (Hamamatsu Photonics K.K.).

Immunostaining. Immunostaining for lacrimal gland tissue using AQP5 antibody was performed as previously described (19). After paraffin removal from paraffin-fixed lacrimal gland tissue sections, the tissue was heat-treated at 121˚C for 5 min for antigen activation. The sections were then incubated with methanol containing 3% H_2O_2 for 5 min to inactivate endogenous peroxidase, followed by washing three times with phosphate-buffered saline (PBS) for 5 min. The sections were then placed in anti-AQP5 antibody (1: 500) diluted in PBS containing 0.1% bovine serum albumin and incubated overnight at 4˚C. After washing with PBS three times for 5 min each, the sections were incubated in the Envision™ Dual Link System-HRP (Dako Japan Inc., Tokyo, Japan) for 5 min at room temperature. After washing with PBS three times for 5 min each, the sections were stained with DAB solution (50 mM Tris-HCl, 1.3 mM DAB, 4.2 mM H_2O_2, pH 7.6), and the reaction was stopped with distilled water. After hematoxylin staining, the samples were treated with ethanol three times for 30 s and three times for

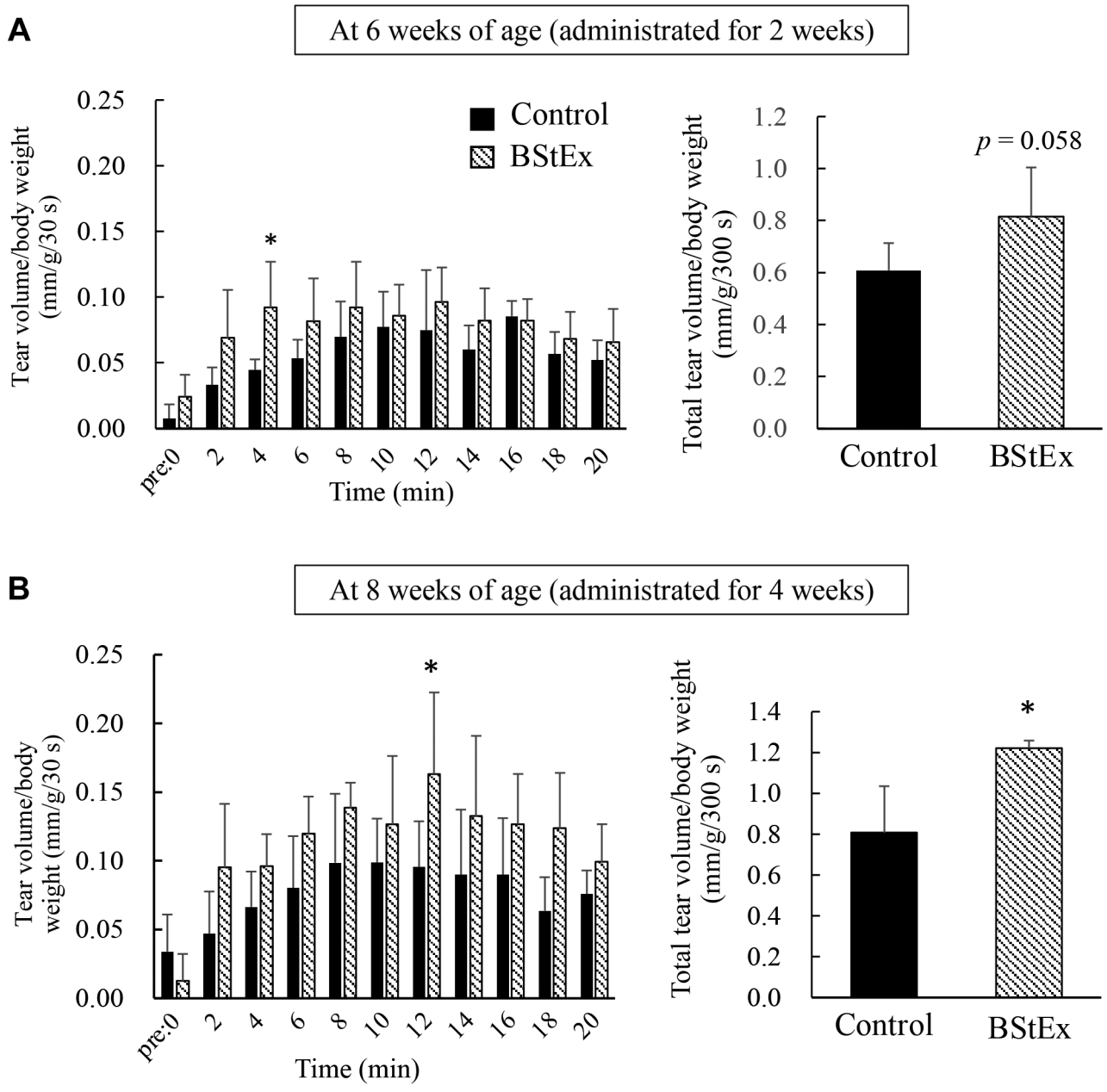

Figure 2. *Continued*

60 s and then permeabilized and sealed with hemody three times for 5 min. Images of lacrimal gland tissue sections were photographed using a NanoZoomer 2.0-RS Digital slide scanner C10730-13 and observed using NDP.View2.

Western blot analysis. The western blot analysis was performed based on our previous report (15). The lacrimal glands were isolated from euthanized mice before and after injection of pilocarpine at 8 weeks of age and immediately homogenized. Protein samples (40 μg for AQP5 and α-tubulin and 4 μg for phospho-AMPK, AMPK, ATG5, LC3AB, and α-tubulin) were used for SDS-PAGE. The blots were blocked at room temperature (15-25˚C) for 60 min in 3% of skim milk (Meiji Seika Pharma Co., Ltd, Tokyo, Japan) for AQP5 and for 30 min in Blocking One-P (Nacalai Tesque, Inc., Kyoto, Japan) for phospho-AMPK, AMPK, ATG5, LC3, and α-tubulin. Then, the blots were probed with the primary antibodies anti-AQP5 (diluted 1:500), anti-phospho-AMPK (diluted 1:1,000), anti-AMPK (diluted 1:1,000), anti-ATG5 (diluted 1:1,000), anti-LC3 (diluted 1:1,000), or α-tubulin (diluted 1:1,000) at 4˚C overnight.

Statistical analysis. Data are presented as mean±standard deviation (SD). Statistical comparisons were made using one-way analysis

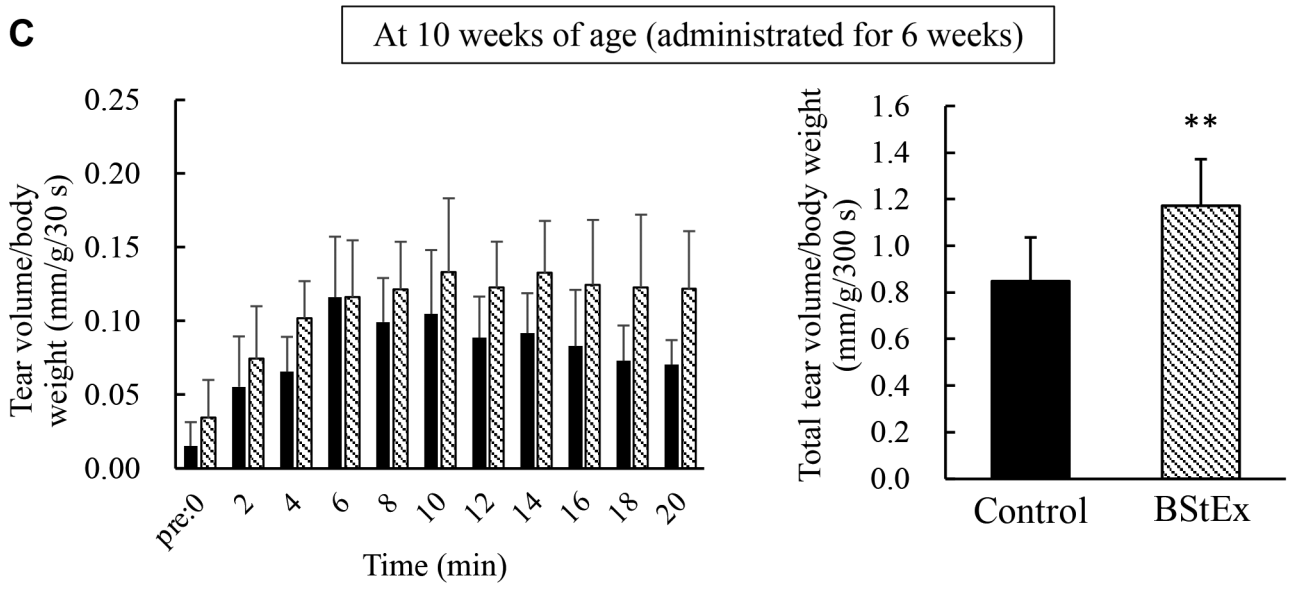

Figure 2. *Effect of BStEx on lacrimal hyposecretion in male non-obese diabetic (NOD) mice. Pilocarpine at a dose of 0.5 mg/kg was intraperitoneally injected into NOD mice at (A) 6, (B) 8, and (C) 10 weeks of age after 2-, 4-, and 6-week feeding of the BStEx contained- or control-pellets, respectively, and the secreted tear volume was measured every 2 min for 30 s. (A) An increased tendency was observed for the total tear volume in the BStEx group at 6 weeks of age compared with that in the control group. (B and C) There were significant increases in the total tear volume in the BStEx group at both 6 and 10 weeks of age compared with the values in the control group. Data are represented as mean±SD (n=5-7). *p<0.05, **p<0.01 compared with the control group. BStEx group: NOD mice fed with an AIN-93G diet containing 1% blueberry stem hot water extract. Control group: NOD mice fed with a control AIN-93G diet.*

of variance (ANOVA) followed by Student's *t*-test and two-way ANOVA followed by Sidak's multiple comparisons test, performed with GraphPad Prism ver. 7 (GraphPad Software, La Jolla, CA, USA). *p*-Values below 0.05 were regarded as statistically significant differences.

Results

BStEx intake inhibited lacrimal hyposecretion in NOD mice. BStEx contains 442 mg/g proanthocyanidin, 6.1 mg/g chlorogenic acid, 3.6 mg/g catechin, 4.5 mg/g epicatechin, and 78.3 mg/g quinic acid. BStEx has less chlorogenic but more quinic acid than BLEx. The amounts of the other components in BStEx were similar to those in BLEx. There were no differences in body weight gain, amount of food, or water intake between the NOD-control and NOD-BStEx groups over the observation period of 6 weeks from 4 weeks of age to 10 weeks of age (Figure 1). There were also no differences in the weight of the lacrimal glands between the NOD-control and NOD-BStEx groups at 6, 8, and 10 weeks of age at each sampling time (data not shown). To evaluate tear volume, 0.5 mg/kg pilocarpine was intraperitoneally injected into NOD mice at 6, 8, and 10 weeks of age after 2-, 4-, and 6-week feeding of BStEx-containing or control pellets, respectively, and the secreted tear volume was measured. The results showed an increasing tendency in the total tear volume in the BStEx group at 6 weeks of age compared with that in the control group, although the difference was insignificant (Figure 2A). In contrast, there were significant increases in the total tear volume in the BStEx groups at both 6 and 10 weeks of age compared with the values of the control groups (Figure 2B and C). These results suggest that the administration of BStEx for at least 4 weeks before the onset of dacryoadenitis prevented lacrimal hyposecretion in NOD mice and that the effect lasted until at least 10 weeks of age if the administration continued.

BStEx intake did not inhibit inflammatory cell infiltration into lacrimal glands in NOD mice. We evaluated the severity of inflammatory cell infiltration into lacrimal glands by performing hematoxylin and eosin staining of lacrimal glands removed from NOD-control and NOD-BStEx mice at 8 weeks of age. Inflammatory cells were observed in the lacrimal glands of both NOD-control and NOD-BStEx mice (Figure 3A). The focus score (number of mononuclear cell infiltrates containing >50 cells per 4 mm^2 area) and the area of inflammatory cell foci (% of the total lacrimal gland area) in the lacrimal glands were the same in the NOD-control and NOD-BStEx groups (Figure 3B and C). We also performed histological analysis of NOD-control and NOD-BStEx mice at 10 weeks of age, but there were no significant differences between these two groups, similar to the result at 8 weeks of

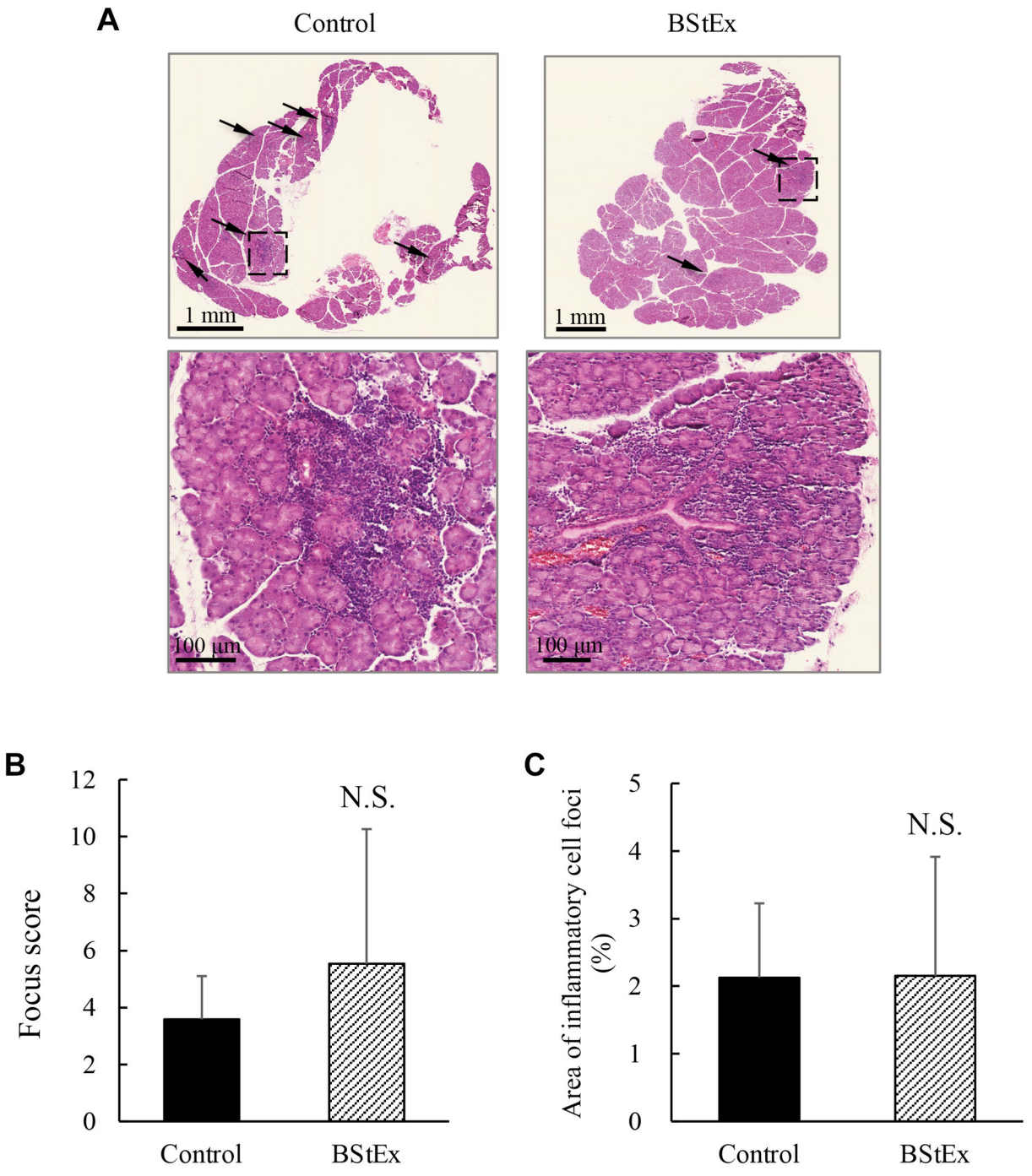

Figure 3. *Continued*

age (data not shown). These results suggest that the preventive effect of BStEx on lacrimal hyposecretion is not due to the inhibition of inflammatory cell infiltration into the lacrimal glands in NOD mice.

We then evaluated the inflammatory cytokines, TNF-α, IL-6, and IFN-γ, which were reported to increase in the lacrimal glands of NOD mice at 8 weeks of age (11). The results showed that there was no significant difference in the amount of TNF-α and IL-6 in the lacrimal glands between the NOD-control and NOD-BStEx groups at 8 weeks of age (Figure 3D and E). In contrast, BStEx significantly reduced IFN-γ levels, but the reduction ratio was only 7.1% (Figure

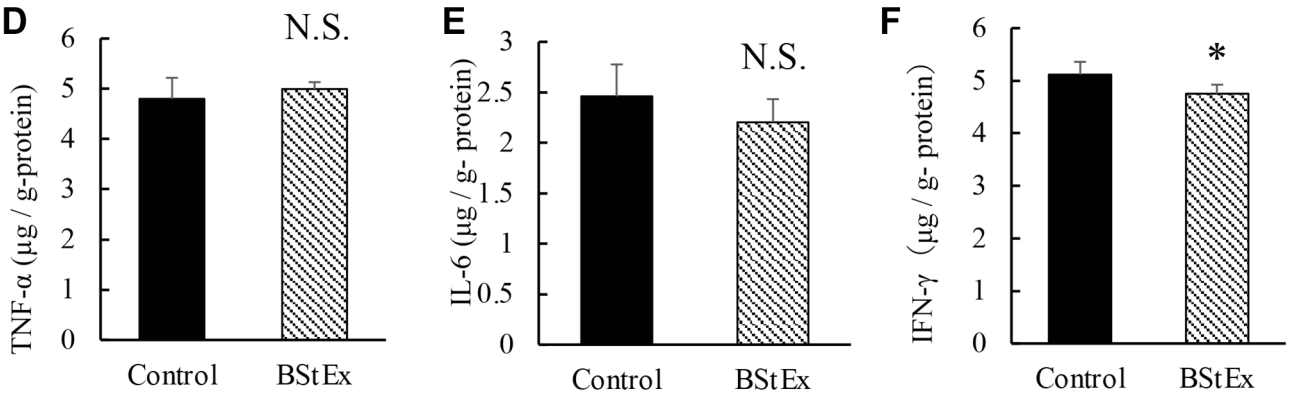

Figure 3. *Histological analysis of inflammatory cytokines in the lacrimal gland of male non-obese diabetic (NOD) mice fed with diets containing BStEx. (A) Representative cross-sections of hematoxylin and eosin (HE)-stained lacrimal glands from NOD mice fed with control diets and those fed with a diet containing 1% BStEx for 4 weeks, at 8 weeks of age. Arrows indicate the inflammatory area showing infiltration of mononuclear leukocytes. Scale bars represent 1 mm in the upper panels and 100 μm in the lower panels. Lower panels are representative images of the "focus" showing infiltration of >50 mononuclear cells. There were no significant differences in the focus score (B) and the ratio of inflammatory cell foci area (%) (C) in lacrimal gland HE sections of NOD mice between the BStEx and control groups. There were no significant differences in the levels of inflammatory cytokines TNF-α (D) and IL-6 (E) in the lacrimal gland of NOD mice measured by ELISA, but IFN-γ was slightly reduced in the BStEx group (F). Data are represented as mean±SD (n=6). *p<0.05 compared with the control group. BStEx group: NOD mice fed with an AIN-93G diet containing 1% blueberry stem hot water extract. Control group: NOD mice fed with a control AIN-93G diet.*

3F). These results suggest that BStEx slightly reduced IFN-γ but did not affect the infiltration of inflammatory cells. Furthermore, it remains unknown how the slight reduction in IFN-γ contributes to the preventive effect of BStEx on lacrimal hyposecretion.

BStEx intake did not alter the autophagy-related protein expression. We then examined the activity of autophagy because BLEx significantly reduced the autophagy-related protein LC3 in a previous study (15). We performed western blotting for ATG5 and LC-I/II in the lacrimal glands removed from NOD-control and NOD-BStEx mice at 8 weeks of age. The results showed no difference in the expression levels of ATG5 and LC-I/II ratio between the control and BStEx lacrimal glands (Figure 4). These results suggested that the increase in tear amount after BStEx intake was not due to a change in autophagy activity in the lacrimal glands of NOD mice.

BStEx intake did not alter the localization and expression level of AQP5. AQP5 is one of the water channels expressed in the apical membrane of acinar cells and some ductal cells in the lacrimal glands and is involved in transcellular water secretion. To confirm the localization of AQP5, we performed an immunohistochemical analysis of AQP5 in lacrimal glands removed from NOD-control and NOD-BStEx mice at 8 weeks of age. Both control and BStEx samples showed apical localization of AQP5 in both the acinar and duct cells, and there was no difference (Figure 5A). Furthermore, western blot analysis revealed that there was no difference in the expression level of AQP5 between the control and BStEx lacrimal glands (Figure 5B and C). These results suggested that the increase in tear amount after BStEx intake was not due to a change in localization of AQP5 or an increase in the expression level of AQP5 in lacrimal glands in NOD mice. In other words, BStEx has no effect on transcellular water secretion.

BStEx intake increased phosphorylation of AMPK. We then evaluated the activity of AMPK, a sensor of energy metabolism, because activation of AMPK has been reported to promote paracellular water transport *via* opening tight junctions (20). Representative western blot data for phosphorylated and total AMPK are shown in Figure 6A, indicating that pilocarpine stimulation itself increased the phosphorylated AMPK/total AMPK ratio, and BStEx also increased the ratio both before and after pilocarpine treatment. Quantitative analysis of western blotting showed that the phosphorylated AMPK/total AMPK ratio was increased in the BStEx group compared with that in the control group without pilocarpine treatment (Figure 6B; n=8 each). However, the rate of increase in the phosphorylated AMPK/total AMPK ratio in the BStEx group compared with that in the control group decreased after pilocarpine treatment, although there was a significant difference between them (Figure 6C; n=6 each). These results that pilocarpine treatment itself largely activates the AMPK and BStEx also activates AMPK suggested that BStEx may promote paracellular water secretion in the lacrimal glands of NOD mice.

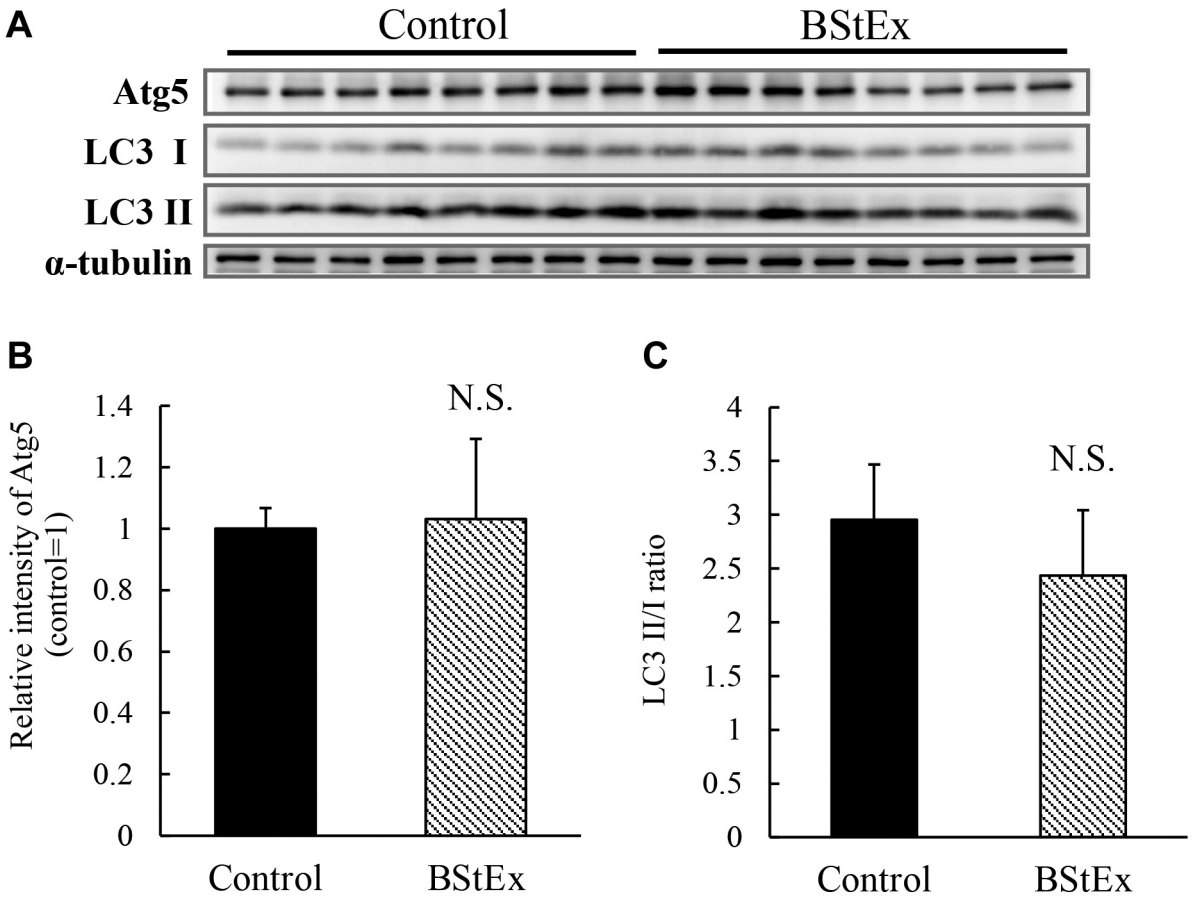

Figure 4. *Expression levels of autophagy-related proteins in the lacrimal gland of male non-obese diabetic (NOD) mice fed with diets containing BStEx. (A) ATG5, LC3 I, LC3 II, and α-tubulin western blot bands of lacrimal glands from NOD mice fed with control diets and those fed with a diet containing 1% BStEx for 4 weeks, at 8 weeks of age. There were no significant differences in the relative intensity of ATG5 (B) and the ratio of LC3 II/I (C) between BStEx and control groups. Data are represented as mean±SD (n=8). BStEx group: NOD mice fed with an AIN-93G diet containing 1% blueberry stem hot water extract. Control group: NOD mice fed with a control AIN-93G diet.*

Discussion

In this study, we found that continuous oral intake of BStEx prevented lacrimal hyposecretion in an SS-like model of male NOD mice. BStEx had no effect on transcellular water transport *via* AQP5, but it may increase paracellular water transport *via* AMPK activation (as illustrated schematically in Figure 7). Furthermore, BStEx did not prevent inflammatory cell infiltration into the lacrimal gland and did not alter the expression levels of autophagy-related proteins.

Water secretion in the acinar cells of exocrine glands occurs through a combination of two pathways: direct secretion from lacrimal cells *via* water channels (transcellular water transport) and water secretion from intercellular spaces caused by the opening of tight junctions (paracellular water transport) (21, 22). The former is the transport pathway usually controlled by aquaporin 5 (AQP5) on the apical membrane in the acinar/duct cells of the lacrimal glands (23-25). We checked the localization and expression levels of AQP5 in the lacrimal gland and found that there were no significant differences between the BStEx and control groups (Figure 5). BLEx, which has similar ingredient contents, also did not alter the expression level of AQP5 in the lacrimal gland of male NOD mice (15). The BLEx study also revealed no significant differences in the expression levels of M3r (muscarinic receptor), Nkcc1 (Na^+-K^+-Cl^- cotransporter), and Tmem16a (Cl^- channel) between the BLEx and control groups, which are involved in water secretion (15). Taken together, these results indicate that BStEx does not affect transcellular water transport, but rather might affect paracellular water transport.

There is less information regarding paracellular water transport than that on intracellular water transport. It is considered to occur when tight junctions are opened and water passes between the acinar cells (26). It has been reported that opening of tight junctions is involved in water secretion in transplanted salivary glands (27), and disruption

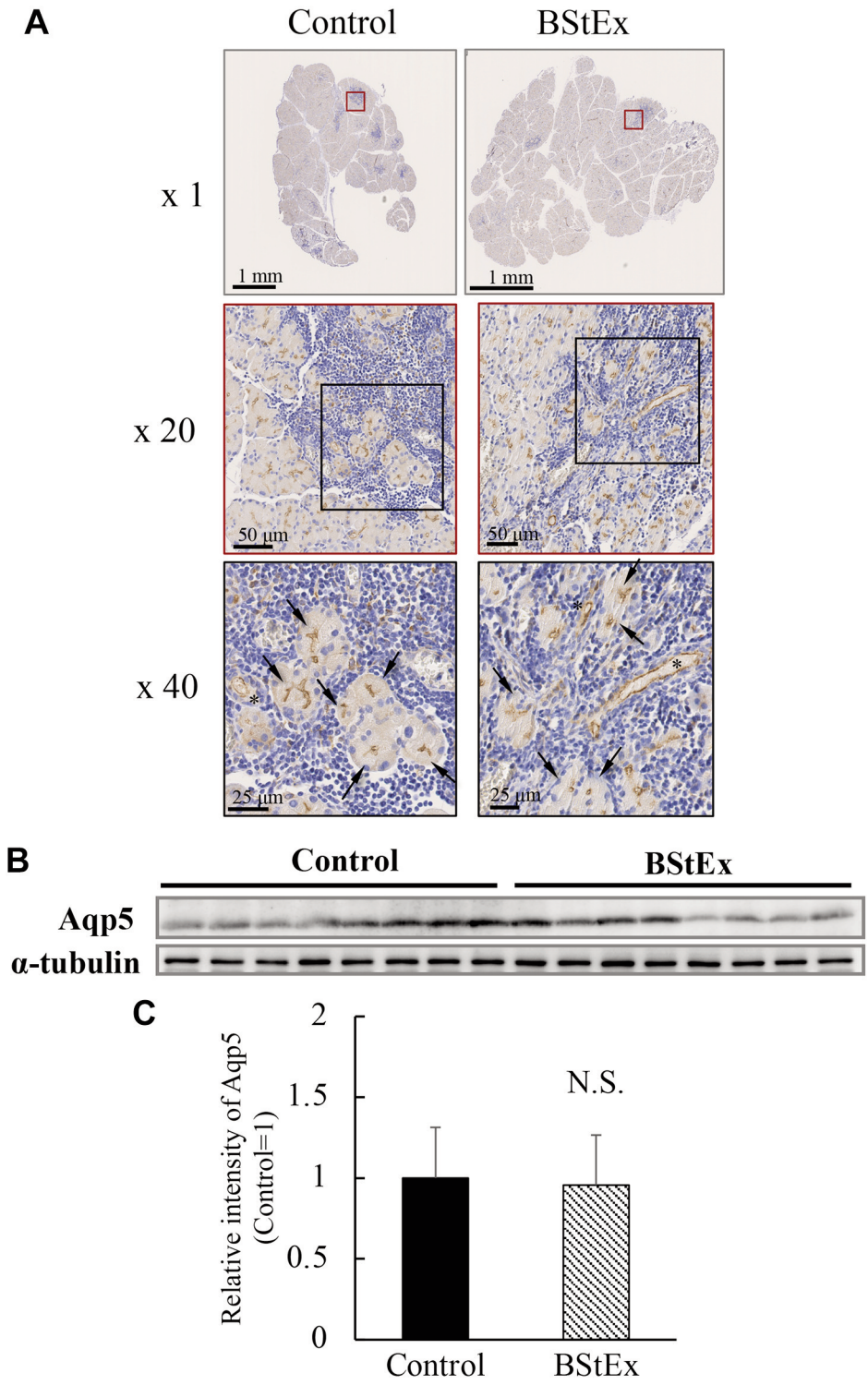

Figure 5. *Localization and expression level of Aquaporin 5 (AQP5) in the lacrimal gland of male non-obese diabetic (NOD) mice fed with diets containing BStEx. (A) Representative AQP5-immunostained cross-sections of lacrimal glands from NOD mice fed with control diets and those fed with a diet containing 1% BStEx for 4 weeks, at 8 weeks of age. AQP5 was expressed on the apical membrane of acinar and duct cells in both BStEx and the control group. Arrows indicate acinar cells, and asterisks indicate ducts. Scale bars represent 1 mm in the upper panels, 50 μm in the middle panels, and 25 μm in the lower panels. (B) AQP5 and α-tubulin western blot bands. (C) There were no significant differences in the relative intensity of AQP5 between BStEx and control groups. Data are represented as mean±SD (A: n=6; B and C: n=8). BStEx group: NOD mice fed with an AIN-93G diet containing 1% blueberry stem hot water extract. Control group: NOD mice fed with a control AIN-93G diet.*

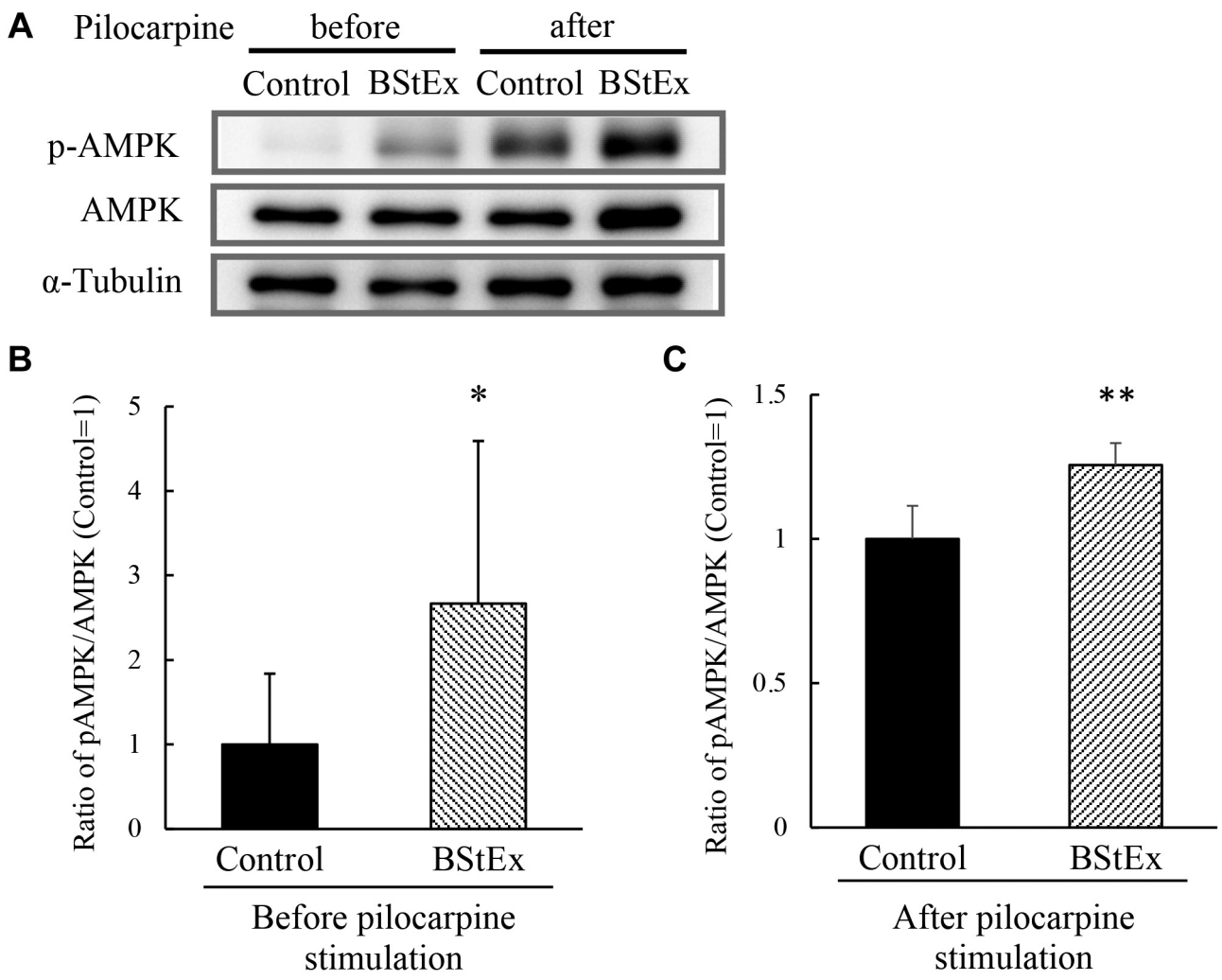

Figure 6. *The expression level of phosphorylated AMPK in the lacrimal gland of male non-obese diabetic (NOD) mice fed with diets containing BStEx. (A) Representative phosphorylated AMPK, total AMPK, and α-tubulin western blot bands of lacrimal glands from NOD mice fed with control diets and those fed with a diet containing 1% BStEx for 4 weeks, at 8 weeks of age, before and after pilocarpine treatment. The ratio of phosphorylated AMPK/total AMPK before (B) and after (C) pilocarpine treatment. Pilocarpine itself seems to increase the ratio largely (A), and BStEx significantly increased the ratio both before (B) and after (C) pilocarpine treatment compared to the control group. Data are represented as mean±SD (B; n=8 and C; n=6). *p<0.05, **p<0.01 compared with the control group. BStEx group: NOD mice fed with an AIN-93G diet containing 1% blueberry stem hot water extract. Control group: NOD mice fed with a control AIN-93G diet.*

of tight junctions can lead to hyposecretion of the salivary glands (28). AMPK activation regulates tight junctions (29). Ding *et al*. reported that adiponectin contributed to the opening of tight junctions *via* AMPK activation, resulting in an increase in water secretion in perfused rat submandibular glands without altering the localization and expression levels of AQP5 (20). In this study, we revealed that pilocarpine stimulation itself seemed to contribute to AMPK activation (Figure 6A), suggesting that opening tight junctions leads to water secretion. In addition, AMPK was activated in the BStEx group compared to the control group, both before and after pilocarpine stimulation (Figure 6B and C). This may suggest that BStEx opens tight junctions and allows water to pass smoothly, resulting in increased tear volume in the BStEx group (Figure 7).

Some studies have shown the prevention of lacrimal hyposecretion in male NOD mice *via* the suppression of inflammation (30-32). In this study, however, BStEx prevented lacrimal hyposecretion but not inflammatory cell infiltration (Figure 3A-C). Therefore, the preventive effect of BStEx on lacrimal hyposecretion does not appear to be derived from the suppression of lacrimal tissue destruction by inflammatory cell infiltration. In our previous study, BLEx also did not change inflammatory cell infiltration but

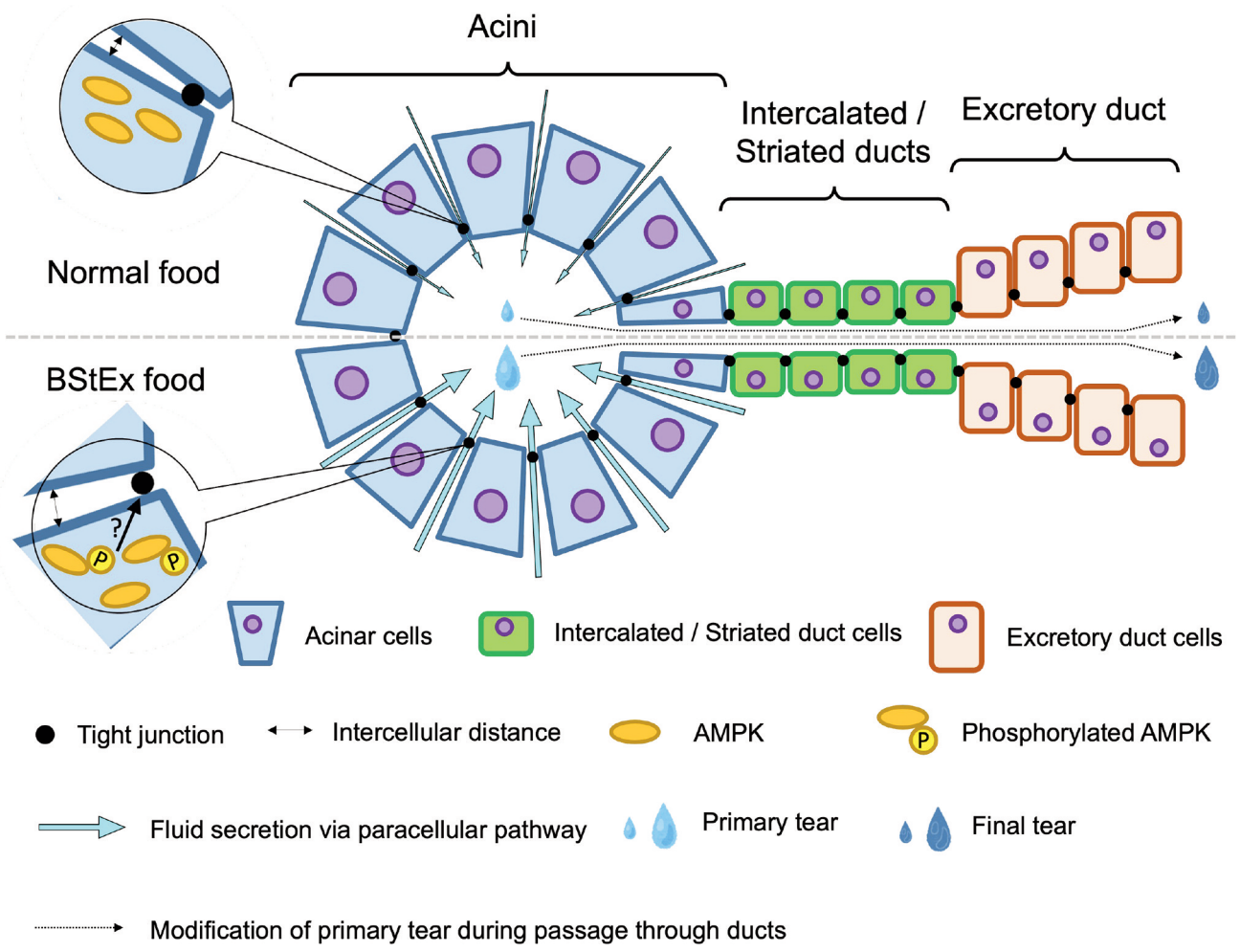

Figure 7. *Putative mechanism of tear fluid-increasing effect of BStEx in male non-obese diabetic (NOD) mice. A primary tear is produced in acini and then modified during passage through ducts. BStEx increased activation of AMPK (phosphorylation of AMPK). This may suggest that BStEx opens tight junctions and lets water pass smoothly, resulting in increased tear volume in the BStEx group.*

significantly reduced TNF-α by approximately 25% compared to the control group (15). Thus, we expected a similar effect of BStEx on TNF-α; however, BStEx group did not show any change in TNF-α concentration (Figure 3D). In addition, there was only a slight difference in IFN-γ concentration and no significant difference in IL-6 concentration between the BStEx and control groups (Figure 3E, F). This suggests that BStEx had little or no effect on the immune system. Furthermore, BLEx inhibited the autophagy activity indicator LC3 II/I ratio (15), whereas BStEx did not alter LC3 II/I ratio or ATG5 expression level. Although differences between BLEx and BStEx appear here, further investigation is required to clarify these differences.

The blueberry leaf or stem extract content in the diets was 1% in both the previous BLEx (15) and the current BStEx study. Generally, the types of ingredients contained in both extracts were very similar, but the contents of each ingredient were different in the two extracts. For example, chlorogenic acid, rutin, and catechins are generally known to have anti-inflammatory effects *via* the inhibitory action of TNF-α (33-35), but the contents of these ingredients are lower in BStEx than those in BLEx (15). Thus, BStEx may not reduce TNF-α levels in the lacrimal glands. Chlorogenic acid has been reported to inhibit autophagy (36). Chlorogenic acid in BStEx is two orders of magnitude less than that in BLEx, and this difference is considered to be the reason why BStEx did not show an inhibitory effect on autophagy. As for the phosphorylation of AMPK, a previous report has shown that oral administration of proanthocyanidins to mice models of type 2 diabetes promotes AMPK activation and has anti-obesity effects (37). Furthermore, an increase in AMPK phosphorylation in a dose-dependent manner has been

reported in C2C12 myotubes and skeletal muscle fibers of BALB/c mice treated with proanthocyanidins (38). Since BStEx and BLEx contain almost equal amounts of proanthocyanidins, we speculated that both BStEx and BLEx have an activating effect on AMPK, although we did not confirm this in our previous study.

Conclusion

In summary, we found that BStEx prevented lacrimal hyposecretion in a SS-like model in male NOD mice. The mechanism might involve opening of tight junctions *via* the activation of AMPK in lacrimal acinar cells, but further studies are needed to confirm this. Blueberry stems are part of the functional food supply that can be consumed daily, but further research on their potential usability as an herbal medicine that helps alleviate the lacrimal hyposecretion of dry eye diseases, including SS, is needed.

Conflicts of Interest

KO received a research grant from Biolabo Co., Ltd. and YG was an employee of Biolabo Co., Ltd.

Authors' Contributions

KO and YO designed the study and drafted the manuscript. KO, AT, and KU performed the tests and analyzed the data. KS, NF, HS, and MI supported the study design. YG provided the blueberry stem extract. HK, KN, and MY supervised the study. All Authors contributed to the manuscript preparation and approved the final paper.

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