in vivo *37*: 149-162 (2023)
doi:10.21873/invivo.13064

Blueberry Leaf Extract Prevents Lacrimal Hyposecretion in Sjögren's Syndrome-like Model of Non-obese Diabetic Mice

KENJIROU OGAWA[1], KARIN URATA[2], SAKI MAEDA[3], YUTA OHNO[4], KEITARO SATOH[5], YOSHIYUKI YAMADA[3], YOSUKE SUZUKI[6], YASUKO KOGA[2], KAZUHIRO SUGAMOTO[6], MAKIKO KAWAGUCHI[7], HISATO KUNITAKE[2,3], KAZUO NISHIYAMA[2,3], YO GOTO[8], TAKAYUKI NAKAYAMA[8] and MASAO YAMASAKI[2,3]

[1]*Institute for Tenure Track Promotion, University of Miyazaki, Miyazaki, Japan;*
[2]*Graduate School of Agriculture, University of Miyazaki, Miyazaki, Japan;*
[3]*Department of Biochemistry and Applied Biosciences, Faculty of Agriculture, University of Miyazaki, Miyazaki, Japan;*
[4]*Department of Pharmacology, Asahi University School of Dentistry, Mizuho, Japan;*
[5]*Department of Pharmacology, Meikai University School of Dentistry, Sakado, Japan;*
[6]*Department of Applied Chemistry, Faculty of Engineering, University of Miyazaki, Miyazaki, Japan;*
[7]*Department of Pathology, Faculty of Medicine, University of Miyazaki, Miyazaki, Japan;*
[8]*Biolabo Co., Ltd., Kobe, Japan*

Abstract. *Background/Aim: This study evaluated the effect of blueberry leaf hot water extract (BLEx) on Sjögren's syndrome (SS)-like lacrimal hyposecretion in male non-obese diabetic (NOD) mice. Materials and Methods: NOD or BALB/c mice were fed 1% BLEx or control (AIN-93G) for 2 weeks from the age of 4 to 6 weeks. Pilocarpine-induced tear volume was measured using a phenol red-impregnated thread. The lacrimal glands were evaluated histologically by H&E staining. The IL-1β and TNF-α levels in the lacrimal gland tissue were measured by ELISA. The mRNA expression levels of secretion-related proteins were measured by real-time PCR. LC3 I/II and arginase 1 expression levels were measured by western blot. Results: After feeding with BLEx, pilocarpine-induced tear secretion in NOD mice was increased. In contrast, the mRNA expression levels of the cholinergic muscarinic M3 receptor, aquaporin 5, and ion channels related to lacrimal secretion were not changed by BLEx administration. In addition, the protein expression of arginase 1, which was recently reported to be involved in tear hyposecretion in NOD mice, was also not improved by BLEx administration. Although infiltration in the lacrimal gland of NOD mice was not decreased, the levels of TNF-α and the autophagy-related protein LC3 were significantly suppressed by BLEx treatment. Conclusion: BLEx treatment may ameliorate lacrimal hyposecretion in NOD mice by delaying the progression of autoimmune disease by suppressing autophagy in lacrimal glands.*

Correspondence to: Kenjirou Ogawa, Ph.D., Institute for Tenure Track Promotion, University of Miyazaki, 1-1 Gakuen Kibanadai-nishi, Miyazaki-shi, Miyazaki 889-2192, Japan. Tel: +81 985587234, e-mail: ogawa.kenjirou.u2@cc.miyazaki-u.ac.jp

Key Words: Dry eye, blueberry leaf, proanthocyanidin, tear secretion, lacrimal gland, Sjögren's syndrome.

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Tear fluid plays an important role in preventing dryness, protecting the ocular surface from external stimuli, and carrying oxygen and nutrients to the cells. Tears are also necessary for light to be refracted correctly for clear vision. Dry eye disease is an ocular disease characterized by chronic multifactorial conditions of the ocular surface, such as dryness and breaking up of a lipid film that induces damage to the ocular surface. Furthermore, the dry eye symptoms can progress to dryness, fatigue, discomfort of the eye, and blurred vision, leading to decreased visual function and work efficiency and a decline in quality of life. Dry eyes may be caused by a decrease in the lacrimal secretory function due to aging, lack of sleep, LASIK surgery, wearing contact lenses, mental stress, environmental influences, such as windy conditions, a very dry climate, smoking, and Sjögren's syndrome (SS). In addition, in recent years, the increase in the amount of time spent working with visual display terminals, such as computers and mobile phones, has also contributed to an increase in the prevalence of dry eyes (1).

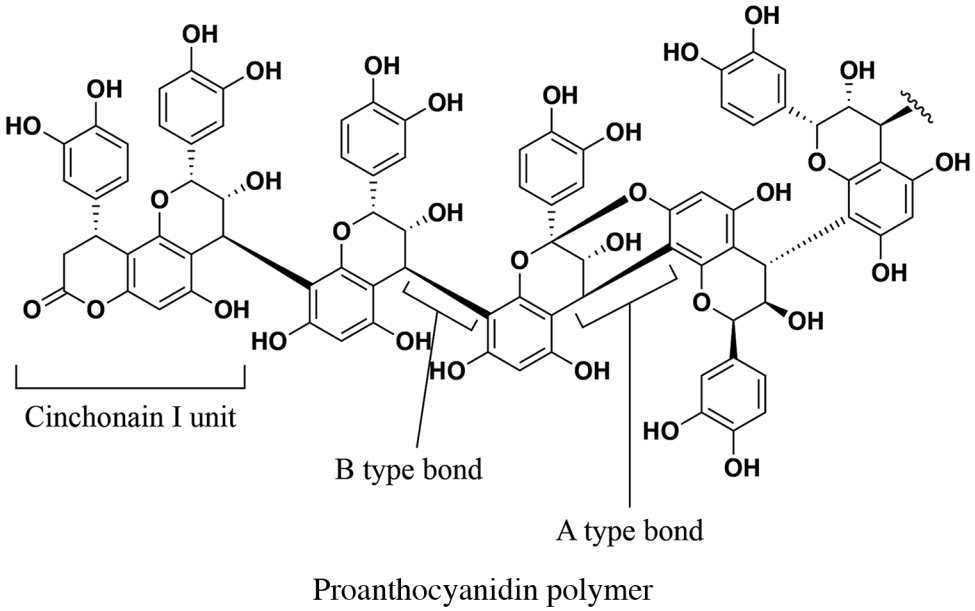

Figure 1. *Structure of the proanthocyanidin polymer found in the rabbiteye blueberry leaf extract. Pro-anthocyanidin polymer is one of the predictive high polymerized structures that includes a cinchonain I unit, an A-type bond, and a B-type bond.*

According to the new TFOS DEWS II diagnostic criteria for dry eye, the global prevalence of dry eyes is estimated to be 29.5±0.8% (2). Abnormalities in tear fluid induce high osmolarity, which in turn, induces inflammation and a vicious cycle of epithelial damage on the ocular surface, worsening tear fluid abnormalities (3).

Primary SS is an autoimmune disease that targets exocrine glands, such as the lacrimal and salivary glands, where lymphocytic infiltration occurs. Inflammation induces lacrimal hyposecretion by destroying the lacrimal gland tissue (4, 5). There has been no causal treatment for dry eye related to SS till date; however, there are symptomatic treatments, such as eye drops, secretagogues, and antibody therapy.

Non-obese diabetic (NOD) mice have been used as SS models because of the similarity in symptoms with those of human primary SS. NOD mice spontaneously develop lymphocytic infiltration in salivary and lacrimal glands, which is associated with hyposecretion (6). Male NOD mice are more susceptible to dacryoadenitis than female mice and begin to infiltrate the lacrimal gland after 4 weeks of age. Infiltration in the lacrimal gland is reported to be confirmed at 6 to 8 weeks of age (7, 8). In addition, pilocarpine-induced lacrimal secretion measurements gradually decrease from 6 to 10 weeks of age compared to BALB/c mice, in which lacrimal secretion does not decrease (7). Moreover, male NOD mice show increased levels of inflammatory cytokines, such as IL-1β and TNF-α, after 6 weeks of age (8). Infiltration of T and B lymphocytes in the lacrimal glands leads to degradation of the lacrimal gland extracellular matrix structures, resulting in decreased aqueous humor secretion in male NOD mice (9-11). Recent studies have shown that activation of autophagy and reduced expression of arginase-1 also affect the decrease in tear secretion in NOD mice (7, 12).

Rabbiteye blueberry (*Vaccinium virgatum* Aiton) leaves are nutritionally important food cultivated in Miyazaki, Japan. It contains rich proanthocyanidins polymerized by flavan-3-ol. As shown in Figure 1, the proanthocyanidins in blueberry leaves are presumably composed of a polymer of cyanidin and catechin with one interflavan B-type linkage and two interflavan A-type linkages, and phenylpropane cinchonain I units (13). Proanthocyanidin has been reported to have antioxidant and anti-inflammatory properties (14, 15). Blueberry leaves have high antioxidant, hepatoprotective, anti-obesity, and immunomodulatory effects (16-18). Moreover, blueberry leaves contain other polyphenols, such as catechin, chlorogenic acid, and rutin, which have been reported to possess anti-inflammatory effects (12, 19-21). Several polyphenols affect exocrine function, such as resveratrol, which increases saliva secretion in NOD mice, and maqui berry (*Aristotelia chilensis*) containing rich anthocyanins and rooibos tea containing periodic-tyol-6-C-glucoside, which improve tear secretion in mice (22-24). However, there are no reports of foods that have ameliorating effects against impaired lacrimal secretory function in NOD mice of the SS-like model.

In this study, we investigated the effect of blueberry leaf hot water extract (BLEx) on lacrimal hyposecretion in NOD mice.

Table I. *Composition of control (AIN-93G) and blueberry leaf extract (BLEx)-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 leaf hot water extract (BLEx)	–	1.0

Materials and Methods

Materials. BLEx was provided by Biolabo Co., Ltd. Fresh leaves of rabbiteye blueberry (*Vaccinium virgatum* Aiton; Kunisato 35 Gou) cultivated in Miyazaki, Japan, were purchased from Nanaha Corporation. The leaves were extracted with boiling water at 98˚C for 60 min. After filtration, the extracted solution was freeze-dried to obtain the extract powder. Medetomidine (Dorbene) was purchased from Kyoritsu Seiyaku (Tokyo, Japan), midazolam (Dormicum injection®) was purchased from Astellas Pharma (Tokyo, Japan). Butorphanol (Vetorphale) was purchased from Meiji Seika Pharma (Tokyo, Japan). Pilocarpine hydrochloride, protease inhibitor cocktail, phosphatase inhibitor cocktail 2, and phosphatase inhibitor cocktail 3 were purchased from Sigma-Aldrich (St. Louis, MO, USA). RIPA lysis buffer was purchased from Fujifilm Wako Pure Chemical (Osaka, Japan). Blue protein loading dye was purchased from New England Biolabs (Hitchin, UK). Anti-arginase-1 and anti-α-tubulin antibodies were purchased from GeneTex (Irvine, CA, USA). Anti-LC3A/B antibody and anti-rabbit IgG Horse-radish peroxidase (HRP)-linked antibody were purchased from Cell Signaling Technology (Danvers, MA, USA). Phenol red-impregnated thread (Zone-Quick) was purchased from Ayumi Pharmaceutical (Tokyo, Japan).

Quantification of proanthocyanidins. HCl/butanol and thiolysis methods. The amount of proanthocyanidins contained in BLEx was quantified using the HCl/butanol method. BLEx [100 μl of BLEx dissolved in n-butanol/H_2O (95:5, v/v)] was mixed with 25 μl of 2% (w/v) $NH_4Fe(SO_4)_2 \cdot 12\ H_2O$ in 2 M HCl and 875 μl of n-butanol/HCl (95:5; v/v), and heated in an oil bath at 105˚C for 30 min. After heating, the mixture was cooled with cold water for 15 min, and the absorbance at 550 nm was measured. Proanthocyanidin content was expressed in mg/g dry weight of cyanidin chloride equivalents. The degree of polymerization of proanthocyanidins in BLEx was estimated using thiolysis (13). BLEx (2.5 mg) was dissolved in 0.5 ml ethanol solution consisting of 5% (v/v) 2-mercaptoethanol, 4% (v/v) 0.5 M HCl, and 32% (v/v) H_2O. The mixture was heated at 70˚C for 7 h. The resulting thiolytic products were filtered, and the filtrate was injected into the HPLC system. HPLC was performed on a Q-Exactive mass spectrometry system (G.L. Sciences, Tokyo, Japan) equipped with a UV visible detector and an Inertsil ODS-HL column (4.6 mm×250 mm, 1.9 μm, Thermo Fisher Scientific, Waltham, MA, USA). The separation conditions were as follows: flow rate, 0.8 ml/min; elution solvent, A (0.1% formic acid in water) and B (acetonitrile), and the gradient program, 10%-28% B from 0 to 10 min, 28%-80% B from 10 to 30 min. UV detection was performed at 280 nm. The mean degree of polymerization (mDP) of proanthocyanidin was calculated as follows: mDP=[sum of (2-hydroxyethylthio adducts • n)+sum of (free flavan-3-ol • n)]/[total free flavan-3-ol], where n represents the degree of polymerization of detected flavan-3-ol by thiolysis. Average percentage of A-type bonds=[sum of (thiolysis compounds containing A-type bonds • n)]/[total free flavan-3-ol]/mDP×100. Average percentage of cinchonain I unit=[sum of (thiolysis compounds containing cinchonain I unit • n)]/[total free flavan-3-ol]/mDP×100.

HPLC analysis of polyphenols. BLEx was dissolved in 80% (v/v) methanol, and the filtrate was injected into the HPLC system. HPLC was performed on a UF-Amino station (Shimadzu, Kyoto, Japan) equipped with a photodiode array detector and an Inertsil ODS3 column (4.6 mm×250 mm, 5 μm, G.L. Sciences). The separation conditions were as follows: flow rate, 1 ml/min; elution solvent A, 20 mM potassium phosphate solution (pH 2.4); solvent B, ethanol; gradient program, 15%-32% B from 0 to 12 min, 32% B from 12 to 15 min, 32%-45% B from 15 to 20 min, 85% B from 20 to 29 min, and column temperature of 40˚C. UV detection was performed at 280 nm for catechin and epicatechin, 320 nm for chlorogenic acid, and 360 nm for rutin. Polyphenols in BLEx were identified and quantified from calibration curves of standards of catechin, chlorogenic acid, and rutin.

Mice. Male non-obese diabetes/ShiJcl mice were purchased from CLEA Japan (Tokyo, Japan). Male BALB/c mice were purchased from Japan SLC (Shizuoka, Japan). All mice were maintained under controlled conditions (23±2˚C, 50±5% humidity, light/dark cycle) in the animal facility at the University of Miyazaki. Mice were divided into two groups and fed ad libitum with AIN-93G formula diet (M.F.; Oriental Yeast, Tokyo, Japan) (as control) or AIN-93G mixed with 1% BLEx for 2 weeks from the age of 4 to 6 weeks. The composition of the diets, which were purchased from Oriental Yeast, is shown in Table I.

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

Measurement of tear secretion volume. Tear volume of mice was determined using the cotton thread test using a phenol red-impregnated thread based on a previous report (7). Mice at 6 weeks of age were anesthetized by intraperitoneal (*i.p.*) administration of an anesthetic agent mixture (0.75 mg/kg medetomidine, 4.0 mg/kg midazolam, and 5.0 mg/kg butorphanol) at a volume of 0.05 ml per 10 g body weight. The volume of tear fluid was measured by carefully placing a phenol red-impregnated thread at the canthus of the left eye of each mouse for 30 s every 2 min for up to 20 min following pilocarpine *i.p.* injection at 0.5 mg/kg. The length of the thread changed color as the tear fluid was absorbed, and the absorption was measured in millimeters. All tear volume values were normalized to the body weight. The total tear volume was

Table II. *Primers used for the analysis of mRNAs by real-time RT-PCR.*

Gene	Primer sequence	Product length (bp)	Tm (˚C)
M3r	Forward: 5'-TGCTGAGCAGATGGACCAAGA-3'	148	61.45
(Chrm3)	Reverse: 5'-CGGCAGCTTGAGTACAATGGAA-3'		61.19
Aqp5	Forward: 5'-GGGTAACCTGGCCGTCAATG-3'	167	61.03
	Reverse: 5'-TGACCGACAAGCCAATGGATAA-3'		60.03
Nkcc1	Forward: 5'-GCGAGAAGGTGCACAATAC-3'	59	56.73
(Slc12a2)	Reverse: 5'-CGACACCATGGATTTTGGTA-3'		57.45
Tmem16a	Forward: 5'-AGGAATATGAGGGCAACCTG-3'	75	56.96
	Reverse: 5'-CGGCAGCTTGAGTACAATGGAA-3'		55.74
Gapdh	Forward: 5'-TGTGTCCGTCGTGGATCTGA-3'	150	60.89
	Reverse: 5'-TTGCTGTTGAAGTCGCAGGAG-3		61.14

calculated by summing up the length of the color-changed thread in each 30 s-measurement segments from 2 to 20 min following pilocarpine injection. The maximum tear volume value (peak tear secretion) was the longest length of the color-changed thread in each 30 s-measurement segment from 2 to 20 min following pilocarpine injection.

Histological analysis. The mice were euthanized under anesthesia after tear secretion was measured. The lacrimal glands were immediately removed and fixed in 10% formalin at 4˚C for at least 24 h. Lacrimal glands were then paraffin-embedded. Sections (5 μm thick) of the center and one quarter of the lacrimal gland were cut and stained with HE. The degree and extent of inflammation in the lacrimal gland were evaluated using the focus score and the ratio (%) of the inflammatory area to the total area, respectively. Focus was defined as infiltration of >50 mononuclear cells, and the focus score was defined as the focus number per 4 mm^2 area. The area ratio was measured and calculated using the Image Processing and Analysis in Java (ImageJ) software. Results are expressed as mean±standard deviation (SD) calculated from the mean values of sections per animal.

ELISA. The lacrimal glands were isolated from NOD mice at 6 weeks of age and immediately homogenized in ice-cold RIPA lysis buffer (50 mM Tris HCl, 150 mM sodium chloride, 0.5% w/v sodium deoxycholate, 0.1% w/v sodium dodecyl sulfate, 1.0% w/v NP 40 substitute) containing 1% (v/v) of protease inhibitor cocktail (aminoethyl ben-zylsulfonyl fluoride or pefabloc SC, aprotinin, bestatin, E-64, leupeptin, and pepstatin A), 1% (v/v) of phosphatase inhibitor cocktail 2 (sodium orthovanadate, sodium mo-lybdate, sodium tartrate, and imidazole), and 1% (v/v) of phosphatase inhibitor cock-tail 3 [cantharidin, (-)-P-bromolebamizole oxalate, and calyculin A]. The supernatant was collected and adjusted to the same protein concentration of 500 μg/ml. IL-1β and TNF-α in the lacrimal gland tissue supernatant were measured using DuoSet® Mouse IL-1β/IL-1F2 and DuoSet® Mouse TNF-α (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's protocol.

Real-time PCR. Mouse lacrimal glands were excised and immediately immersed in RNAlater™ Stabilization Solution (Thermo Fisher Scientific K.K., Tokyo, Japan), overnight at 4˚C, and stored at –20˚C until total RNA extraction. The lacrimal glands were homogenized with the TRIzol® Reagent (Thermo Fischer Scientific) to isolate total RNA from tissues. Reverse transcription was performed using 1 μg of extracted RNA with the ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan) according to the manufacturer's protocol. The reaction was incubated at 37˚C for 15 min and then terminated by incubation at 98˚C for 5 min. Real-time PCR was performed using the AriaMX real-time PCR system G8830A (Agilent Technologies, Santa Clara, CA, USA) to evaluate the gene expression of differentiation markers. The primer sequences of muscarinic acetylcholine receptor M3 (M3r; Chrm3), aquaporin-5 (Aqp5), Na-K-Cl cotransporter 1 (Nkcc1), transmembrane protein 16A (Tmem16a; Ano1), and Gapdh used are shown in Table II. Five μl of QPCR Master Mix (Brilliant III Ultra-Fast SYBR Green QPCR Master Mix; Agilent Technologies), 0.6 μl forward primer (100 nM), 0.6 μl reverse primer (100 nM), 2.8 μl nuclease-free water, and 1 μl cDNA were mixed in a 96-well plate (Inaoptica Corporation, Osaka, Japan). The reaction conditions included initial activation for 2 min at 95˚C, followed by 45 cycles of denaturation at 95˚C for 15 s and annealing/elongation at 60˚C for 30 s. After the reaction, the expression of each gene was calculated using Pfaffl's formula based on the obtained Ct values. The Ct value of Gapdh was used as an internal control.

Table III. *The amounts of polyphenols and proanthocyanidin in blueberry leaf hot water extract (BLEx).*

Polyphenol	Amount (mg/g-BLEx)
Proanthocyanidin	444.4
Chlorogenic acid	119.9
Rutin	20.9
Catechin	12.3
Epicatechin	5.6

Western blot analysis. The lacrimal glands were isolated from mice at 6 weeks of age, and immediately homogenized in ice-cold RIPA lysis buffer containing 1% (v/v) protease inhibitor cocktail, 1% (v/v) phosphatase inhibitor cocktail 2, and 1% (v/v) phosphatase inhibitor cocktail 3. The supernatant was collected and mixed with blue protein loading dye [62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% glycerol, 0.01% (w/v) bromophenol blue, and 1.25 M

Table IV. *Structural composition of blueberry leaf hot water extract (BLEx) calculated by thiolysis of proanthocyanidins.*

Sample	mDP*	Cinchonain I unit (%)	A-type bond (%)	B-type bond (%)
BLEx	7.70	13.4	3.9	82.7

*mDP: The mean degree of polymerization.

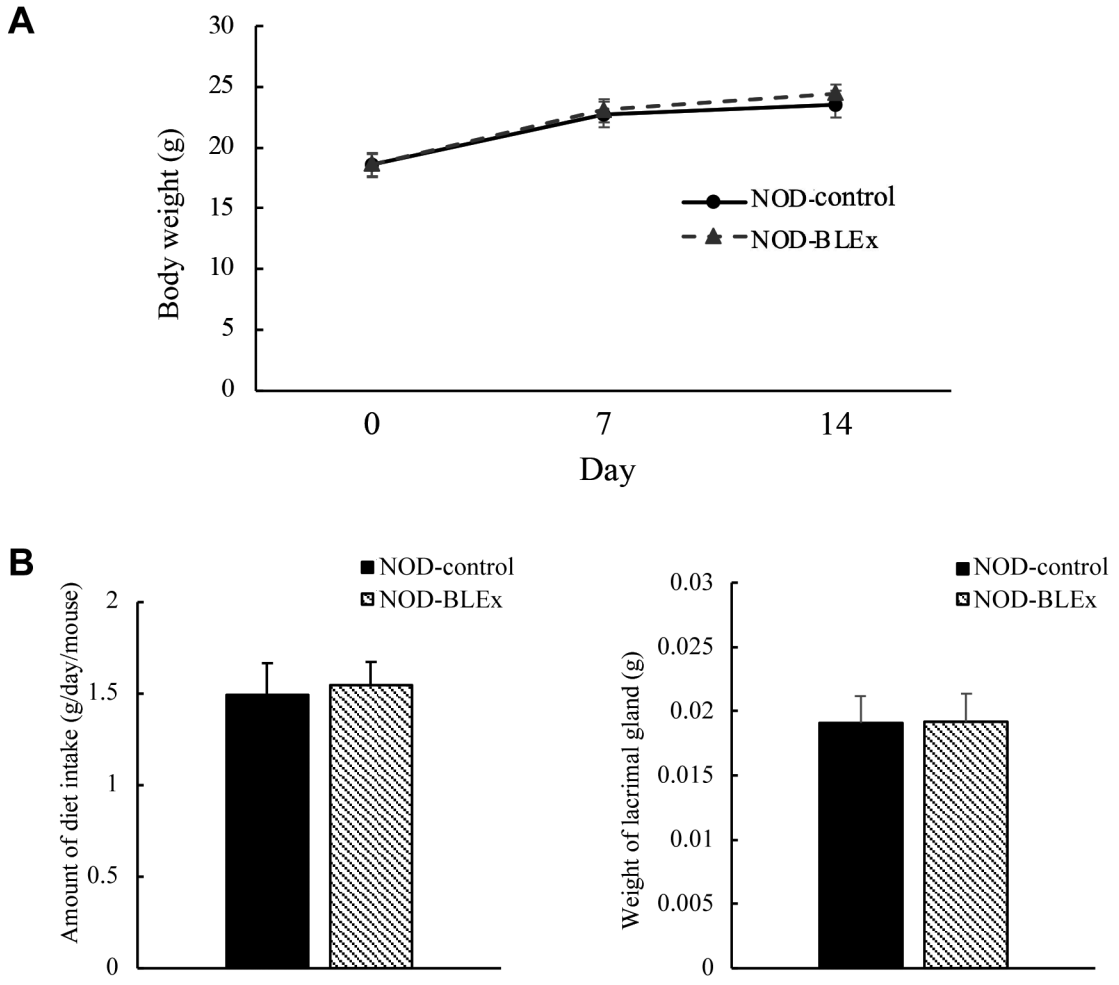

Figure 2. *Body weight change and amount of food intake, and lacrimal gland tissue weight of non-obese diabetic (NOD) mice. No significant difference in (A) body weight and (B) amount of diet intake for 2 weeks, and (C) weight of lacrimal gland at 6 weeks age of between NOD-control and NOD-BLEx mouse groups. Data are represented as mean±SD (n=8). BLEx: Blueberry leaf hot water extract; Control: the group of NOD mice fed with a control AIN-93G diet; BLEx: the group of NOD mice fed with an AIN-93G diet containing 1% BLEx.*

dithiothreitol], and the mixture was boiled at 100˚C for 5 min. Protein samples (4 μg) were separated by SDS-PAGE using an EasySeparator system (Fujifilm Wako Pure Chemical Co., Osaka, Japan). After electrophoresis, the separated proteins were transferred onto PVDF membranes using a Wet/Tank Blotting System (Bio-Rad Laboratories, Hercules, CA, USA). The blots were blocked at room temperature; 15-25˚C for 50-60 min in 3% of skim milk (Meiji Seika Pharma Co., Ltd, Tokyo, Japan) for arginase-1 and α-tubulin, and 30 min in Blocking One-P (Nacalai Tesque, Inc., Kyoto, Japan) for LC3. Then, the blots were probed with a primary antibody, anti-arginase 1 (diluted 1:1,000), anti-LC3 (diluted 1:1,000), or α-tubulin (diluted 1:2,000) at 4˚C overnight. The blots were washed three times with Tris-buffered saline (pH 7.6) containing 0.1% Tween 20. Subsequently, the blots were probed with an anti-rabbit IgG HRP-linked antibody (diluted 1:2,000) for 60-90 min. After washing the blots, immunoreactivity was determined using Clarity Max Western ECL Substrate detection reagents (Bio-Rad). Immunoblot images were acquired,

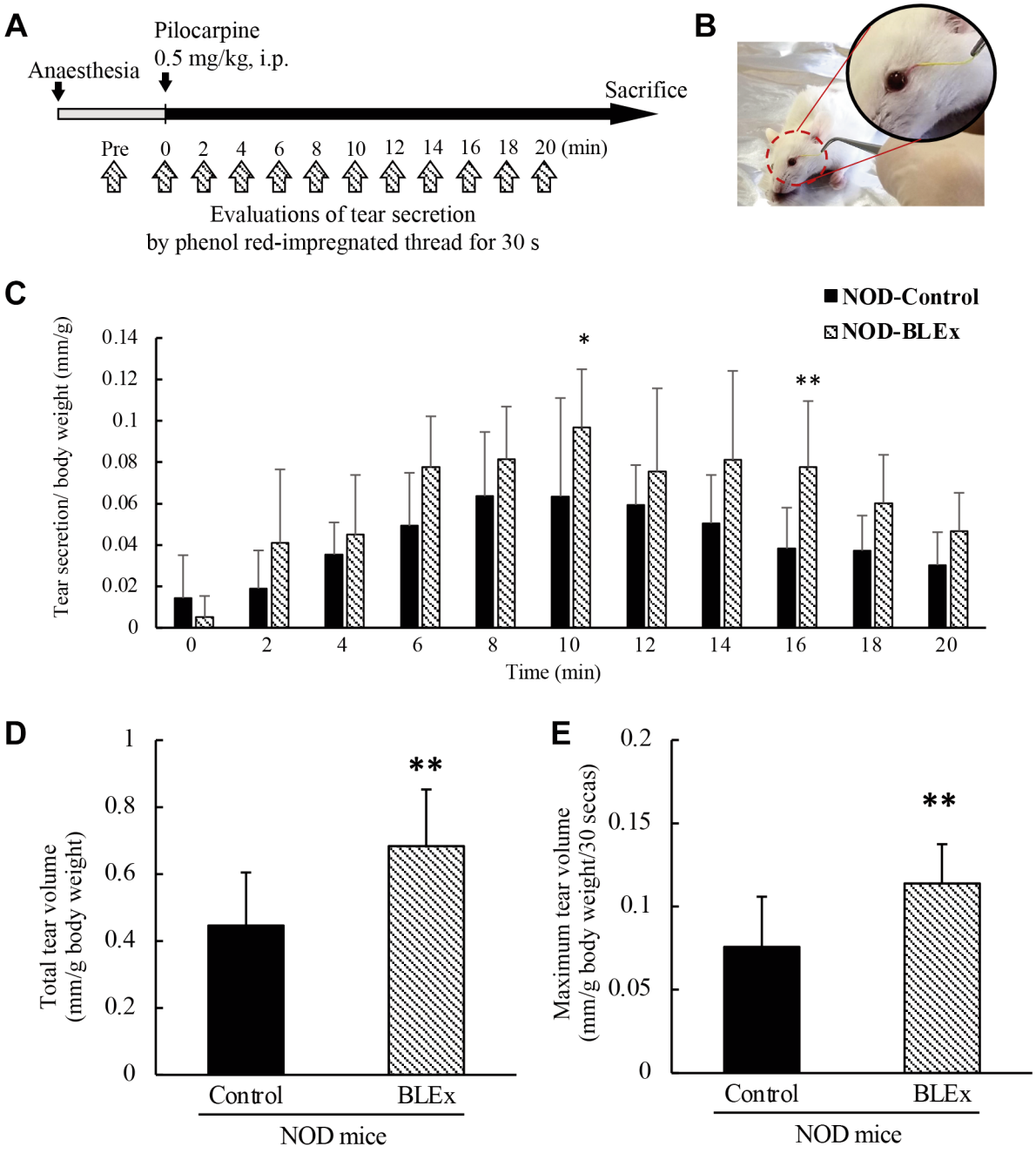

Figure 3. *Continued*

and the quantification of immunoblot bands was measured using the ImageQuant LAS 4 000 system (GE Healthcare, IL, USA).

Statistical analysis. Data are presented as the mean±SD. Statistical comparisons were made using one-way analysis of variance (ANOVA) followed by the Student's *t*-test and two-way ANOVA followed by Sidak's multiple comparisons test using GraphPad Prism ver. 7 (GraphPad Software, La Jolla, CA, USA). Differences were considered statistically significant at $p<0.05$.

Results

Polyphenol ingredients in blueberry leaf extract (BLEx). Of the ingredients found in BLEx, proanthocyanidins were analyzed using butanol-hydrochloric acid assay and chlorogenic acid, and rutin, catechin, and epicatechin were analyzed using high-performance liquid chromatography (HPLC). BLEx used in this study contained 444.4 mg/g of

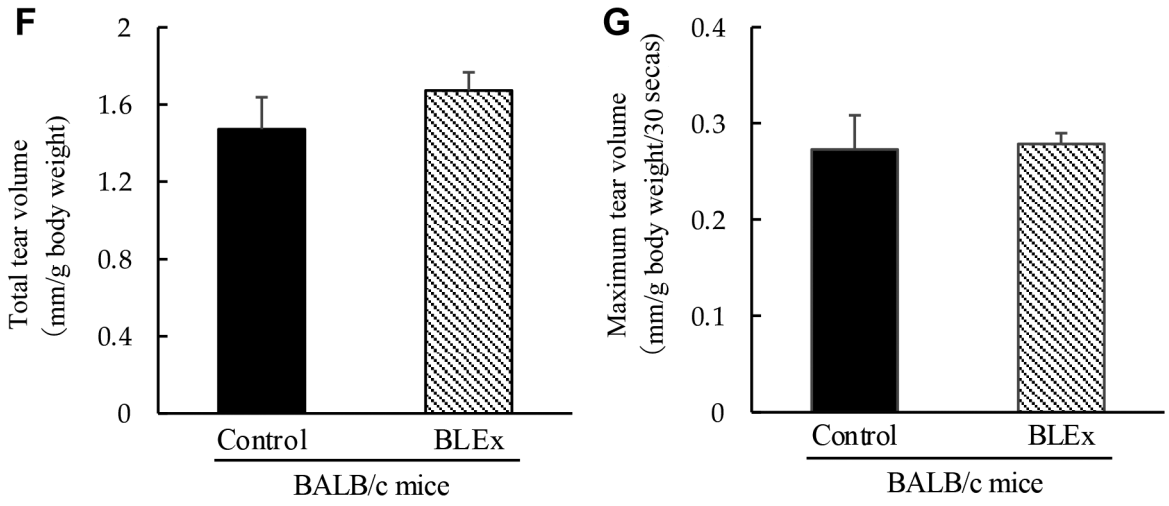

Figure 3. *Restorative effect of blueberry leaf hot water extract (BLEx) on pilocarpine-induced tear secretion in 6-week-old non-obese diabetic (NOD) mice. (A) Schematic representation of the experiment to measure pilocarpine-induced tear secretion in mice. (B) The measurement of tear secretion as the length of color-changed (to red) phenol red-impregnated thread due to the absorption of tear fluid from the left eye of NOD mice. (C) Tear volumes adjusted by body weight in NOD mice at each time point following pilocarpine intraperitoneal injection. (D) Total volume of tear secretion for 20 min, and (E) maximum tear volume in 30 s during a 20 min measurement period in NOD mice. (F) Total volume of tear secretion for 20 min, and (G) maximum tear volume in 30 s during a 20 min measurement period in BALB/c mice. Data are represented as mean±SD (n=10 or 12 in NOD mice; n=6 in BALB/c mice). *p<0.05, **p<0.01 compared with the control group. Control, group of NOD or BALB/c mice with a regular intake of AIN-93G diet; BLEx, group of NOD or BALB/c mice with a regular intake of AIN-93G diet containing 1% BLEx.*

proanthocyanidin, 119.9 mg/g of chlorogenic acid, 20.9 mg/g of rutin, 12.3 mg/g of catechin, and 5.6 mg/g of epicatechin (Table III). The average degree of polymerization of proanthocyanidins analyzed by the thiolysis LC/UV method is shown in Table IV. BLEx contain highly polymerized proanthocyanidins, with an average degree of polymerization of 7.70, and composed of 13.4%, 3.9%, and 82.7% of cinchonain I unit, A-type bond, and B-type bond structures.

The effect of BLEx on tear secretion in NOD mice. No differences in body weight gain were observed over a 14-day period between the NOD-control and NOD-BLEx groups (Figure 2). To evaluate tear secretion volume, pilocarpine was injected into NOD mice 2-weeks after feeding of the BLEx contained- or control-pellets, and tear volume was measured (Figure 3A and B). Tear secretion was increased in a time-dependent manner up to 10 min following pilocarpine injection in both groups. BLEx-fed NOD mice showed significantly higher tear secretion than the control group mice at 10 and 16 min (Figure 3C). By comparing the cumulative and maximum secretion between the control and BLEx groups over a time period of 2-20 min, it was found that BLEx increased both total and maximum tear volume in NOD mice (Figure 3D and E). In contrast, BLEx did not further increase tear secretion in BALB/c mice without lacrimal hyposecretion (Figure 3F and G). These results suggest that BLEx prevented lacrimal hyposecretion in NOD mice.

The effect of BLEx on infiltration and inflammatory cytokines of lacrimal glands in NOD mice. The lacrimal gland tissue sections were stained with hematoxylin and eosin (HE) to evaluate the effect of BLEx on lymphocyte infiltration into the lacrimal gland of NOD mice at 6 weeks of age. The infiltration of mononuclear leukocytes, mostly lymphocytes, was observed in both NOD-control and NOD-BLEx mice (Figure 4A). We then measured the focus score (the number of mononuclear cell infiltrates containing >50 cells per 4 mm^2 area) and the ratio (%) of inflammatory area to the total area. Neither the focus score nor the inflammatory area ratio was altered by BLEx (Figure 4B and C).

We then used ELISA to evaluate the inflammatory cytokines IL-1β and TNF-α, which were reported to increase early in the lacrimal glands of NOD mice (8). As shown in Figure 4D, IL-1β level was not significantly altered in the lacrimal glands of NOD-BLEx mice compared to that in the NOD-control mice. However, the expression of TNF-α was significantly decreased in the lacrimal gland of the NOD-BLEx mice group compared to that of the NOD-control group (Figure 4E). These results suggest that BLEx did not inhibit lymphocyte infiltration into the lacrimal gland, although it slightly reduced the release of the proinflammatory cytokine TNF-α in the lacrimal gland of NOD mice.

mRNA expression of genes associated with tear secretion in NOD mice fed BLEx. To investigate the expression levels

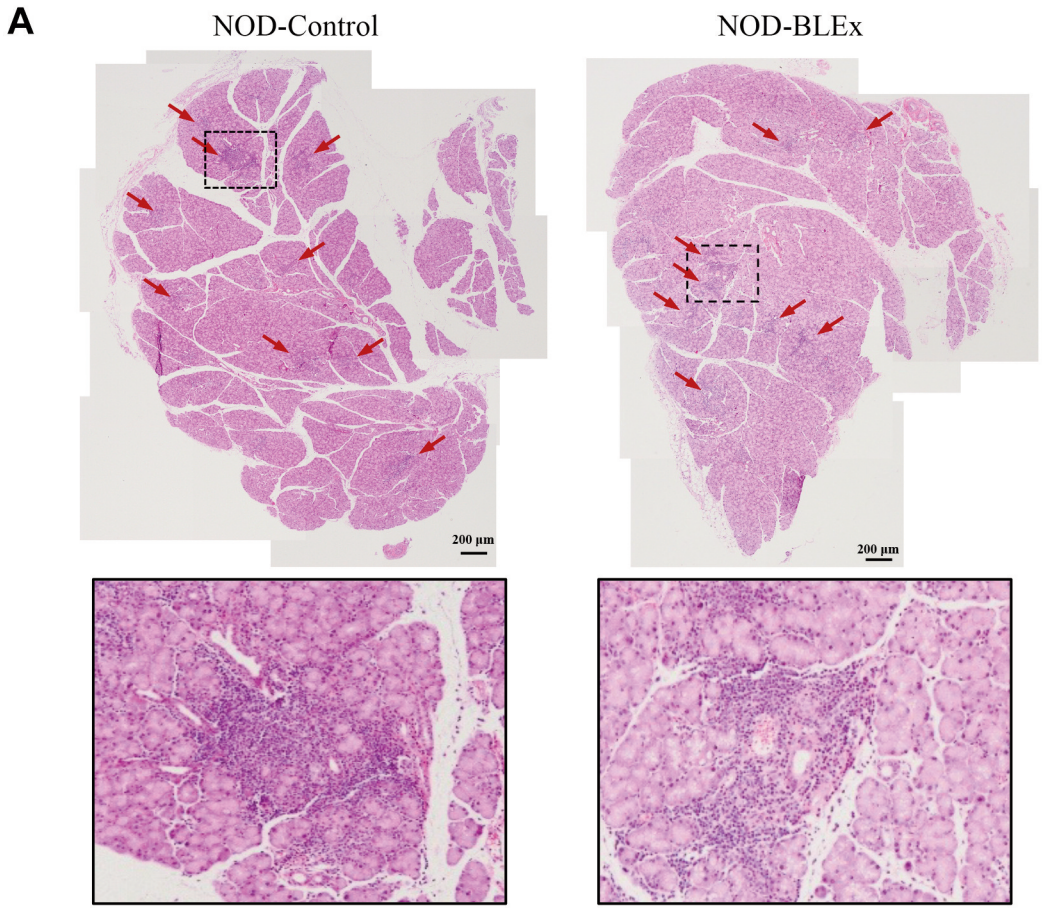

Figure 4. *Continued*

of tear secretion-related mRNA, we analyzed the expression of M3r, Aqp5, Nkcc1, and Tmem16a mRNA using real-time PCR. There are no reports on the expression of these genes in the lacrimal gland of SS-like NOD mice. There was no significant difference in the expression of these genes between the NOD-control and NOD-BLEx mouse groups. These results suggest that BLEx did not alter the expression levels of major tear secretion-related genes (Figure 5).

The expression of arginase-1 in the lacrimal gland in NOD mice fed with BLEx. Arginase-1 was recently reported to be involved in lacrimal hyposecretion in male NOD mice (7). The protein expression level of arginase 1 was investigated using western blotting (Figure 6A). In the lacrimal gland of the NOD-BLEx group mice, the expression level of arginase 1 was increased in some individuals, but did not change in others, resulting in no significant change compared to the control group mice (Figure 6B). These results suggest that BLEx did not affect the expression level of arginase 1 in NOD mice.

The effect of BLEx on autophagy in the lacrimal gland of NOD mice. To investigate the involvement of autophagy in lacrimal hyposecretion in NOD mice, LC3 II/I expression was measured using western blotting (Figure 7). The LC3 II/I ratio indicates the formation rate of autophagosomes. LC3 activation was significantly suppressed in the lacrimal glands of BLEx-treated NOD mice compared to that in the control NOD mice. These results indicate that BLEx may have inhibited autophagy activation in the lacrimal glands of NOD mice.

Discussion

In this study, we examined the ameliorative effects of a continuous oral intake of BLEx on dry eye disease in the SS-like model of NOD mice and found that the BLEx prevented the lacrimal hyposecretion. The TNF-α level in the lacrimal gland was decreased, though BLEx did not alter the severity of lymphocyte infiltration into the lacrimal glands. Furthermore, we revealed BLEx might suppress autophagy activity.

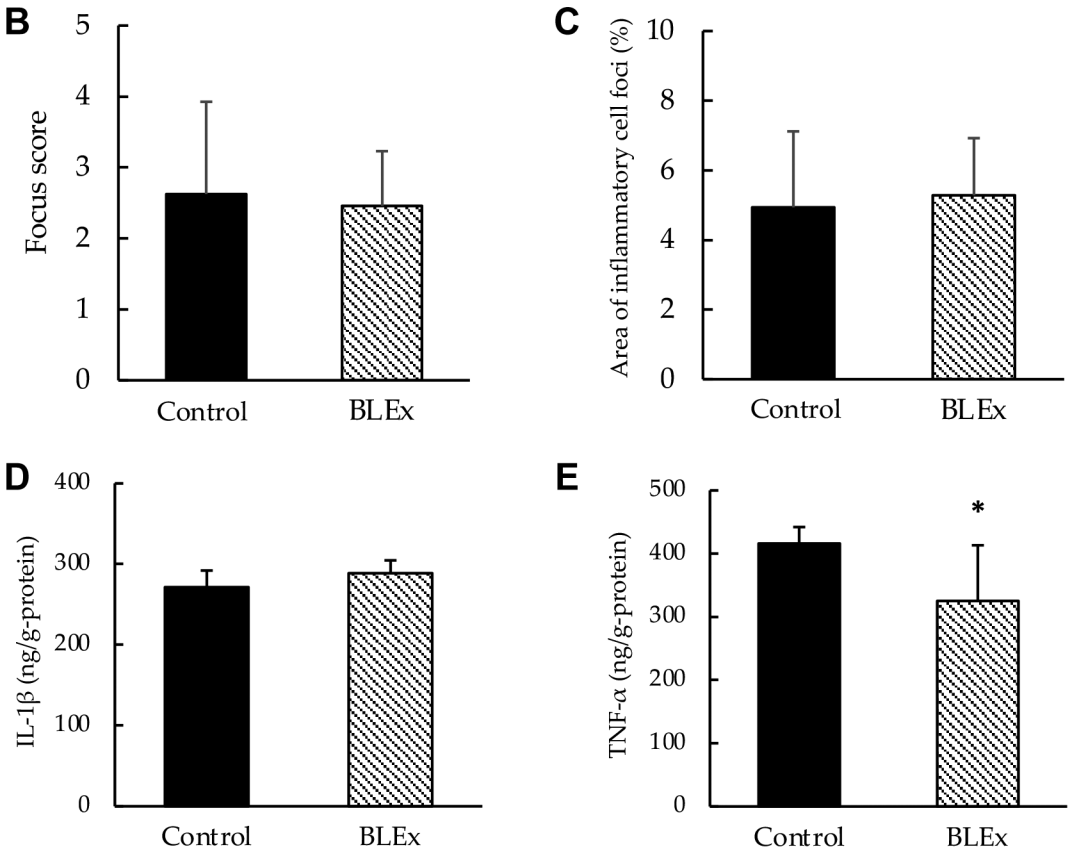

Figure 4. *The histological analysis and inflammatory cytokines on the lacrimal gland of non-obese diabetic (NOD) mice fed with diets containing blueberry leaf extract. (A) Representative cross-sections of hematoxylin and eosin (HE)-stained lacrimal glands from NOD mice fed with regular diets and those fed with a diet containing 1% blueberry leaf extract for 2 weeks, at 6 weeks of age. Red arrows indicate the inflammatory area showing infiltration of mononuclear leukocytes. Scale bars represent 200 μm. Lower panels are representative images of the "focus" showing infiltration of >50 mononuclear cells. (B) Focus score and (C) the ratio of inflammatory area (%) in lacrimal gland HE sections of NOD mice. (D) IL-1β and (E) TNF-α as inflammatory cytokines in the lacrimal gland of NOD mice measured by ELISA. Data are represented as mean±SD (n=6, 8). BLEx: Blueberry leaf hot water extract; Control: the group of NOD mice fed with a control AIN-93G diet; BLEx: the group of NOD mice fed with an AIN-93G diet containing 1% BLEx.*

BLEx prevented the lacrimal hyposecretion in NOD mice, since it increased tear secretion in NOD mice with hyposecretion (Figure 3C-E) but did not further increase tear secretion in BALB/c mice with normal secretion (Figure 3F and G). On the other hand, contrary to our expectation, there was no ameliorative effect of BLEx on the lymphocyte infiltration (Figure 4A-C). Thus, BLEx did not inhibit the destruction of lacrimal gland tissue by lymphocyte infiltration itself. There is similar previous report that administration of resveratrol in NOD mice leads to improvement in saliva secretion without inhibiting infiltration into the salivary gland (22), indicating our result is possible.

Our ELISA analysis showed BLEx reduced the inflammatory cytokine TNF-α in the lacrimal glands of NOD mice (Figure 4E). TNF-α has been reported to increase in SS and induce apoptosis in human salivary gland ductal cells (25). In the LPS-stimulated inflammatory response to macrophage RAW 264.7, BLEx has shown immunomodulatory effects by suppressing TNF-α production (26). Moreover, the polyphenolic components in BLEx, such as proanthocyanidins, rutin, chlorogenic acid, and catechins, have been reported to have anti-inflammatory effects *via* suppression of TNF-α production (12, 14, 15, 19-21). Therefore, it is speculated that the up-regulated TNF-α might negatively affect the lacrimal secretion (such as apoptosis in lacrimal gland cells) besides from inducing inflammation. In other words, BLEx may possibly decrease the negative non-inflammatory effect of TNF-α and prevent lacrimal hyposecretion in NOD mice. In addition, since the suppression of TNF-α production ultimately leads to suppression of the promotion of immune cell infiltration, longer-term of BLEx administration may reduce the infiltration in NOD mice. Further investigation is warranted

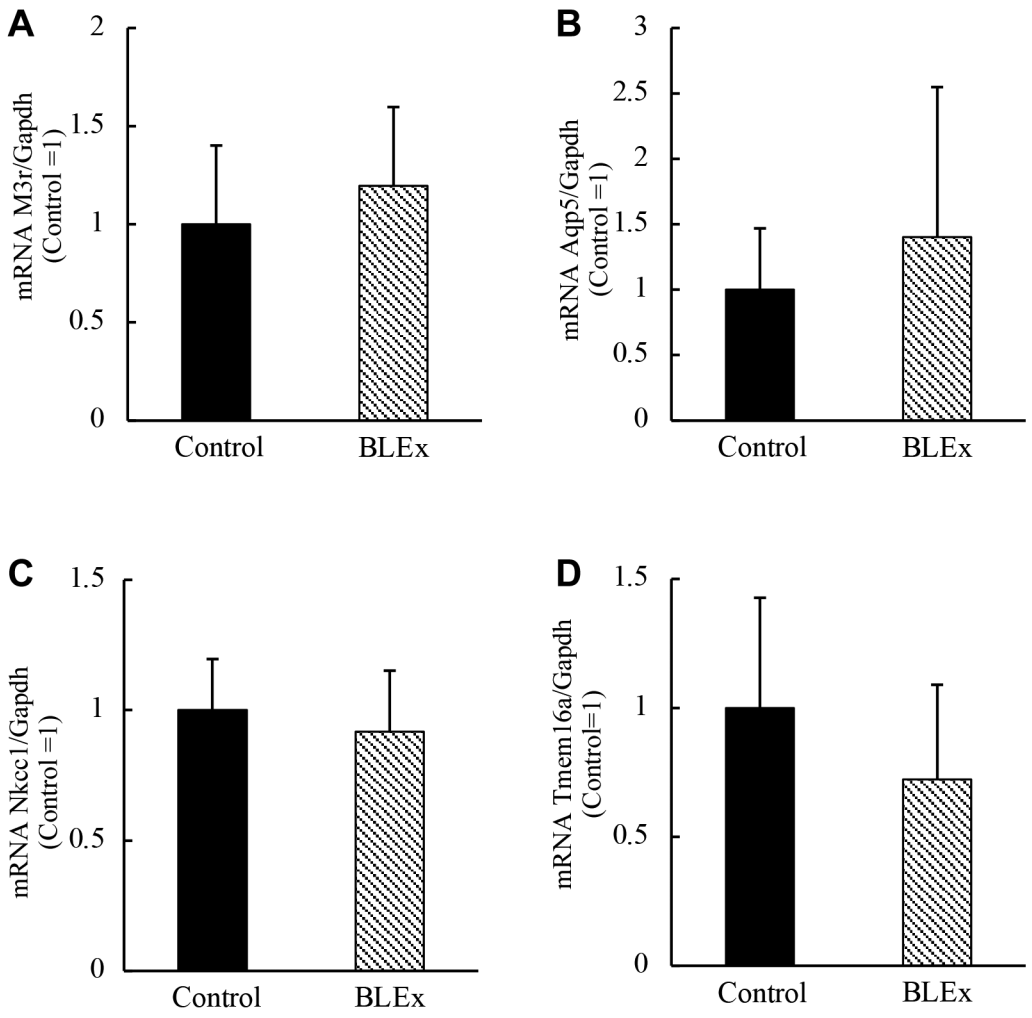

Figure 5. *mRNA expression of genes associated with tear secretion in the lacrimal gland of non-obese diabetic (NOD) mice at 6 weeks of age. (A) Muscarinic acetylcholine receptor M3 (M3r), (B) aquaporin 5 (Aqp5), (C) Na-K-Cl cotransporter 1 (Nkcc1), and (D) anoc-tamin-1 (Tmem16a) mRNA adjusted by glyceraldehyde-3-phsphate dehydrogenase (Gapdh) by real-time PCR analysis. Data are represented as mean±SD (n=8) and compared with the control. BLEx: Blueberry leaf hot water extract; Control: the group of NOD mice fed with a normal AIN-93G diet; BLEx: the group of NOD mice fed with an AIN-93G diet containing 1% BLEx.*

to determine whether infiltration and apoptosis in the lacrimal gland are suppressed by a more long-term BLEx treatment *via* inhibition of inflammatory cytokines.

The mechanism of exocrine secretion by pilocarpine hydrochloride is that pilocarpine acts on M3R and stimulates water secretion mainly from AQP5 *via* Ca^{2+} ions (27-29). We measured the expression levels of M3r and Aqp5 mRNA in the lacrimal gland of NOD mice using real-time PCR but found that the expression levels were not affected by BLEx treatment (Figure 5). Furthermore, AQP5 showed no difference in protein expression as determined by western blot analysis (data not shown). It has been reported that anti-M3R antibodies (anti-M3 muscarinic acetylcholine receptor antibodies), which antagonize M3R, increase in SS patients and NOD mice, and anti-M3R antibodies inhibit AQP5 membranous localization of acinar cells for water secretion in NOD mice (30). However, it remains a challenge to examine the effects of BLEx on the localization of AQP5 in lacrimal gland tissues. In addition, other Na^+ and Cl^- ion channels, such as NKCC1 and TMEM16A, are involved in the induction of aqueous humor secretion of salivary glands (31, 32). In the present study, we examined the effects of BLEx administration in NOD mice using real-time PCR and found no effect on the expression levels (Figure 5). Therefore, it is unlikely that BLEx affects the expression of ion channels in lacrimal gland acinar cells. As Ca^{2+} ions are essential for the exocrine secretion of glandular acinar cells, detecting Ca^{2+} mobilization in lacrimal gland tissues is also

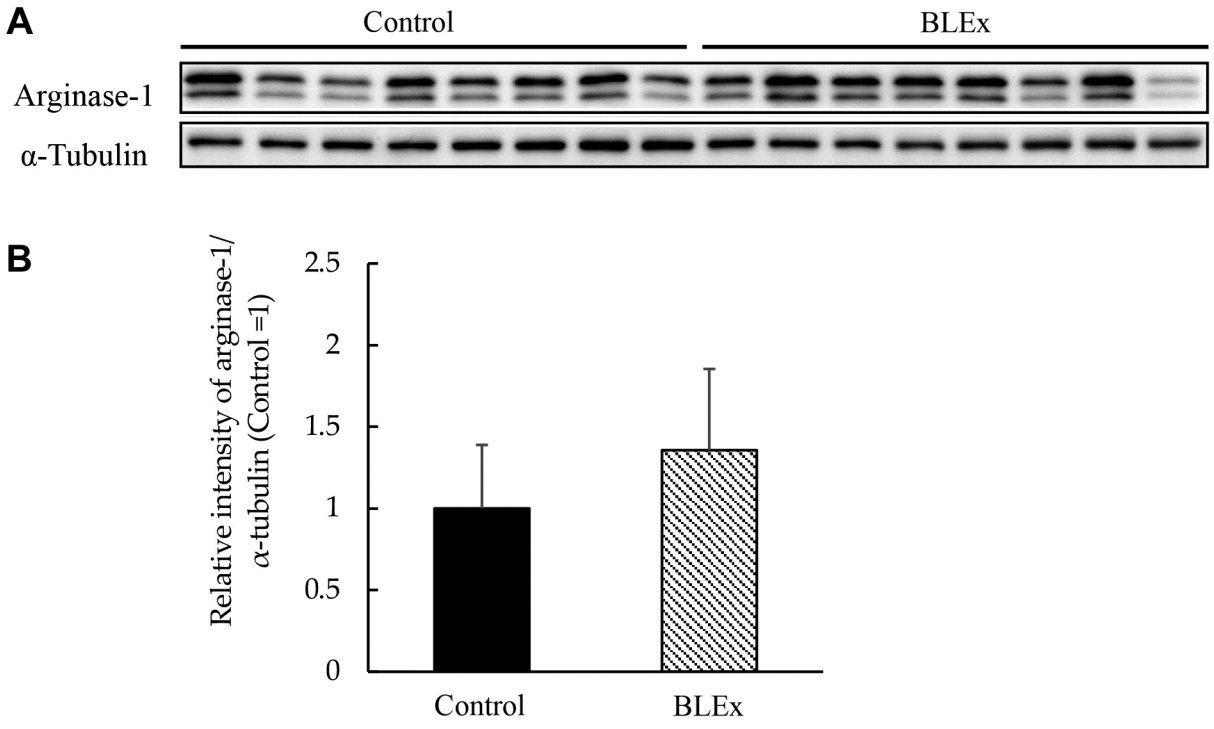

Figure 6. *Arginase-1 protein expression by western blot analysis on the lacrimal gland in non-obese diabetic (NOD) mice at 6 weeks of age. (A) Arginase-1 and α-tubulin bands by western blot and (B) quantification graphs of arginase-1 adjusted by α-tubulin. Data are represented as mean±SD (n=8) and compared with control groups. BLEx: Blueberry leaf hot water extract; Control: the group of NOD mice fed with a normal AIN-93G diet; BLEx: the group of NOD mice fed with an AIN-93G diet containing 1% BLEx.*

an issue to be addressed, as there are other membrane proteins involved in Ca^{2+} ion release.

As a non-inflammatory mechanism, arginase-1 expression is decreased from 6 weeks of age in male NOD mice, which affects the decrease in lacrimal secretion separately from the progression of inflammation (7). Arginase-1 is involved in ATP synthesis under aerobic conditions, and ATP is important for Na^{+}-K^{+} ATPase to drive ion transport indirectly, which induces primary tear secretion in acinar cells (33). In this study, we investigated the effect of BLEx administration on the expression of arginase-1 in NOD mice at 6 weeks of age and found that BLEx administration increased the expression of arginase-1 in some lacrimal glands of NOD mice, although not significantly overall (Figure 6). A previous report suggested that rutin increased the expression of arginase-1 mRNA in adipocytes of high-fat-fed mice (34). Although rutin in BLEx treatment might promote the increase in arginase-1 expression in the lacrimal gland of NOD mice, it was not significantly up-regulated at the dose used in this study. Thus, it was suggested that arginase-1 was not involved in the increase in tear secretion volume of NOD mice administered with BLEx, suggesting that the suppression of TNF-α and autophagy by BLEx may contribute more to the increase in tear secretion.

The role of autophagy in SS is still known to be limited. The activation of autophagy, indicated by increased expression of LC3 and autophagy related gene 5 (ATG5), has been found in the lacrimal fluid of patients with SS and the lacrimal tissue of NOD mice, and involves a decrease in lacrimal secretory function (35). In addition, the administration of chloroquine, an autophagy inhibitor, in NOD mice, improved tear secretion *via* suppression of autophagy activation through inflammation (36). In our experiment, autophagy activation induced by LC3 II/I upregulation was observed in the lacrimal gland tissues of NOD mice, and it was significantly suppressed by BLEx administration (Figure 7B). TNF-α is known to be one of the causes of autophagy activation and TNF-α is able to induce autophagy in different cell types (37, 38). Additionally, TNF-α inhibitors seem able to modulate the autophagy pathway in rheumatoid arthritis as a systemic autoimmune disease (39). In the present study, the suppression of both the autophagy-related protein LC3 II/I and TNF-α were simultaneously observed in the lacrimal gland of NOD mice administered with BLEx. Thus, BLEx may have contributed to autophagy regulation *via* suppression of TNF-α production. In BALB/c mice, without pathology, neither activation nor inhibition of autophagy was detected (data not

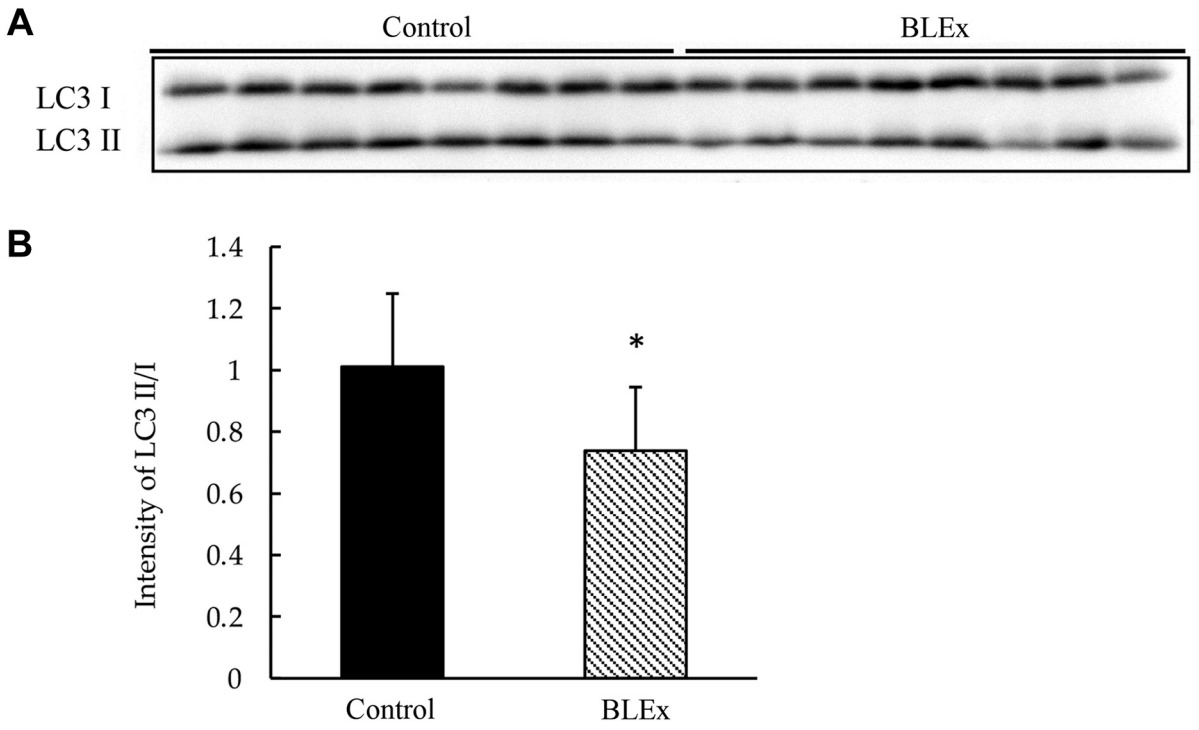

Figure 7. *LC3 I and II protein expression by western blot analysis on the lacrimal gland in mice at 6 weeks of age. (A) LC3 I and LC3 II bands by western blot and (B) intensity of LC3 II/I on the lacrimal gland in non-obese diabetic (NOD) mice. Data are represented as mean±SD (n=8). *p<0.05 compared with the control. BLEx: Blueberry leaf hot water extract; Control: the group of NOD mice fed with a control AIN-93G diet; BLEx: the group of NOD mice fed with an AIN-93G diet containing 1% BLEx.*

shown). Previous study has shown that chlorogenic acid, an ingredient in BLEx, has an inhibitory effect on autophagy (40). The inhibition of autophagy activation by BLEx administration might be related to the inhibition of lacrimal hyposecretion in NOD mice. Although it is necessary to investigate other factors related to autophagy, such as autophagy related genes (ATGs), in the future, the present study suggests that inhibition of autophagy activation by BLEx administration may be involved in the suppression of lacrimal gland tissue deterioration and the maintenance of lacrimal secretion.

The BLEx used in this study had a high polymerization degree of proanthocyanidins with an average of 7.70 (Table IV), and its molecular structure is assumed to be as shown in Figure 1. As for the disposition of highly polymerized proanthocyanidins in the body, only a small percentage is absorbed from the small intestine. It is known that highly polymerized proanthocyanidins from apples are degraded by intestinal bacteria, absorbed from the colon as monomeric or dimeric catechins, proanthocyanidins, and other degradation products, and transferred to the bloodstream (41, 42). In the future, it will be necessary to further investigate the pharmacokinetics of BLEx administration to determine which metabolites are transferred from the blood to the lacrimal gland, thus affecting the immune response and lacrimal secretion.

Conclusion

In summary, we found that BLEx prevented the lacrimal hyposecretion in Sjögren's syndrome-like model of NOD mice at 6 weeks of age. The mechanism of BLEx in improving lacrimal secretory function might be inhibition of autophagy activation *via* the reduction in cytokine TNF-α. In the future, it will be necessary to investigate the mechanism of action in more detail. Blueberry leaves are foods that can be consumed daily, and we suggest that their intake may be useful in relieving the symptoms of lacrimal hyposecretion associated with Sjögren's syndrome.

Conflicts of Interest

K. Ogawa received a research grant from Biolabo Co., Ltd.; YG and TN are employees of Biolabo Co., Ltd.

Author's Contributions

KO designed the study and drafted this paper. KU, SM, YY, YS, and YK performed the tests and analyzed the data obtained. YO, KSa,

KSu, and MK supported the design of the study. YG and TN provided the blueberry leaf extract. HK, MY, and KN supervised the execution of the study. All Authors contributed to the manuscript preparation and approved of the final paper.

Acknowledgements

The Authors thank Hiroaki Kataoka and Junko Kurogi at Department of Pathology, Faculty of Medicine, University of Miyazaki for technical guidance on histological evaluation. The Authors would like to thank Editage (www.editage.com) for English language editing.

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Received November 1, 2022
Revised November 8, 2022
Accepted November 9, 2022