Original Article

Effects of polyphenols in non-centrifugal cane sugar on saliva secretion: *in vitro* and *in vivo* experiments and a randomized controlled trial

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This study examined the bioactivities and mechanisms of the non-centrifugal cane sugar polyphenols saponarin, schaftoside, and isoschaftoside in the salivary gland and their effects on salivation. In acute isolated C57BL/6N mouse submandibular gland cells, these polyphenols led to a higher increase in intracellular calcium after stimulation with the muscarinic agonist carbachol. Stimulation of these cells with polyphenols enhanced ATP production, aquaporin-5 translocation to the plasma membrane and eliminated intracellular reactive oxygen species generated by H_2O_2. In addition, phosphorylation of endothelial nitric oxide synthase and increased nitric oxide production in vascular endothelial cells were observed. *In vivo* administration of these polyphenols to C57BL/6N male mice resulted in significantly increased blood flow (saponarin, $p = 0.040$; isoschaftoside, $p = 0.010$) and salivation (saponarin, $p = 0.031$). A randomized controlled trial showed that intake of non-centrifugal cane sugar significantly increased saliva secretion compared with placebo ($p = 0.003$). These data suggest that non-centrifugal cane sugar polyphenols affect several pathways that support salivation and increase saliva secretion by enhancing vasodilation. Hence, non-centrifugal cane sugar polyphenols can be expected to maintain saliva secretion and improve reduced saliva flow.

***Key Words*: polyphenols, non-centrifugal cane sugar, salivation, randomized controlled trial**

Japan is known for being the country with the longest average life expectancy.[1] In particular, between the mid-1970s and early 2000s, the Japanese prefecture Okinawa gained worldwide attention as one of the Blue Zones® (regions with the highest numbers of centenarians).[1,2] People living in Okinawa traditionally eat food that is low in calories, high in dietary fibers, and rich in bioactive compounds such as polyphenols.[1] This unique food culture is assumed to explain the longevity of people living in Okinawa, and it has been shown also to decrease the incidence of heart disease and death due to cancer.[3]

Among the numerous beneficial components of the Okinawan food, we focused on non-centrifugal cane sugar (NCS), commonly known as brown sugar, or "Kokuto" in Japanese. NCS is richer in minerals, vitamins, and polyphenols than refined sugar.[4,5] Furthermore, it contains compounds that reduce oxidative stress and have antitoxic, cytoprotective, and anticarcinogenic effects.[4] Several studies revealed a preventive effect of NCS on neurodegenerative and cardiovascular diseases, diabetes, obesity, and anemia.[4,5] In addition, NCS manufactured in Okinawa contains more polyphenols, i.e., saponarin, schaftoside, and isoschaftoside, than other brown sugars.[6]

Polyphenols are naturally occurring bioactive compounds found abundantly in plants and fruit-based diets that contain one or more aromatic rings and two or more hydroxyl groups.[7] They have several health benefits, including anti-inflammatory and antioxidant effects against reactive oxidant species (ROS) scavenging activity.[8] Thus, interest in polyphenols has surged in pharmacology and medicine.[9]

Excess ROS production causes oxidative damage to cells and severely affects homeostasis. An imbalance in ROS generation can induce oxidative damage, which is responsible for several diseases, including dysfunction of saliva secretion.[4,5,9]

Saliva is essential to maintain a healthy oral environment. However, several diseases and conditions, e.g., diabetes, Sjogren's syndrome (SS), administration of medications with adverse effects on saliva production, psychological stress, and radiation therapy, can reduce saliva production.[10,11] In turn, decreased saliva secretion may increase the risk of various systemic and infectious diseases, such as aspiration pneumonia.[11,12] Hyposalivation also affects daily activities and food intake and can severely compromise quality of life.[12,13]

Previously, we investigated the effects of plant-derived polyphenols on salivary secretion and reported that isoflavone improved secretion and subjective symptoms related to impaired secretion in elderly women.[14] Also, quercetin prevented radiation-induced salivary dysfunction in a mouse model through its anti-inflammatory and antioxidative effects and enhanced expression of the water channel protein aquaporin 5 (AQP5).[15] Furthermore, resveratrol exerted anti-inflammatory effects in non-obese diabetic (NOD) mice (a model of Sjogren's syndrome, salivary gland dysfunction caused by an inflammatory autoimmune disease) through increased expression of sirtuin 1, and these effects prevented the reduction of saliva secretion.[16] Triptolide, a bioactive compound, has also been reported to be a potential treatment for Sjogren's syndrome because in NOD mice it decreased the expression of inflammatory factors by inhibiting an activator of the JAK/STAT and NF-κB pathways.[17]

In the present study, we performed *in vitro*, *in vivo* studies in mice and a randomized controlled trial (RCT) to investigate the effects of polyphenols abundant in NCS on salivary secretion and their mechanisms of effect.

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Materials and Methods

Animals. The experiments in mice were approved by the Tsurumi University Animal Experiment Committee (approval number: 20A020) and performed according to the related guidelines, laws, and regulations, including national guidelines for animal usage in research. The study is reported in accordance with the ARRIVE guidelines (https://arriveguidelines.org).[18] C57BL/6N male mice aged 6- to 12-weeks (Clea Japan Inc., Tokyo, Japan) were reared in a husbandry facility with controlled temperature and humidity under a 12-h light cycle. The mice had free access to feed and water. Simple but not blinded randomization was used to allocate animals to groups.

Cell cultures. A normal salivary gland SV40-transformed acinar cell line (NS-SV-AC) was kindly provided by Prof. M. Azuma, Tokushima University, Tokushima, Japan. Cells were cultured in keratinocyte-SFM growth medium (Gibco, Carlsbad, CA) with the provided supplements.[19]

Human umbilical vein endothelial cells (HUVECs) purchased from PromoCell (Heidelberg, Germany) were cultured in KBM VEC-1 growth media (Kohjin Bio Co., Ltd., Saitama, Japan) with vascular endothelial growth factor (VEGF) supplements and serum.

All the cultures were maintained at 37°C in a humidified atmosphere containing 95% air and 5% CO_2.

Acute isolation of mouse salivary gland cells. Mouse submandibular glands (mSMG) were excised from 6- to 12-weeks old male mice weighing 20 to 25 g. mSMG tissues were washed, dissected, and minced into 0.5-mm squares. Balanced salt solution [BSS; 20 mM HEPES, 150 mM NaCl, 5.4 mM KCl (pH 7.4)] supplemented with 0.18% D-glucose, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.5% bovine serum albumin (BSA) to prepare 0.5% BSA/BSS. Minced mSMG was suspended in 0.5% BSA/BSS with 3.2 mg/ml collagenase II (Worthington Biochemical Corp., Lakewood, NJ), 1.14 mg/ml hyaluronidase (Sigma Aldrich, St. Louis, MO), and 1 U/ml DNase I (Roche Diagnostics GmbH, Mannheim, Germany). The suspension was incubated for 20 min at 37°C in a water bath, filtered through a 200-µm nylon mesh, and centrifuged to collect the mSMG cells. The cells were resuspended in 0.5% BSA/BSS and kept on ice until use.

Cell viability and proliferation assay. The polyphenols saponarin, schaftoside, and isoschaftoside were purchased from Extrasynthese (1238S, 1369, 1354, Genay Cedex, France), and dimethyl sulfoxide (DMSO) was purchased from Sigma Aldrich. NS-SV-AC cells were cultured on a 100-mm culture dish. The cells were stimulated with 1, 10, and 100 µM of three different polyphenols and DMSO as the vehicle control for 48 h. A trypan blue exclusion test was performed to evaluate cytotoxicity of polyphenols.

To examine cell viability, NS-SV-AC cells were seeded onto 6-well plates at a density of 8.0×10^5 cells/well and stimulated with the previously mentioned three concentrations of polyphenols for 48 h. The cell proliferation assay was performed with the Cell Count Reagent SF (Nacalai Tesque Inc., Kyoto, Japan) according to the manufacturer's recommended procedure. The absorbance of the water-soluble formazan produced in proportion to the number of viable cells was measured with a plate reader (Wallac 1420 ARVO SX; PerkinElmer, Boston, MA).

***In vitro* experiments.**

Measurement of intracellular calcium. Fura 2-AM (Dojindo Laboratories, Kumamoto, Japan) loaded acute isolated mSMG cells were treated with 50 µM of the three different polyphenols for 30 min and washed twice with 0.5% BSA/BSS. Cells were stimulated with the muscarinic acetylcholine receptor agonist carbachol (30 µM), and intracellular calcium ions ($[Ca^{2+}]i$) were determined by measuring fluorescence intensities with a plate reader (Wallac 1420 ARVOSX); a dual excitation wavelength of 340 and 380 nm was used and an emission wavelength of 510 nm. The fluorescence ratio (F340/F380) was recorded every second for 120 s, and the fold change from baseline was plotted. The tests were conducted in the presence or absence of 1 mM H_2O_2.

ATP production. A suspension of acutely isolated mSMG cells with 1×10^6 cells/ml was added to 1.5-ml tubes. The cell suspensions were stimulated with 50 µM of the three polyphenols and the vehicle control for 30 min. An equivalent mixture of cell suspension and 100 µl of Cellno ATP assay reagent (TOYO B-Net Co., Ltd., Tokyo, Japan) was transferred into a 96-well clear-bottomed white plate. The luminescence intensity associated with ATP was measured with a plate reader (Wallac 1420 ARVOSX).

Immunohistochemistry. Mice SMG tissue was cut into 200-µm thick slices with a tissue slicer (DSK, linear Slicer, PRO7; Dosaka EM Co., Ltd., Kyoto, Japan). The sliced tissues were kept in a 6-well culture dish with DMEM containing 10% FBS and 1% penicillin/streptomycin and stimulated with 50 µM of saponarin, schaftoside, and isoschaftoside and vehicle control for 10 min. After stimulation, tissues were fixed with 4% paraformaldehyde, and paraffin-embedded tissue sections were prepared. Immunohistochemistry was performed with mouse anti-E-cadherin (ab53033, 1:200 dilution; Abcam, Cambridge, UK) and rabbit anti-AQP5 antibody (ab15858, 1:200 dilution; Millipore Co., Ltd., Darmstadt, Germany) as primary antibodies and TRITC conjugated mouse anti-rabbit IgG (1:200 dilution; Santa Cruz Biotechnologies, CA) and AlexaFluor® 488 conjugated chicken anti-mouse IgG (1:200 dilution; Gibco Life Technologies) as secondary antibodies. DAPI (1:1,000 dilution; Dojindo Laboratories) was used for nuclear staining. The degree of colocalization between AQP5 and the plasma membrane marker E-cadherin was calculated as Pearson's correlation coefficient with ImageJ Coloc2 plugin.[20]

Western blot analysis. HUVECs were cultured on a 100-mm culture dish until 80% confluence. The cells were then starved for 6 h by using a serum- and VEGF-free medium. This step was followed by incubation with 50 µM of three different polyphenols for 5 min at 37°C. The cells were solubilized in RIPA buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, and 1% Nonidet P-40] containing a protease inhibitor cocktail (Takara Bio., Shiga, Japan) and phosphatase inhibitor (Roche Diagnostics GmbH). Acutely isolated mSMG cells were then stimulated with 50 µM of three different polyphenols and the vehicle control for 10 min at 37°C. EzSubcell extract kit (WSE-7421; ATTO Co., Ltd., Tokyo, Japan) was used to separate the cytosolic and plasma membrane protein fractions from cells. Western blot analysis was performed with anti-endothelial nitric oxide synthase (eNOS) antibody (M221; ab76198, 1:1,000; Abcam), anti-phosphorylated eNOS (p-eNOS; S1177) antibody (EPR20991; ab230158, 1:1,000; Abcam), and anti-AQP-5 antibody (AB15858, 1:200 dilution; Millipore Co., Ltd.) as the primary antibodies. Expression of target proteins was detected with ECL™ Prime Western Blot Detection Reagents (Cytiva, Buckinghamshire, UK), and relative band intensities were digitalized with a C-DiGit Blot scanner (LI-COR Inc., Lincoln, NE). The band intensities of the target proteins were normalized to total protein levels stained by EzStainAQua MEM (WSE-7160; ATTO Co., Ltd.).

Intracellular ROS generation. Intracellular ROS generation was determined with a ROS indicator dye dichlorodihydrofluorescein diacetate (DCFH-DA) kit (R252; Dojindo Laboratories). Acutely isolated mSMG cells were loaded with DCFH-DA ROS dye at 37°C for 30 min. Then, cells were stimulated with 50 µM of the three polyphenols and the vehicle control for 30 min, then 1 mM H_2O_2 was added, and cells were incubated at 37°C for 30 min. The cell suspension was added in triplicate to a 96-well clear-bottomed black plate, and fluorescence was measured at 490 nm excitation and 535 nm emission.

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Nitric oxide production. HUVECs were starved for 6 h in a serum- and VEGF-free culture medium. Then, 10 μM of NO indicator dye, Diaminofluorescein-FM diacetate (DAF-FM-DA) dye (SK1004-01; Goryo Chemical, Inc., Sapporo, Japan), was added to the cells, and the cells were incubated in the dark for 30 min. Next, cells were stimulated with 50 μM the three polyphenols and the vehicle control for 10 min at 37°C. The relative fluorescence of DAF-FM, which is dependent on nitric oxide (NO) production, was measured at 488 nm excitation and 530 nm emission by BD FACS Jazz (BD Bioscience, Becton and Dickinson and Co., Franklin Lakes, NJ). The percentages of DAF-FM–positive cells were calculated.

***In vivo* experiments.**

Measurement of blood flow and saliva secretion in mice. Mice were anesthetized with 3 types of mixed anesthetic agents (0.3 mg/kg medetomidine, 4.0 mg/kg midazolam, and 5.0 mg/kg butorphanol).[21] Mice were administered 8 mg/kg of saponarin, isoschaftoside, or vehicle control and 0.5 mg/kg pilocarpine via

Table 1. Ingredients of test food

Ingredients (%)	NCS candy	Placebo candy
Brown sugar	74.13	—
Granulated sugar	—	74.13
Syrup	24.71	24.71
Caramel color	0.84	0.84
Emulsifier	0.31	0.31

NCS, non-centrifugal cane sugar.

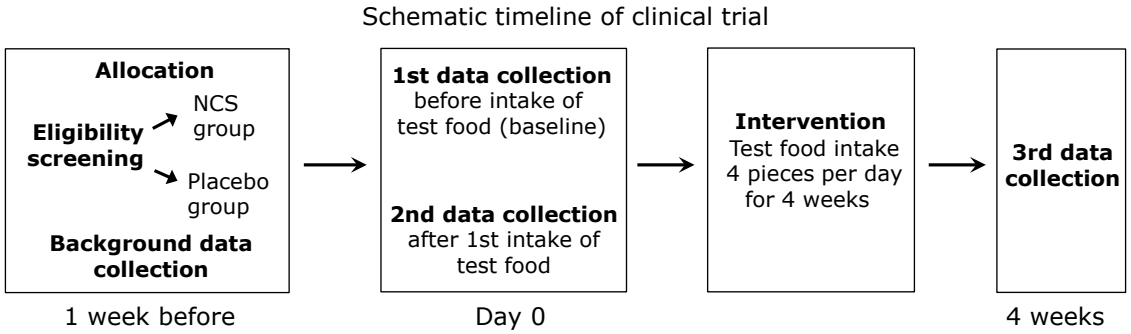

Fig. 1. Timeline of randomized controlled trial.

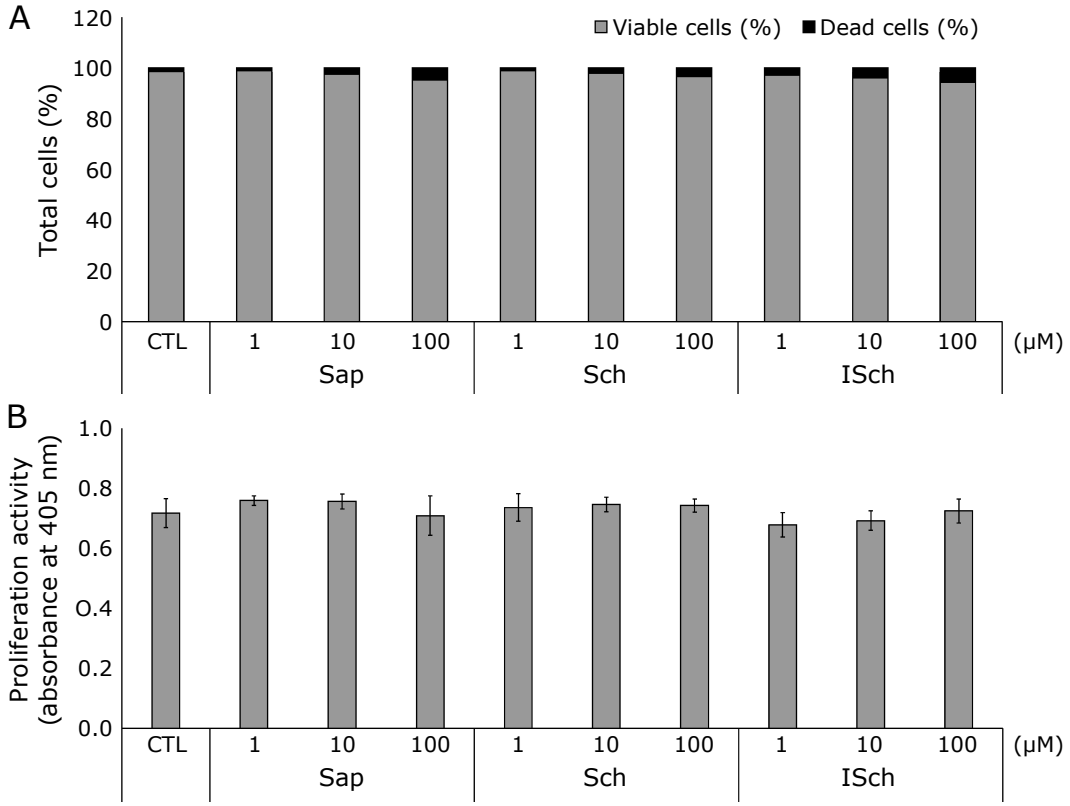

Fig. 2. Cytotoxicity and cell proliferation activity. Normal salivary gland SV40-transformed acinar cells were treated with 1 μM, 10 μM, and 100 μM saponarin (Sap), schaftoside (Sch), and isoschaftoside (ISch) and DMSO as vehicle control (CTL) for 48 h. (A) Percentage of viable and dead cells in trypan blue exclusion test. Dead cells did not exceed 5% in the presence of any of the polyphenols. (B) Proliferation activity of the cells after stimulation with non-centrifugal cane sugar polyphenols. None of the culture conditions showed a significant difference in proliferative activity compared with control. All experiments were performed in triplicate and analyzed by ANOVA with a post hoc Dunnet's test.

the intraperitoneal route. The blood flow rate was measured with the laser Doppler flowmeter RBF-101 (Pioneer Co., Tokyo, Japan) in the capillaries of the skin in the submandibular region for 20 min. Secreted saliva was collected from the oral cavity of mice with a filter paper for 30 min. The amount of saliva obtained was calculated as the difference in the weight of the filter paper before and after collection of saliva and was normalized per body weight of the mice.

Randomized controlled trial. To examine the effect of the polyphenols abundant in NCS from Okinawa on saliva secretion, we conducted a randomized, double-blind, placebo-controlled, parallel-group comparative study. The study was in accordance with the ethical standards laid down in the Declaration of Helsinki and its later amendments. It was approved by the Institutional Review Board of the Chiyoda Paramedical Care Clinic (IRB number: 15000088; IRB approval number: KSG22C1) and registered in UMIN-CTR (UMIN000048129). Informed consent was obtained from all participants. The study period from registration to the end of follow-up was June 28, 2022, to August 19, 2022. The protocol was not amended after the study had started. The RCT is reported in accordance with the CONSORT 2010 statement.

The study enrolled healthy individuals aged 40 to 63 with symptoms such as a mild sensation of a decrease in saliva. Candidates were screened at the trial agency, Chiyoda Paramedical Care Clinic (Tokyo, Japan), one week before starting the intervention; the following information was thereby collected: health status, height, weight, body mass index, systolic and diastolic blood pressure, pulse rate, saliva flow measured by the Saxon test,[22] blood flow, and length of the blood vessel near the cuticle of the left, fourth fingernail. The main exclusion criteria were use of any medications or supplements, salivary secretion of less than 2 g or more than 6 g at screening, difficulty measuring blood flow, and being deemed ineligible by the investigators. Candidates who met the eligibility criteria were stratified by sex, age, and saliva flow by the investigator and randomized into two groups that received either a candy containing NCS or a placebo candy without NCS as test food provided by the funding organization, Kasugai Seika Co., Ltd. (Nagoya, Japan). The person responsible for distributing the test food to participants was not involved with screening, randomization, or outcome assessment.

Table 1 shows the ingredients of the test food. Both candies were brown, weighed 4 g, and were indistinguishable based on their color, taste, smell, and packaging. The randomization code was kept by the principal investigator and was not disclosed to participants or the people responsible for randomizing patients and collecting and analyzing data until the analysis was complete. Participants were instructed to consume four of the assigned test foods per day for four consecutive weeks and to record their intake daily. The trial agency verified participant adherence to the instructions by checking the remaining amount of test food at the end of the study. Outcomes were assessed 1 week before (background) and after 4 weeks intervention, and just before and after the first administration of a candy (Fig. 1). The primary outcome was saliva flow, and the secondary outcome was blood flow near the cuticle of the nail and in the gingiva and the survey of subjective symptoms.[23] The outcomes were not changed after the start of the study.

Statistical analysis. Statistical analyses of *in vivo* and *in vivo* experiments were performed with StatPlus:mac (Analystsoft Inc., Walnut, CA). For the *in vitro* experiments, the parametric data were analyzed by one-way analysis of variance (ANOVA) with a post hoc Dunnett test and the non-parametric data, by the Mann–Whitney *U* test. For the *in vivo* study, Student's *t* test was used to compare the two independent groups, and Cohen's d with 95% CI was calculated as an index of effect size.[24] Statistical analysis of the RCT was performed with SPSS (IBM Corp.,

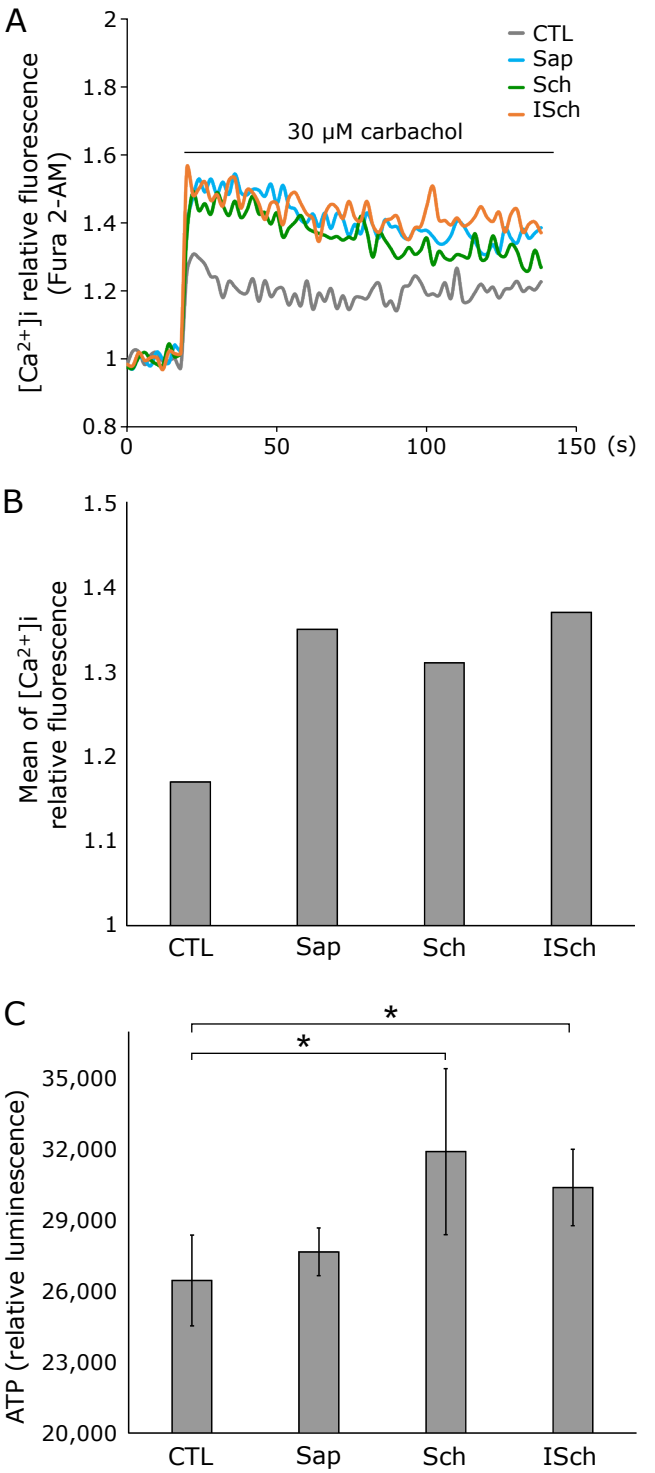

Fig. 3. Intracellular calcium increases dependent on muscarinic receptor stimuli and adenosine triphosphate production. Mouse submandibular gland (mSMG) cells were pretreated with 50 μM saponarin (Sap), schaftoside (Sch), isoschaftoside (ISch) and DMSO as vehicle control (CTL) for 30 min. (A) Changes in intracellular calcium ($[Ca^{2+}]i$) upon stimulation with 30 μM carbachol. (B) Mean $[Ca^{2+}]i$ relative fluorescence. In response to carbachol stimulation, $[Ca^{2+}]i$ increased more with the polyphenols than with the control. (C) Adenosine triphosphate (ATP) production in mSMG cells. ATP production increased significantly more in cells treated with Sch ($p = 0.043$) and ISch ($p = 0.043$) than in control cells. All experiments were performed in triplicate. Statistical analysis was performed by post hoc Dunnett's test. *$p<0.05$.

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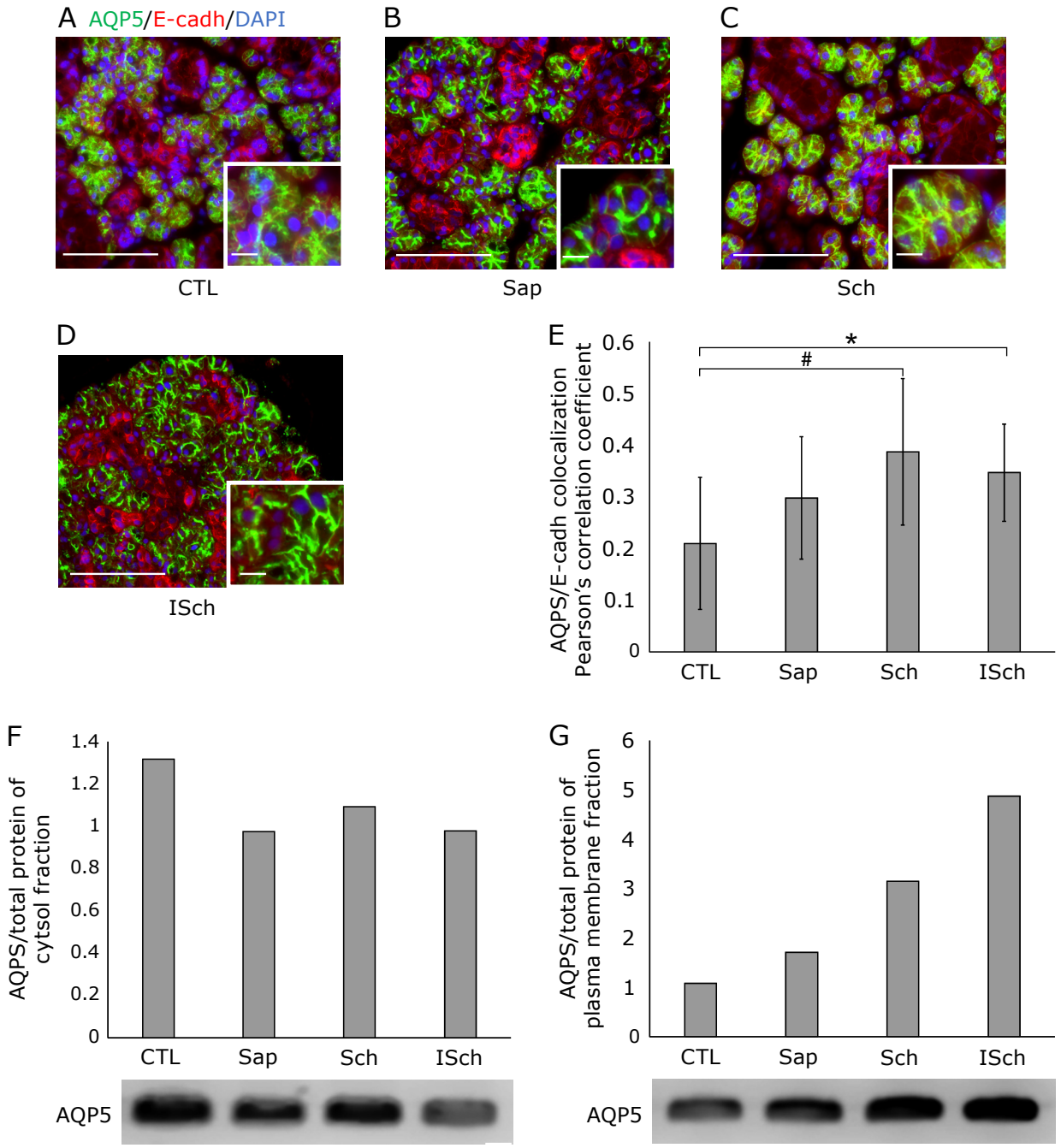

Fig. 4. Localization of aquaporin 5 in the mouse submandibular gland. Representative immunohistochemistry images of mouse submandibular gland (mSMG; A–D). mSMG cells were treated with saponarin (Sap), schaftoside (Sch), isoschaftoside (ISch), or DMSO as vehicle control (CTL) for 10 min. (E) Aquaporin 5 (AQP5) and E-cadherin (E-cadh) colocalization was analyzed in immunohistochemical tests. Colocalization was significantly higher with Sch ($p = 0.003$) and ISch ($p = 0.029$) than with control. (F, G) Western blot analysis of AQP5 protein expression normalized with total protein obtained from cell fractions of the cytosol (F) and plasma membrane (G). The amount of protein carried per lane was 5 µg. Translocation of AQP5 protein was observed, with a decrease in the cytosol fraction and an increase in the plasma membrane of mSMG cells treated with polyphenols. All experiments were performed in triplicate and analyzed by Mann–Whitney *U* test. $^{*}p<0.05$, $^{\#}p<0.01$. Scale bar: 100 µm; scale bar (inset): 10 µm. See color figure in the on-line version.

Armonk, NY) and BellCurve for Excel (Social Survey Research Information Co., Ltd., Tokyo, Japan) according to the statistical analysis plan. In the RCT, participants whose data included extreme outliers or could not be measured at some time points were excluded from the analysis.

Results

Cytotoxicity and cell proliferation. NS-SV-AC cells were stimulated with 1 µM, 10 µM, and 100 µM NCS polyphenols for 48 h. The 1 µM and 10 µM doses of all three polyphenols resulted in 1% to 2% of dead cells, and the 100 µM dose, in 3% to 5%, indicating that none of the polyphenols affected cell growth at the low doses studied (Fig. 2A). The absorbance of the

formazan produced in proportion to the number of viable cells was 0.6 to 0.8, with no significant difference between culture conditions, showing that polyphenols do not have a severe effect on cell viability (Fig. 2B).

Increase in [Ca^{2+}]i dependent on muscarinic receptor stimulation and ATP production. A transient increase of [Ca^{2+}]i by 30 μM carbachol stimulation was plotted as a fold change from the mean value before stimulation (Fig. 3A). The mean fold change 120 s after carbachol stimulation was compared for each test condition. The [Ca^{2+}]i increase dependent on carbachol was higher with saponarin (1.35), schaftoside (1.31), and isoschaftoside (1.37) than with control (1.17) (Fig. 3B). When cells were treated with a 50 μM dose of the different polyphenols, the relative luminescence intensity of ATP production was significantly higher with schaftoside ($p = 0.043$) and isoschaftoside ($p = 0.043$) than with the control (Fig. 3C).

Localization of AQP5. Fluorescence microscopy images of mSMG showed that AQP5 (green fluorescence) located on the plasma membrane were more concentrated on the apical side of the acinar cells in the polyphenol-treated cells compared with the control. For quantitative assessment of the translocation of AQP5 to the plasma membrane, the degree of co-localization between AQP5 and E-cadherin, a plasma membrane marker, was evaluated by Pearson's correlation coefficient. Compared with the control (0.21 ± 0.12), the mean ± SD Pearson's correlation coefficient was significantly higher for schaftoside (0.38 ± 0.14, $p = 0.003$) and isoschaftoside (0.34 ± 0.36, $p = 0.029$); no significant difference was observed for saponarin 0.298 ± 0.12 ($p = 0.54$) (Fig. 4A–E).

To confirm the translocation of AQP5, Western blot was performed with the protein fractions of the cytosolic and plasma membrane extract from mSMG cells. The relative band intensities of AQP5 protein in the cytosol were as follows: isoschaftoside, 0.98; saponarin, 0.97; schaftoside, 1.09; and control, 1.31. In the plasma membrane, the following values were measured: isoschaftoside, 4.87; schaftoside, 3.14; saponarin, 1.71; and control, 1.07 (Fig. 4F and G).

Evaluation of antioxidant properties. To verify the effect of polyphenols against oxidative stress, [Ca^{2+}]i was measured in the presence of H_2O_2. The results showed that oxidative stress reduced the increase in [Ca^{2+}]i caused by stimulation with the muscarinic receptor agonist carbachol. However, the presence of polyphenols increased [Ca^{2+}]i (saponarin, 1.19; isoschaftoside, 1.21) compared with the control (1.00) (Fig. 5A and B). To clarify the inhibitory effect of polyphenols against H_2O_2-induced intracellular ROS in mSMG cells, a DCFH-DA fluorescence assay was performed. The fluorescence intensity of DCFH-DA was increased when cells were treated with 1 mM H_2O_2. However, when polyphenols were present in the culture system, intracellular ROS generation was significantly reduced (saponarin, $p = 0.006$; schaftoside, $p = 0.00005$; and isoschaftoside, $p = 0.00012$) (Fig. 5C). Both experiments showed that all three polyphenols reduced ROS generation in the mSMG cells compared with the control.

Phosphorylation of eNOS and NO production. The expression level of eNOS protein in the presence of polyphenols indicated no significant difference between each polyphenol and the control. However, compared with the control (51,767.6 ± 4,335.8), the relative band intensity (mean ± SD) of p-eNOS was significantly higher with saponarin (77,601.9 ± 14,345.2, $p = 0.019$) followed by isoschaftoside (63,850.9 ± 5,012.7, $p = 0.011$) (Fig. 6A–C).

Flow cytometry analysis showed that the percentage of DAF-FM–positive cells with enhanced NO production was increased in the presence of saponarin (20.7%) and isoschaftoside (14.3%) compared with the control (12.8%) (Fig. 6D and E).

Measurement of blood flow and saliva secretion in mice. Saponarin and isoschaftoside were used for the *in vivo* studies

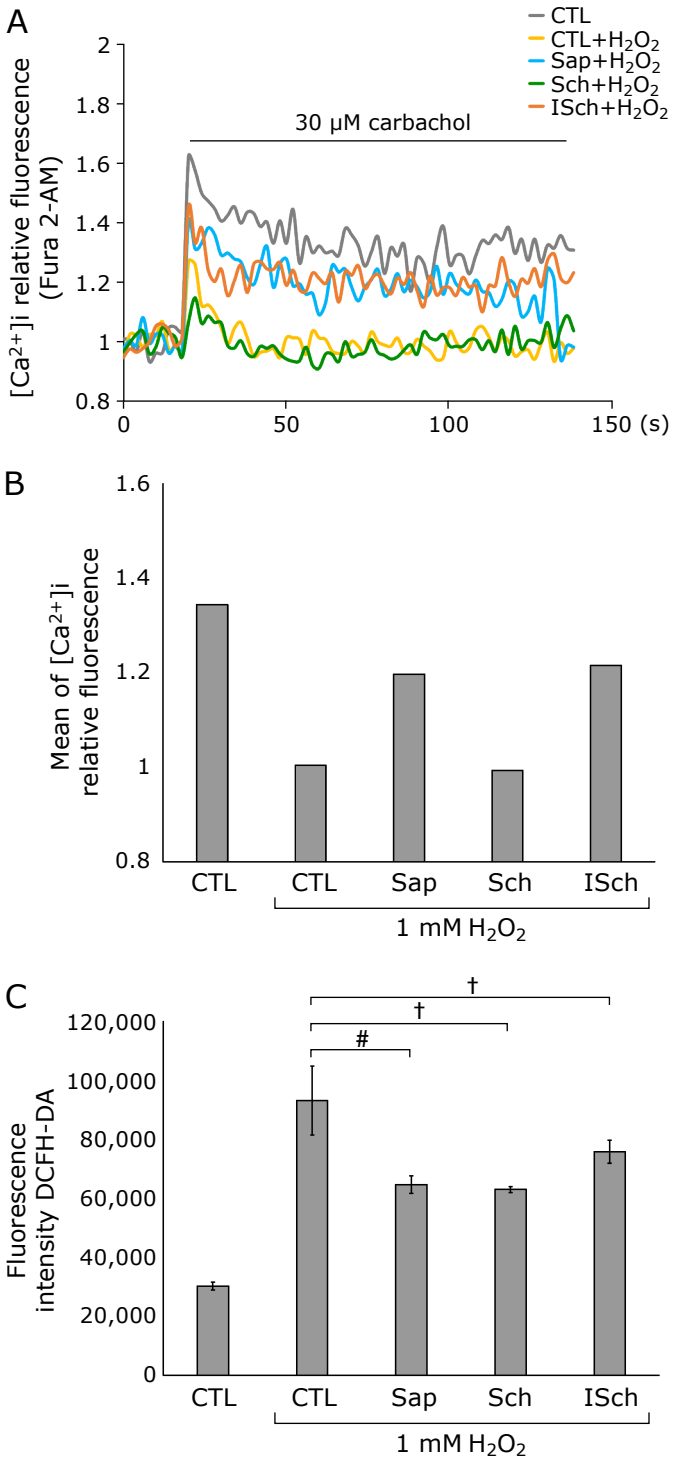

Fig. 5. Antioxidant effects of non-centrifugal cane sugar polyphenols. Mouse submandibular gland (mSMG) cells were pretreated with 50 μM saponarin (Sap), schaftoside (Sch), isoschaftoside (ISch), and DMSO as vehicle control (CTL) and then exposed to 1 mM H_2O_2 for 30 min. (A) Changes in intracellular calcium ([Ca^{2+}]i) upon stimulation with 30 μM carbachol. (B) Mean [Ca^{2+}]i relative fluorescence. Treatment with H_2O_2 decreased [Ca^{2+}]i in dependence on carbachol. However, pre-treatment of the mSMG cells with non-centrifugal cane sugar (NCS) polyphenols Sap and ISch prevented the decrease in [Ca^{2+}]i compared with CTL. (C) Intracellular ROS generation indicated by dichlorodihydrofluorescein diacetate (DCFH-DA) on exposure to H_2O_2 was decreased compared with CTL when pretreated with the NCS polyphenols Sap ($p = 0.0065$), Sch ($p = 0.00005$), and ISch ($p = 0.00012$). Each experiment was performed in triplicate and analyzed by ANOVA using post hoc Dunnett's test. $^{\#}p<0.01$, $^{\dagger}p<0.005$.

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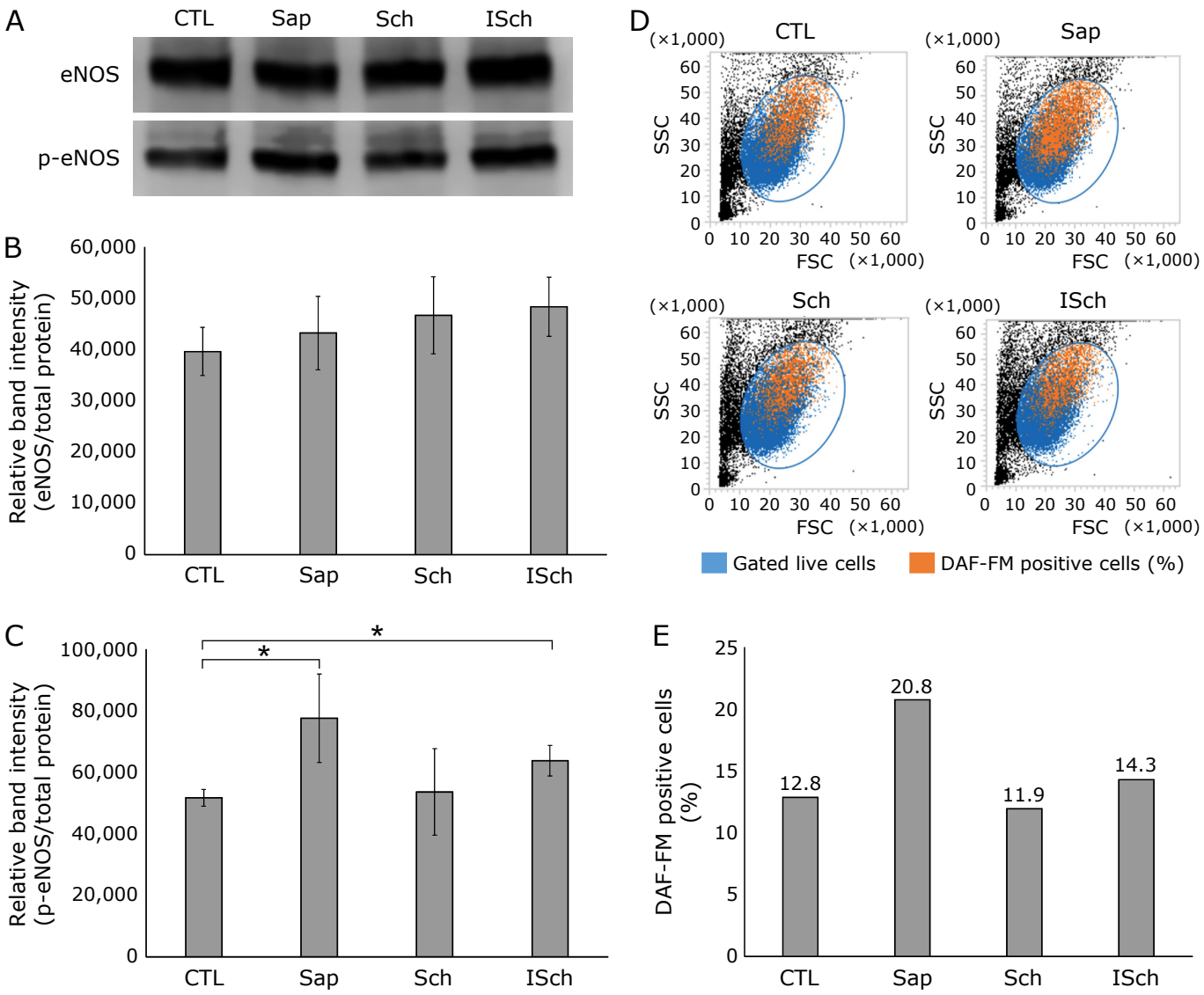

Fig. 6. Phosphorylation of endothelial nitric oxide synthase and nitric oxide production. Human umbilical vein endothelial cells (HUVECs) were stimulated with 50 μM saponarin (Sap), schaftoside (Sch), isoschaftoside (ISch), or DMSO as vehicle control (CTL) for 5 min. (A) Western blot analysis for endothelial nitric oxide synthase (eNOS) and phosphorylated eNOS (p-eNOS). The amount of protein carried per lane was 25 μg. The target protein level was normalized with the total protein. (B) eNOS level of the cells in the presence of non-centrifugal cane sugar polyphenols. No significant difference was found compared with the CTL. (C) p-eNOS level of the cells in the presence of polyphenols. A significant increase was observed with Sap ($p = 0.019$) and ISch ($p = 0.011$) compared with the CTL. (D) Flow cytometry analysis of nitric oxide production. The percentage of diaminofluorescein-FM diacetate (DAF-FM)–positive cells (indicated by orange) increased with Sap and ISch compared with the CTL. (E) Percentage of DAF-FM–positive cells represented as a graph. Each experiment was performed in triplicate and analyzed by Student's *t* test. *$p<0.05$.

since these polyphenols increased muscarinic stimulation-dependent $[Ca^{2+}]i$, ATP production, translocation of AQP5, the phosphorylation level of p-eNOS, and NO production and decreased intracellular ROS in the *in vitro* experiments. The fold change in blood flow rate in the submandibular region showed a significantly larger increase in the saponarin group ($n = 5$; $p = 0.040$; Cohen's d = 1.39; 95% CI, 0.011 to 2.774) and isoschaftoside group ($n = 5$; $p = 0.010$; Cohen's d = 2.01; 95% CI, 0.491 to 3.534) compared with the control group ($n = 5$). Likewise, saliva secretion was significantly increased in the saponarin group ($n = 11$) compared with the control group ($n = 12$; $p = 0.031$; Cohen's d = 0.82; 95% CI, −0.0291 to 1.675). There was no significant difference between the control and isoschaftoside group ($n = 10$; $p = 0.110$; Cohen's d = 0.54; 95% CI, −0.311 to 1.398) (Fig. 7A and B).

Regarding the comparisons with statistically significant differences, a sample size analysis by G*power[25] showed that some comparisons slightly failed to meet the required sample size, except for the comparison of blood flow rates between control and saponarin.

RCT. The flow chart of participants through the study is shown in Fig. 8. Thirty-five of the 65 enrolled candidates were excluded according to exclusion criteria, so the study was performed in 30 participants ($n = 15$, two groups). A pre-intervention observation showed no significant differences in demographic or clinical characteristics between the two groups (Table 2).

Table 3 shows the data on salivary secretion and blood flow. Mean salivary flow rate increased in both groups immediately after the first intake of the test food, but it increased significantly

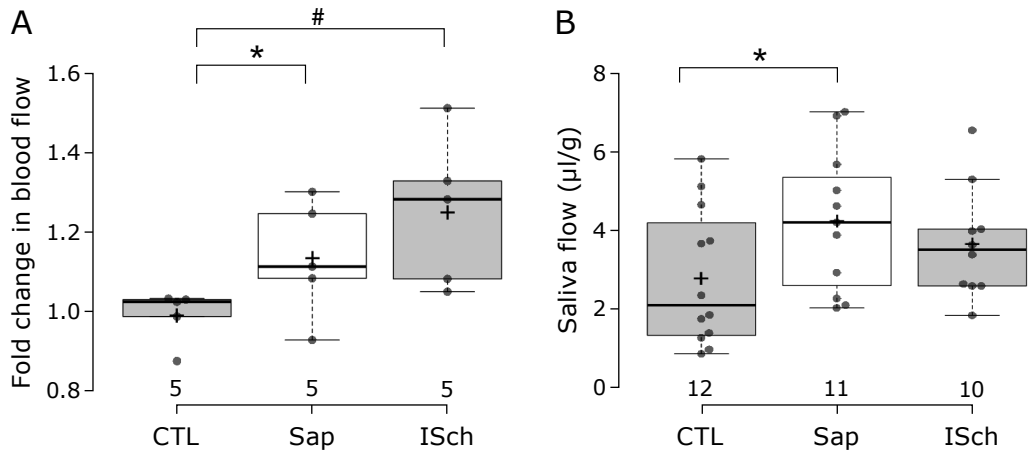

Fig. 7. Blood flow and salivation in mice. Mice were administrated 8 mg/kg of saponarin (Sap), isoschaftoside (ISch) or control (CTL) along with 0.5 mg/kg pilocarpine. (A) Fold changes in blood flow before and after administration. Compared with the CTL group (0.98, $n = 5$), the mean fold change was significantly higher in the Sap group (1.13; $n = 5$; $p = 0.040$; Cohen's d = 1.39; 95% CI, 0.011 to 2.774) and in the ISch group (1.25; $n = 5$; $p = 0.0099$; Cohen's d = 2.01; 95% CI, 0.491 to 3.534). (B) Saliva flow 30 min after administration. Compared with the CTL group ($n = 12$), a significant increase was seen in the Sap group ($n = 11$; $p = 0.031$; Cohen's d = 0.82; 95% CI, –0.0291 to 1.675) and a nonsignificant change with a moderate effect size was seen in the ISch group ($n = 10$; $p = 0.110$l Cohen's d = 0.54l 95% CI, –0.311 to 1.398). Student's t test was used for analysis. *$p<0.05$, #$p<0.01$.

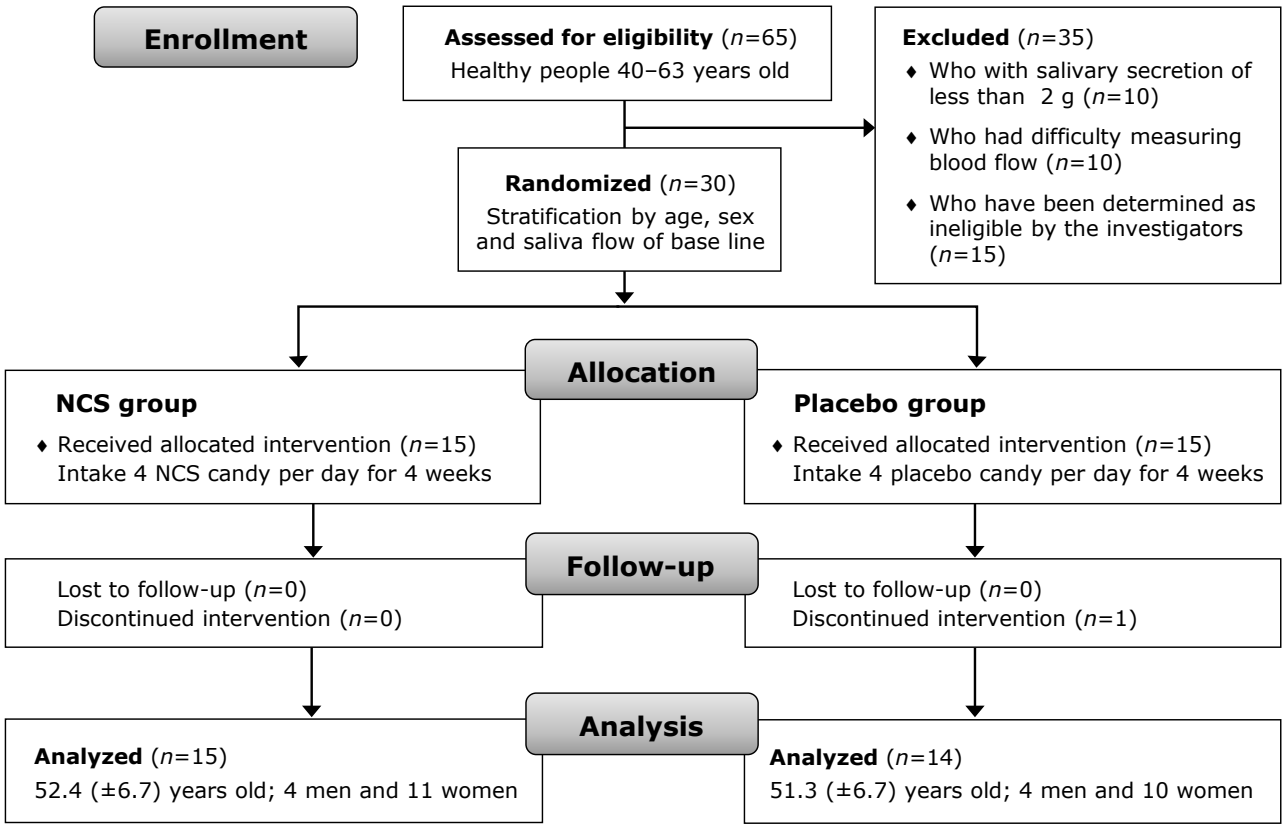

Fig. 8. Flow chart of randomized controlled trial. NCS, non-centrifugal cane sugar. Blood flow rate as secondary outcome was analyzed with $n = 13$ per group, excluding participants with extreme outliers or missing data from the analysis.

more in the NCS group ($p = 0.003$). At the end of the 4-week intervention trial, the mean saliva flow rate in the NCS group was numerically higher than in the placebo group (NCS, 4.508 ± 1.372; placebo, 4.303 ± 1.170), but the difference was not significant. At the start of the trial, the mean blood flow rate was significantly higher in the placebo group than in the NCS group. The NCS group showed an increase in the mean blood flow rate after the first intake from 6.959 ± 3.664 to 7.665 ± 6.220, but the difference was not significant ($p = 0.861$). On the other hand, the mean blood flow rate in the placebo group decreased from

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Table 2. Background characteristics of participants

	NCS group	Placebo group	*p* value
Sex distribution	4 men, 11 women	4 men, 10 women	0.909
Age	52.4 ± 6.7	51.3 ± 6.7	0.656
Salivary flow rate (g/2 min)	4.1 ± 0.9	4.0 ± 1.4	0.804
Body height (cm)	160.4 ± 6.9	159.9 ± 10.5	0.871
Body weight (kg)	56.0 ± 11.4	54.1 ± 11.4	0.485
Body mass (kg/m^2)	21.6 ± 3.2	21.0 ± 2.8	0.59
Systolic blood pressure (mmHg)	113.2 ± 15.7	115.8 ± 18.0	0.683
Diastolic blood pressure (mmHg)	71.5 ± 12.8	72.7 ± 9.7	0.771
Pulse rate (bpm)	77.5 ± 10.1	77.7 ± 6.5	0.939

NCS, non-centrifugal cane sugar. Data shown are means ± SD.

Table 3. Saliva and blood flow rate on RCT trial

	NCS group			Placebo group			Comparison between groups
	Baseline	Immediately after	4 weeks	Baseline	Immediately after	4 weeks	
Saliva flow rate (g/2 min)	4.440 ± 1.428	4.915 ± 1.458	4.508 ± 1.372	4.427 ± 1.294	4.716 ± 1.266	4.303 ± 1.170	Baseline $p = 0.979$
	Baseline vs immediately after $p = 0.003^{\dagger}$		Baseline vs 4 weeks $p = 0.768$	Baseline vs immediately after $p = 0.268$		Baseline vs 4 weeks $p = 0.643$	4 weeks $p = 0.670$
Blood flow rate (ml/min)	6.959 ± 3.664	7.665 ± 6.220	7.410 ± 4.06	12.055 ± 8.461	10.971 ± 7.522	10.295 ± 5.038	Baseline $p = 0.086$
	Baseline vs immediately after $p = 0.861$		Baseline vs 4 weeks $p = 0.506$	Baseline vs immediately after $p = 0.297$		Baseline vs 4 weeks $p = 0.410$	4 weeks $p = 0.069$

NCS, non-centrifugal cane sugar. Values are the mean ± SD. $^{\dagger}p<0.005$ in paired two-sample *t* test (2-tailed). Saliva flow rates: All comparisons were performed with Student's *t* test. NCS, $n = 15$; placebo: $n = 14$. Blood flow rates: Within-group comparisons in the placebo group were performed with Student's *t* tests and the other comparisons were performed with parametric methods ($n = 13$ per group).

12.055 ± 8.461 to 10.971 ± 7.522 after the first intake; this difference was also not significant ($p = 0.297$). At the end of the 4-week intervention, no significant difference in the mean blood flow rate was found between the NCS and placebo groups ($p = 0.069$). The required total sample size calculated by G*power for the saliva flow rate comparison before and after the first intake of NCS candy was 73. No items in the subjective symptom survey improved significantly with NCS ingestion compared with placebo. No serious adverse events were observed.

Discussion

We investigated the effects and mechanisms of action of the NCS polyphenols saponarin, schaftoside, and isoschaftoside on saliva secretion. Our results showed that these polyphenols enhanced the increase in $[Ca^{2+}]i$ after muscarinic receptor stimulation of mSMG cells. Also, NCS polyphenols increased ATP production, suppressed oxidative stress, and induced AQP5 translocation to the plasma membrane of mSMG cells. In addition, they increased eNOS phosphorylation and NO production in HUVECs. *In vivo* experiments in mice showed that NCS polyphenols significantly increased blood flow and saliva secretion. The RCT found a significant increase in salivary secretion immediately after intake of NCS candy.

$[Ca^{2+}]i$ is an important second messenger in saliva secretion. Stimulation of muscarinic, purinergic, and alpha-adrenergic receptors activates inositol triphosphate signaling pathways that induce release of $[Ca^{2+}]i$ from the endoplasmic reticulum.[11,26,27] The increased $[Ca^{2+}]i$ regulates various ion channels and leads to fluid secretion via paracellular or transcellular pathways.[28] In this study, direct stimulation of mSMG cells with NCS polyphenols did not increase $[Ca^{2+}]i$ (data not shown); however, the presence of NCS polyphenols enhanced the increase in $[Ca^{2+}]i$ after muscarinic receptor stimulation. This phenomenon is similar to that found in our previous study on quercetin.[15] Taken together, our findings indicate that NCS polyphenols do not activate $[Ca^{2+}]i$ signals but can contribute to salivary secretion by enhancing the associated pathway (Fig. 9A).

ATP provides energy for the contraction of myoepithelial cells in the salivary glands. It is also responsible for activating the Na^+/K^+ pump, which plays a role in water secretion by transporting ions to create an osmotic gradient between the lumen, the acinar cells, and the interstitium.[29,30] Moreover, ATP activates the phospholipase C/inositol triphosphate signaling pathway via the purinergic receptor pathway to mobilize $[Ca^{2+}]i$ from the endoplasmic reticulum.[28,31] Our previous study demonstrated that ubiquinol improves dry mouth by increasing ATP production.[12] In the present study, NCS polyphenols increased ATP production in mSMG cells, implying that polyphenols may influence saliva secretion by enhancing ATP production (Fig. 9A).

AQP5 is a water channel protein located in salivary gland acinar cells that mediates transcellular water movement.[32–34] Muscarinic receptor stimulation also induces translocation of AQP5 from the cytoplasm to the apical side of the acinar cells.[35] Previous studies reported that an antioxidant found in green tea and some plant-derived compounds promote salivation via AQP5 translocation.[36,37] Our immunohistochemical study showed that NCS polyphenols promoted colocalization of AQP5 with E-cadherin, a plasma membrane marker. These data were supported by Western blot analysis, which showed that NCS polyphenols increased AQP5 expression in the plasma membrane fraction and decreased it in the cell cytosol fraction. These findings indicate that NCS polyphenols can contribute to saliva secretion by enhancing the water permeability of salivary gland cells by

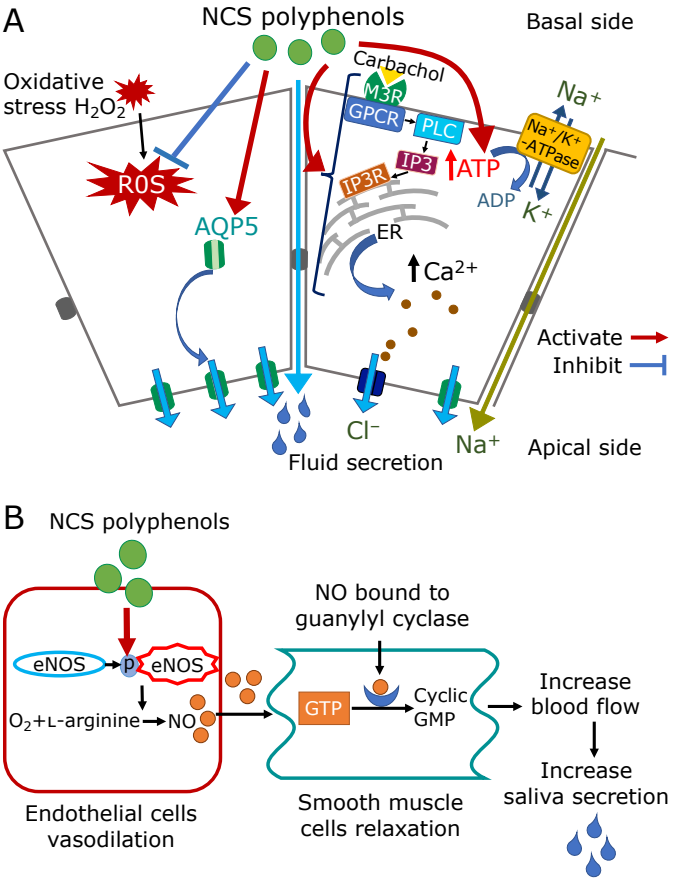

Fig. 9. Hypothetical mechanism by which non-centrifugal cane sugar polyphenols affect saliva secretion. (A) Diagram of possible mechanism by which non-centrifugal cane sugar (NCS) polyphenols act in salivary gland acinar cells. The presence of NCS polyphenols enhances production of intracellular calcium ([Ca^{2+}]i) via a pathway dependent on the muscarinic acetylcholine receptor (mAChR) and an increase in adenosine triphosphate (ATP) production. Furthermore, NCS polyphenols influence aquaporin 5 (AQP5) translocation from the cytosol to the plasma membrane. NCS polyphenols can prevent oxidative stress by reactive oxygen species (ROS). (B) Diagram of possible mechanism by which NCS polyphenols act in vascular endothelial cells. NCS polyphenols enhance phosphorylation of endothelial nitric oxide synthase (eNOS), leading to formation of nitric oxide (NO). NO produced in the endothelial cells binds to guanylyl cyclase to activate production of cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP) in peri-vascular smooth muscle cells. cGMP relaxes smooth muscle cells, causing vasodilation of blood vessels. Vasodilation increases the flow of blood that contains the water required to enhance fluid secretion by salivary glands. Thus, NCS polyphenols contribute to salivary secretion through multiple effects. ADP, adenosine diphosphate; ER, endoplasmic reticulum; GPCR, G protein-coupled receptors; IP3, inositol triphosphate; IP3R, inositol triphosphate receptor; PLC, phospholipase C.

inducing AQP5 translocation to the apical membrane. Translocation of AQP5 is known to be induced by protein kinase A through cyclic adenosine monophosphate signaling and the mitogen-activated protein kinase (MAPK) signal pathway.[32,38,39] Hence, NCS polyphenols may affect the MAPK pathway or cyclic adenosine monophosphate/protein kinase A pathway to enhance AQP5 translocation (Fig. 9A).

Polyphenols are dietary antioxidants that protect cells from oxidative damage.[4,5] Our previous study showed that quercetin intake significantly augmented saliva secretion in irradiated mice.[15] In the present study, the presence of H_2O_2 increased ROS generation and suppressed [Ca^{2+}]i mobility dependent on carbachol. In contrast, the presence of NCS polyphenols in the culture medium significantly decreased ROS generation and protected the cells from oxidative stress. These results suggest that NCS polyphenols exert protective effects against ROS and prevent salivary hypofunction induced by oxidative stress (Fig. 9A).

NO is a free radical with an array of biological functions, including signal transduction in the vascular system.[40] In endothelial cells, NO synthesized by eNOS phosphorylation induces relaxation of vascular smooth muscles, causing vasodilation and an increase in blood flow via the NO-cyclic guanosine monophosphate (cGMP) pathway.[40,41] Therefore, increased NO production and eNOS phosphorylation indicate vasodilation and blood flow enhancement.[42] Our experiment on HUVECs found increased phosphorylation levels of eNOS and increased NO production in the presence of NCS polyphenols, especially saponarin and isoschaftoside. The *in vivo* experiments showed that saponarin and isoschaftoside increased blood flow in the submandibular region of the mice. Moreover, saponarin significantly enhanced saliva secretion induced by pilocarpine. The water in saliva is derived from blood plasma that is transferred to the lumen through the paracellular or transcellular pathway of acinar cells;[29] thus, blood flow and salivary secretion are correlated.[39] These findings suggest that NCS polyphenols also contribute to salivary secretion by increasing NO production and thus promoting blood flow, resulting in vasodilation. Some polyphenols are known to induce eNOS phosphorylation via activation of MAPK signaling.[43] Therefore, saponarin and isoschaftoside may bind directly to protein kinases involved in eNOS phosphorylation (Fig. 9B); however, this hypothesis needs to be tested in future research. The limitation of the *in vivo* studies is that some of the sample sizes were smaller than required.

In our RCT to clarify whether consumption of NCS polyphenols has beneficial effects on saliva secretion, we found that the first intake of NCS candy immediately resulted in a significant increase in salivation. At the end of the intervention period, the mean increase in salivation was higher than at the initial intervention, although the difference was not significant. The required sample size calculated from the comparison of saliva flow before and after the first intake of NCS candy was approximately twice the sample size tested, so the study was underpowered to show a difference. Many clinical trials of polyphenols showed increased blood flow in interventions lasting 8 weeks or more,[44] and the shorter intervention period was a limitation of the present study. Hence, future studies need to use a longer intervention to evaluate the potential benefits of NCS on salivation and blood flow. The difference in blood flow rates between groups was nearly twice as large at the beginning of the study than at the end, which may have affected the results. Gingival blood flow could not be measured because of the sensitivity of the measurement system, but we may have obtained different results if we had been able to measure blood flow in a region closer to the salivary glands than the fingers, as in the animal study. Salivary gland massage therapy, i.e., repeated physical stimulation of the salivary glands, is known to improve salivary secretion, and administration of pilocarpine restores salivary secretion after radiotherapy-induced hyposalivation.[45,46] Therefore, NCS, which showed an immediate saliva-stimulating effect in this study, may also promote salivary secretion with continued repeated administration.

In conclusion, NCS polyphenols exhibit several beneficial properties on salivary glands in that they enhance salivation and protect the glands from oxidative stress. These new findings support the notion that NCS polyphenols may maintain salivation and improve an oxidative stress-related reduction in saliva flow.

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Author Contributions

IS, SS, and RU-N designed the study; SS, RU-N, TY, DO, NM, KA, and HI contributed to the collection and analysis of study data; SS, RU-N, TY, DO, KA, and HI interpreted the data; and IS, SS, RU-N, and NM wrote the article. All authors critically revised the manuscript and approved the final version and agree to accept responsibility for its scientific accuracy and consistency.

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Conflict of Interest

No potential conflicts of interest were disclosed.

Data Availability

All the study data and the clinical trial protocol are available from the corresponding author.

References

1 Dominguez LJ, Veronese N, Baiamonte E, *et al*. Healthy aging and dietary patterns. *Nutrients* 2022; **14**: 889.
2 Meccariello R, D'Angelo S. Impact of polyphenolic-food on longevity: an elixir of life. An overview. *Antioxidants* 2021; **10**: 507.
3 Suzuki M, Wilcox BJ, Wilcox CD. Implications from and for food cultures for cardiovascular disease: longevity. *Asia Pac J Clin Nutr* 2001; **10**: 165–171.
4 Jaffé WR. Health effects of non-centrifugal sugar (NCS): a review. *Sugar Tech* 2012; **14**: 87–94.
5 Rao GP, Singh P. Value addition and fortification in non-centrifugal sugar (jaggery): a potential source of functional and nutraceutical foods. *Sugar Tech* 2022; **24**: 387–396.
6 Ogi T, Maeda T. Flavone glycoside contained in brown sugar from Okinawa. *Bulletin of Okinawa Industrial Technology Center* 2008; **10**: 7–11.
7 de Araújo FF, de Paulo Farias D, Neri-Numa IA, Pastore GM. Polyphenols and their applications: an approach in food chemistry and innovation potential. *Food Chem* 2021; **338**: 127535.
8 Higbee J, Solverson P, Zhu M, Carbonero F. The emerging role of dark berry polyphenols in human health and nutrition. *Food Front* 2022; **3**: 3–27.
9 Liu X, Subedi KP, Zheng C, Ambudkar I. Mitochondria-targeted antioxidant protects against irradiation-induced salivary gland hypofunction. *Sci Rep* 2021; **11**: 7690.
10 Guggenheimer J, Moore PA. Xerostomia: etiology, recognition and treatment. *J Am Dent Assoc* 2003; **134**: 61–119.
11 Pedersen AML, Sørensen CE, Proctor GB, Carpenter GH, Ekström J. Salivary secretion in health and disease. *J Oral Rehabil* 2018; **45**: 730–746.
12 Ushikoshi-Nakayama R, Ryo K, Yamazaki T, *et al*. Effect of gummy candy containing ubiquinol on secretion of saliva: a randomized, double-blind, placebo-controlled parallel-group comparative study and an *in vitro* study. *PLoS One* 2019; **14**: e0214495.
13 Petti S, Scully C. Polyphenols, oral health and disease: a review. *J Dent* 2009; **37**: 413–423.
14 Ryo K, Takahashi A, Tamaki Y, Ohnishi-Kameyama M, Inoue H, Saito I. Therapeutic effects of isoflavones on impaired salivary secretion. *J Clin Biochem Nutr* 2014; **55**: 168–173.
15 Takahashi A, Inoue H, Mishima K, *et al*. Evaluation of the effects of quercetin on damaged salivary secretion. *PLoS One* 2015; **10**: e0116008.
16 Inoue H, Kishimoto A, Ushikoshi-Nakayama R, *et al*. Resveratrol improves salivary dysfunction in a non-obese diabetic (NOD) mouse model of Sjögren's syndrome. *J Clin Biochem Nutr* 2016; **59**: 107–112.
17 Guo Y, Ji W, Lu Y, Wang Y. Triptolide reduces salivary gland damage in a non-obese diabetic mice model of Sjögren's syndrome via JAK/STAT and NF-κB signaling pathways. *J Clin Biochem Nutr* 2021; **68**: 131–138.
18 Percie du Sert N, Hurst V, Ahluwalia A, *et al*. The ARRIVE guidelines 2.0: updated guidelines for reporting animal research. *PLoS Biol* 2020; **18**: e3000410.
19 Azuma M, Tamatani T, Kasai Y, Sato M. Immortalization of normal human salivary gland cells with duct-, myoepithelial-, acinar-, or squamous phenotype by transfection with SV40 ori- mutant deoxyribonucleic acid. *Lab Invest* 1993; **69**: 24–42.
20 Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 2012; **9**: 671–675.
21 Kawai S, Takagi Y, Kaneko S, Kurosawa T. Effect of three types of mixed anesthetic agents alternate to ketamine in mice. *Exp Anim* 2011; **60**: 481–487.
22 Kohler PF, Winter ME. A quantitative test for xerostomia. The Saxon test, an oral equivalent of the Schirmer test. *Arthritis Rheum* 1985; **28**: 1128–1132.
23 Oguma Y, Iida K, Yonei Y, Satoh T. Significance evaluation of anti-aging QOL common questionnaire. *Glycative Stress Res* 2016; **3**: 177–185.
24 Nakagawa S, Cuthill IC. Effect size, confidence interval and statistical significance: a practical guide for biologists. *Biol Rev Camb Philos Soc* 2007; **82**: 591–605.
25 Faul F, Erdfelder E, Lang AG, Buchner A. G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behav Res Methods* 2007; **39**: 175–191.
26 Ambudkar I. Calcium signaling defects underlying salivary gland dysfunction. *Biochim Biophys Acta Mol Cell Res* 2018; **1865 (11 Pt B)**: 1771–1777.
27 Ambudkar IS. Ca^{2+} signaling and regulation of fluid secretion in salivary gland acinar cells. *Cell Calcium* 2014; **55**: 297–305.
28 Ambudkar IS. Dissection of calcium signaling events in exocrine secretion. *Neurochem Res* 2011; **36**: 1212–1221.
29 Proctor GB. The physiology of salivary secretion. *Periodontol 2000* 2016; **70**: 11–25.
30 Maclaren OJ, Sneyd J, Crampin EJ. Efficiency of primary saliva secretion: an analysis of parameter dependence in dynamic single-cell and acinus models, with application to aquaporin knockout studies. *J Membr Biol* 2012; **245**: 29–50.
31 Nakamoto T, Brown DA, Catalán MA, Gonzalez-Begne M, Romanenko VG, Melvin JE. Purinergic $P2X_7$ receptors mediate ATP-induced saliva secretion by the mouse submandibular gland. *J Biol Chem* 2009; **284**: 4815–4822.
32 Hosoi K, Yao C, Hasegawa T, Yoshimura H, Akamatsu T. Dynamics of salivary gland AQP5 under normal and pathologic conditions. *Int J Mol Sci* 2020; **21**: 1182.
33 Delporte C, Steinfeld S. Distribution and roles of aquaporins in salivary glands. *Biochim Biophys Acta* 2006; **1758**: 1061–1070.
34 Jaiboonma A, Kaokaen P, Chaicharoenaudomrung N, *et al*. Cordycepin attenuates salivary hypofunction through the prevention of oxidative stress in human submandibular gland cells. *Int J Med Sci* 2020; **17**: 1733–1743.
35 Ishikawa Y, Iida H, Ishida H. The muscarinic acetylcholine receptor-stimulated increase in aquaporin-5 levels in the apical plasma membrane in rat parotid acinar cells is coupled with activation of nitric oxide/cGMP signal transduction. *Mol Pharmacol* 2002; **61**: 1423–1434.
36 Saito K, Mori S, Date F, Hong G. Epigallocatechin gallate stimulates the neuroreactive salivary secretomotor system in autoimmune sialadenitis of MRL-*Fas^{lpr}* mice via activation of cAMP-dependent protein kinase A and inactivation of nuclear factor κB. *Autoimmunity* 2015; **48**: 379–388.
37 Bhattarai KR, Lee HY, Kim SH, Park JS, Kim HR, Chae HJ. Potential application of *Ixeris dentata* in the prevention and treatment of aging-induced dry mouth. *Nutrients* 2018; **10**: 1989.
38 D'Agostino C, Elkashty OA, Chivasso C, Perret J, Tran SD, Delporte C. Insight into salivary gland aquaporins. *Cells* 2020; **9**: 1547.
39 Kitchen P, Öberg F, Sjöhamn J, *et al*. Plasma membrane abundance of human aquaporin 5 is dynamically regulated by multiple pathways. *PLoS One* 2015; **10**: e0143027.
40 Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G. Endothelium-

derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc Natl Acad Sci U S A* 1987; **84**: 9265–9269.
41 Ignarro LJ. Nitric oxide is not just blowing in the wind. *Br J Pharmacol* 2019; **176**: 131–134.
42 Yamazaki T, Ushikoshi-Nakayama R, Shakya S, *et al*. The effects of bathing in neutral bicarbonate ion water. *Sci Rep* 2021; **11**: 21789.
43 Lacroix S, Klicic Badoux J, Scott-Boyer MP, *et al*. A computationally driven analysis of the polyphenol-protein interactome. *Sci Rep* 2018; **8**: 2232.
44 Potì F, Santi D, Spaggiari G, Zimetti F, Zanotti I. Polyphenol health effects on cardiovascular and neurodegenerative disorders: a review and meta-analysis. *Int J Mol Sci* 2019; **20**: 351.
45 Taniguchi A, Susa T, Kogo H, Iizuka-Kogo A, Yokoo S, Matsuzaki T. Long-term pilocarpine treatment improves salivary flow in irradiated mice. *Acta Histochem Cytochem* 2019; **52**: 45–58.
46 Kim HW, Ahn BC, Lee SW, Lee J. Effect of parotid gland massage on parotid gland Tc-99m pertechnetate uptake. *Thyroid* 2012; **22**: 611–616.

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