Biochemical and Biophysical Research Communications 503 (2018) 1098–1102

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Benefits of long-term pilocarpine due to increased muscarinic acetylcholine receptor 3 in salivary glands

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ARTICLE INFO

Article history:
Received 14 June 2018
Accepted 21 June 2018
Available online 3 July 2018

Keywords:
Salivary gland
Pilocarpine
Sjögren's syndrome
Dry mouth
Xerostomia

ABSTRACT

Hypofunction of the salivary gland causes several life-disrupting side effects such as dental caries, oral candidiasis, loss of taste, and swallowing disorders. No satisfactory therapy has been established to treat salivary hypofunction. Pilocarpine represents a potential treatment for dry mouth due to Sjögren's syndrome (SS). Although subjective improvement was consistently observed with pilocarpine therapy, the mechanism was unclear. In this study, we investigated the mechanism of recovery in salivation following treatment with pilocarpine. We first examined the effectiveness of pilocarpine in SS patients as quantified by the Saxon test and the visual analogue scale average. We found that salivation ability and subjective symptoms improved by continuous administration of pilocarpine. These results demonstrated that long-term medication for dry mouth patients was more effective. However, as the mechanism remained unclear, molecular biological mechanisms were analyzed based on the effects of continuous administration of pilocarpine using model mice. In the molecular biological analysis, continuous administration of pilocarpine was effective in both ICR and SS model mice. Gene and protein expression of muscarinic acetylcholine receptor 3 (M3R) increased in salivary glands following continuous administration of pilocarpine compared with single administration. Therefore, continuous administration of pilocarpine effectively induced M3R expression, thereby activating salivation.

1. Introduction

Saliva is secreted by salivary glands and has many physiological functions [1,2]. Serious salivation disorders associated with radiation therapy or Sjögren's syndrome (SS) impair oral function. SS affects 4 million Americans and half a million Japanese, and is the third most common rheumatic autoimmune disorder after rheumatoid arthritis and systemic lupus erythematosus [3]. SS dominantly affects premenopausal women with a female to male ratio of 9:1 [4].

Pilocarpine hydrochloride (pilocarpine) is used to treat increased pressure inside the eye as well as dry mouth [5]. Pilocarpine promotes physiological salivation by binding the muscarinic acetylcholine receptor 3 (M3R) in the acinus cells of the salivary glands. Oral pilocarpine is administered to SS patients to treat dry mouth [6]. In the oral cavity, it is used for dry mouth resulting from SS or radiation therapy.

To examine the effects of the continuous administration of pilocarpine in SS patients, we investigated changes in salivation using the Saxon test and visual analogue scale (VAS) referenced from medical records at our hospital. In addition, to evaluate the effects of the continuous administration of pilocarpine and its mechanisms, a molecular biological study was conducted in mice by orally administering pilocarpine. The severe salivation disorder associated with SS causes oral cavity function disorders, but there is no effective treatment to regenerate the glands [7]; therefore, the establishment of a regenerative approach is highly desired.

2. Materials and methods

2.1. Patients

Fourteen adult patients with SS were recruited from Osaka University Dental Hospital, Japan, between April 2011 and March 2017. All patients were selected based on the presence of dry mouth symptoms. All patients were diagnosed with SS, followed-up and came to Osaka University Dental Hospital for dry mouth treatment.

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https://doi.org/10.1016/j.bbrc.2018.06.125
0006-291X/

Data: age, saliva (Saxon test), dosage of pilocarpine and symptoms with VAS were collected from clinical records. The inclusion criteria were as follows: (1) All patients with secondary SS based on the revised classification standards (2002) by the American-European Consensus Group. (2) All patients who took pilocarpine for more than three months; their clinical characteristics are shown in Table 1. (3) Any patient who experienced dry mouth as a side effect of another drug was excluded. SS patients; N = 17 (1 man and 16 women) Age range; 64 ± 11.0 years, with dry mouth who received pilocarpine. Patients were evaluated when the quantity of saliva increased to 2 g/2 min and the Saxon test was considered normal [8]. This study was performed after approval from the Osaka University Ethics Committee (Osaka University Dental Hospital, No. H21-E23, No. H27-E10).

2.2. Saxon test

Two sterile 10 × 10-cm gauze sponges were first folded twice at 90°angles and placed in a sterile, 60-ml plastic tube (Thermo Fisher scientific), and both the dry gauze sponges and tube were weighed. First, patients swallowed to remove any preexisting oral fluid, and saliva was collected by having the individual vigorously chew on the gauze for 2 min. After chewing the gauze for exactly 2 min, patients replaced it in the same tube, and the amount of produced saliva was calculated by subtracting the original weight from the weight obtained after chewing.

2.3. The visual analogue scale (VAS)

Patients evaluated their symptoms using VAS. VAS can be compared with other linear scales by individuals. After taking pilocarpine, patients were asked the same questions at one month and three months.

2.4. Animals

Animal experiments were performed in strict accordance with the guidelines by the Committee of the Faculty of Dentistry, Osaka University (Permit Number: 25-004-0). All animal surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize pain. SMGs were obtained from female ICR mice from Japan SLC Inc. An animal model for primary SS was previously established using NFS/sld mutant mice [9]. NFS/sld mutant mice were obtained from the central institute for experimental animals in Japan. Thymectomy for NFS/sld mice was performed on day 3 after birth. Female mice were given 1 mg/10 mL/kg of pilocarpine (Wako) orally twice a day for two weeks. Distilled water was used for the control group. Each experiment was repeated at least three times.

2.5. The collection of secreted saliva

The mice were weighed and anesthetized with an intraperitoneal injection mixture of xylazine (24 mg/kg) and ketamine (36 mg/kg). The amount of saliva secreted into the oral cavity during 1-min intervals after the injection of pilocarpine (0.1 mg/kg) was measured. The total amount of saliva produced over 30 min was then divided by the weight of the mice.

Table 1
Baseline patients characteristics.

	Average ± S.E.
Sex (male: female)	1:16
Age	64 ± 11.0
Saxon	0.49 ± 0.42

2.6. Measurement of water intake

Mice were housed in plastic cages with free access to food and water. Water consumption was measured for 5 days using a water supply bottle (Shinano manufacturing Co., Ltd) for daily drinking water measurement. The average volume of water per mouse per day was calculated [10].

2.7. SDS-page

Saliva samples from mice were processed immediately after collection as described in the sample pretreatment section. Saliva was mixed with protease and phosphatase inhibitors (Nacalai tesque) with sample buffer (BioRad Laboratories). Following SDS-PAGE, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes (BioRad Laboratories) run at 100 V with running buffer (Nacalai tesque). Proteins were visualized with Ez Stain Aqua (Atto Corporation) after washing in water and incubating overnight at room temperature.

2.8. Histological analysis and immunohistochemistry (IHC)

Salivary glands were fixed in 4% paraformaldehyde-PBS at 4 °C. Paraffin-embedded salivary gland specimens were examined using 4-μm thick sections. We deparaffinized the tissue sections, and antigen retrieval was carried out by autoclaving in instant antigen retrieval H buffer at 121 °C for 5 min. The slides were washed in phosphate-buffered saline (PBS). The samples were first incubated with M.O.M. Mice Ig Blocking Reagent (Vector Laboratories) and then with primary antibodies in diluent (1 × PBS, containing 8% protein concentrate; M.O.M.™ Kit; Vector Laboratories, Inc.) overnight at room temperature. The antibodies were anti-E-cadherin (E-cad) (dilution 1:100; BD Biosciences), anti-M3R (dilution 1:200; Alomone labs) and anti-AQP5 (dilution 1:100; Alomone labs, Jerusalem, Israel). After washing with PBS, the tissues were incubated with Cy2-labeled donkey anti-mouse, Cy3-labeled donkey anti-rabbit and Cy5-labeled donkey anti-rat or anti-goat IgG for 3 hr at room temperature (dilution 1:100; Life Technologies).

2.9. Western blot analysis

The salivary glands were lysed in RIPA buffer (Nacalai tesque) supplemented with protease and phosphatase inhibitors (Nacalai tesque). Cell lysates were centrifuged (15,000 × *g*) for 10 min, and each samples was heated at 95°C for 5 min in denaturing 2 × Laemmli sample buffer (BioRad). Proteins were separated by SDS-PAGE, and then transferred to 0.2-mm pore size PVDF membranes (BioRad). The membranes were blocked with blocking one (Nacalai tesque), and then incubated with either anti-M3R (1:1,000, Cell Signaling Technology), anti-AQP5 (1:1,000, Alomone labs), anti-αSMA (1:1,000, Sigma-aldrich) or anti-β Actin (1:1,000, Sigma-aldrich).

2.10. Quantitative real-time reverse transcription-polymerase chain reaction (qPCR)

The SMGs and SLGs were evaluated using quantitative real-time RT-PCR. Total RNA was extracted from each tissue using Trizol (Life Technologies) and treated with the PureLink® DNase kit (Thermo Scientific) to prevent DNA contamination. For cDNA synthesis, we performed reverse transcription using the Prime-Script RT Reagent Kit (Takara Bio Inc.). RNA samples were stored at −80°C until use for

PCR experiments. qPCR was performed using the MyiQ real-time detection system (BioRad Laboratories) with iQ SYBR Green Supermix (BioRad Laboratories). The amplification program comprised 40 cycles of denaturation at 95°C for 5 sec, annealing at 55°C for 20 sec, and extension at 72°C. Gene expression was normalized to *glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*), and reactions were performed in triplicate. The primer sequences used are shown in Table 2.

2.11. Statistical analyses

The differences among means were evaluated by Bonferroni and Student's two-tailed unpaired t-tests. *P*-values of <0.05 and < 0.01 were considered significant.

3. Results

3.1. Continuous administration of pilocarpine for patients

Salivation ability and subjective symptoms were improved by the continuous administration of pilocarpine, as demonstrated by the results of the Saxon test and VAS. If the amount of saliva increases to 2 g/2 min, the Saxon test is considered normal [8]. The Saxon test score at the start of medication was 0.55 ± 0.05 g in SS patients. Salivation was significantly increased by pilocarpine (Fig. 1A). There were fewer doses than previously recommended [11], and doses per day did not gradually increase. The average dose during continuous administration for longer than 3 months was 1.62 ± 0.20 tablets per day. The VAS was employed to form a clinical opinion of each patient's symptoms over time [12]. Most patients stated that dry and sticky mouth conditions were improved, but there was a large individual difference in tingling sensation (Fig. 1B–D).

3.2. Effects of the continuous administration of pilocarpine on salivation in WT mice

SMGs and SLGs coexist with each other, but their structure and functions are different [13]. After separating SMGs and SLGs, there was no significant difference in weight in the continuation group given pilocarpine. Furthermore, on histological analysis, there was no difference in salivary glands between the continuous administration (CA) group and control group (Fig. 2A–D). In the WT and SS mice, the increase in total salivation in the continuous pilocarpine administration group during 15 min was significantly greater than that in the control group (Fig. 3A and B). Moreover, the amount of salivation was greater with CA than with single administration (SA). The amount of water drank per day did not differ between WT and SS mice (Fig. 3C). We extracted proteins from saliva by SDS-PAGE, but found no differences in protein components between groups (Fig. 3D).

3.3. Protein and gene expression during continuous administration of pilocarpine in WT salivary glands

M3R is a cell receptor for numerous proteins that is located many places in the body such as smooth muscles, the endocrine glands, lungs, pancreas and the brain [14]. The expression of M3R with CA was significantly higher than with the control (Fig. 4A). M3R is a muscarinic acetylcholine receptor encoded by the gene *Chrm3*. In both SMGs and SLGs, SA caused no change in the salivary gland receptor *Chrm3*, whereas CA significantly increased the markers compared with the control (Fig. 4B). On IHC, there was no difference in expression between CA and the control (Fig. 4C–F).

Table 2
In PCR analysis, the sets of synthetic primers that were used as follows.

Gapdh	5′-CCATCACCATCTTCCAGGAG-3′
	5′-GCATGGACTGTGGTCATGAG-3′
M3r	5′-TCGGTAGAGCGGACTGGACA -3′
	5′-TCCACTGAGCAAGTCAGAAGTGAAG -3′

4. Discussion

Pilocarpine is currently used to treat dry eyes and mouth [6]. Pilocarpine may increase salivation and symptomatically relieve patients with xerostomia who have reduced salivary gland function. Pilocarpine promotes salivation by stimulating M3R in the acinus cells of the salivary glands [15]. Moreover, pilocarpine was reported to have similar effects and side effects with cevimeline hydrochloride hydrate [11]. However, its affinity for the muscarinic acetylcholine receptor, pharmacokinetics and properties are different [16]. In this study, we only investigated patients who received pilocarpine. Regarding side effects, pilocarpine caused less nausea than cevimeline, but it was difficult to continuously administer for many patients [17]. Many countermeasures have been reported for side effects. We decide to leave the dosing to the patients' discretion at our hospital. The dosage of pilocarpine was less than the recommended amount. In a survey of SS patients, salivation ability was increased and subjective symptoms decreased with continuous administration of pilocarpine, as demonstrated by the results of the Saxon test and VAS. After pilocarpine treatment, there was significant improvement in the feeling of oral dryness and mouth comfort. This study clarified the effectiveness of continuous administration of pilocarpine. One limitation of this survey is that most subjects were women. Although this was a limitation, SS is more prevalent among women [4].

In addition, to evaluate the effects of continuous administration of pilocarpine and its mechanisms, a molecular biological study was conducted using mice that received oral pilocarpine [18]. A previous study reported that the continuous administration of pilocarpine is effective for dryness [19]. However, as its mechanism remained unknown, the molecular biological mechanisms were analyzed based on the effects of continuous administration of pilocarpine using model mice.

In the mice experiment, the CA mice group had increased salivation compared with the SA and control mice groups. In this study, the expression of AQP5 and other salivary gland markers did not change, but the expression of M3R increased during the continuous administration of pilocarpine. Furthermore, in NFS/sld mice, SS model mice, the M3R protein level was increased in CA mice groups (Data not shown). It was previously reported that the expression of AQP5 did not change in mouse salivary glands with the continuous administration of pilocarpine [20]. Some studies found that operation medicines can influence receptors themselves by binding receptor agonists that to receptors repeatedly for a long time [21]. When receptors are affected by agonists for a long time, receptors were up-regulated and sensitivity was increased [22,23]. Our results and prior studies demonstrated that the expression of M3R was increased by the continuous administration of pilocarpine.

Of note, not only ICR mice but also NFS/sld mice exhibited increased salivation by the continuous administration of pilocarpine. NFS/sld mice have an autosomal recessive gene causing sublingual gland arrest. Significant inflammatory changes spontaneously developed in the exocrine glands of NFS/sld mutant mice that underwent thymectomy 3 days after birth. Thymectomy was performed on day 3 after birth on the NFS/sld female mice to establish and characterize an animal model of primary SS [24]. In this survey, we analyzed patients with secondary SS; therefore, we adopted NFS/sld female mice as a model of SS [25].

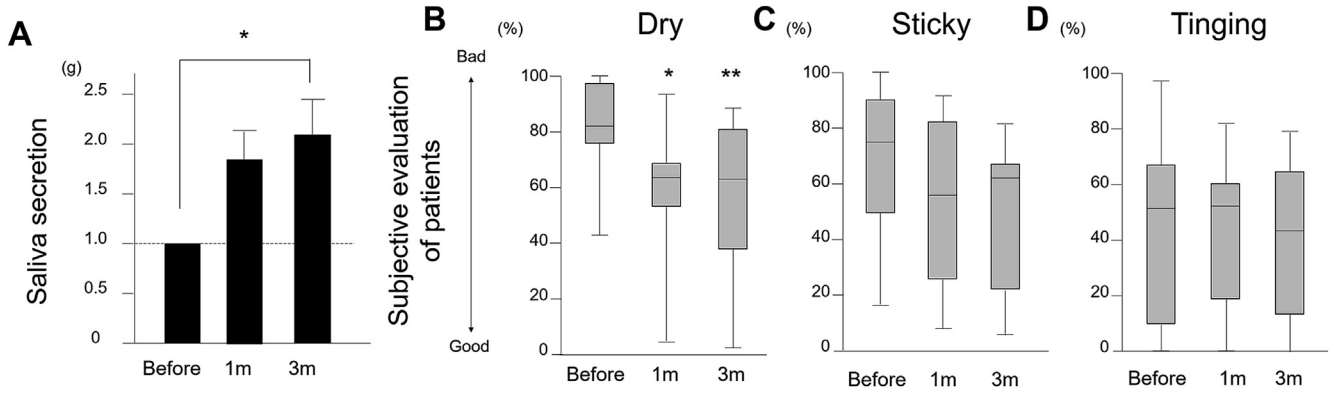
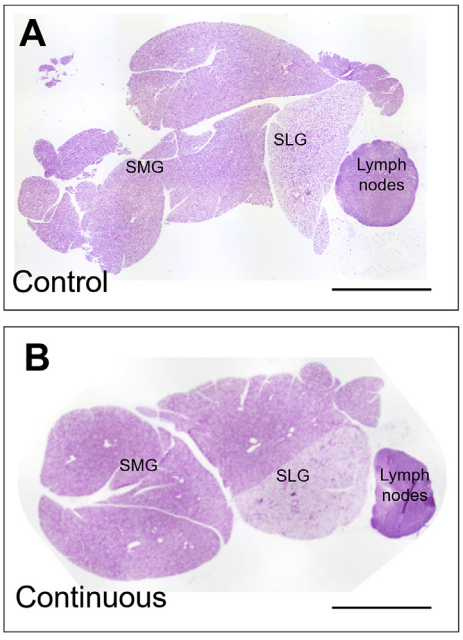


Fig. 1. Improvement of salivation and reduction of oral dryness in xerostomia patients. (A) Amount of salivation (Saxon test). Saliva evaluation consisted of chewing on folded gauze for 2 min. Saliva production was quantified by weighing the gauze before and after chewing. (B–D) Mean symptom scores for dry mouth on the visual analogue scale for Sjogren's syndrome patients. Box plot comparing visual analogue scale scores between before treatment and during treatment with pilocarpine at 1 month and 3 months. Dry (B), Sticky (C) and Tingling (D). $^{*}P < 0.05$, $^{**}P < 0.01$ respectively by the Bonferroni method.



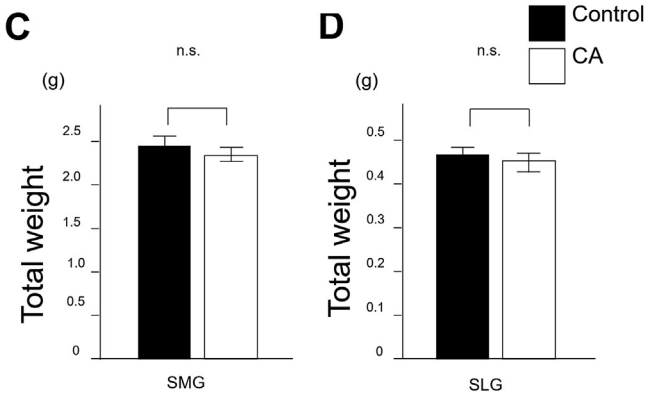


Fig. 2. Histological analysis and weights of the salivary gland tissues. (A, B) Histological analysis of SMGs and sublingual glands (SLGs). There was no difference between the control group and pilocarpine treatment group in hematoxylin-eosin staining images. (C, D) Weights of SMGs and SLGs. There were no differences between the control group and pilocarpine treatment group.

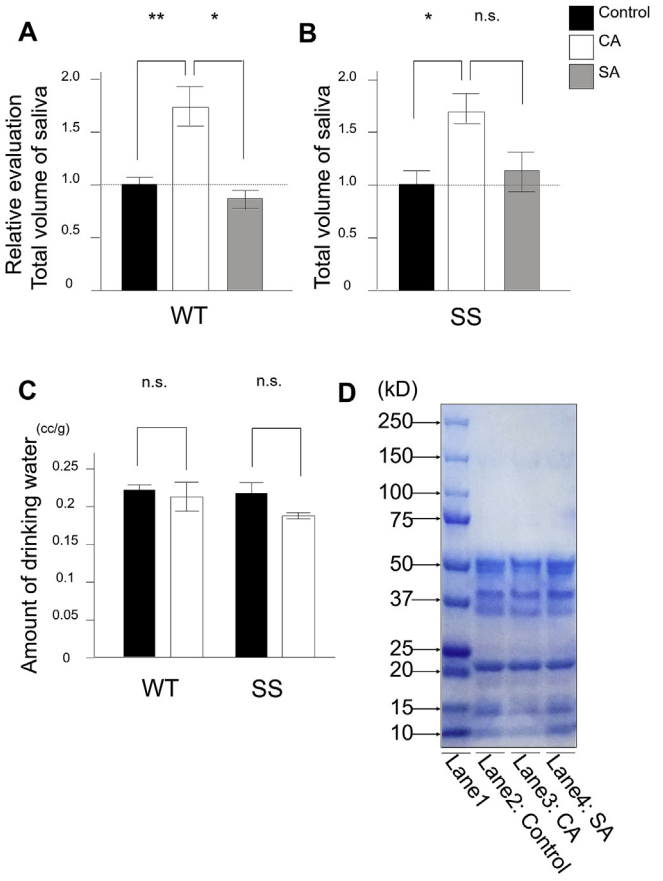


Fig. 3. Effects of the continuous administration of pilocarpine on salivation. (A, B) The total volume of saliva secreted in 15 min in WT mice (A) and in SS mice (B). $^{*}P < 0.05$, $^{**}P < 0.01$, respectively by the Bonferroni method. CA, continuous administration; SA, single administration. (C) The amount of drinking water was evaluated using water supply bottles from WT mice and SS mice treated with pilocarpine or control. (D) SDS–PAGE of mouse saliva. Lane 1 represents the molecular weight markers. Lane 2 represents proteins in control mouse saliva. Lane 3 represents proteins present after CA of pilocarpine and Lane 4 is proteins after SA of pilocarpine.

Salivation and the relief of symptoms were maintained during pilocarpine administration even at lower doses than recommended. In addition, the mouse experiments clarified that continuous administration is critical for maintaining salivation, suggesting the involvement of increases in the receptor M3R. Thus,

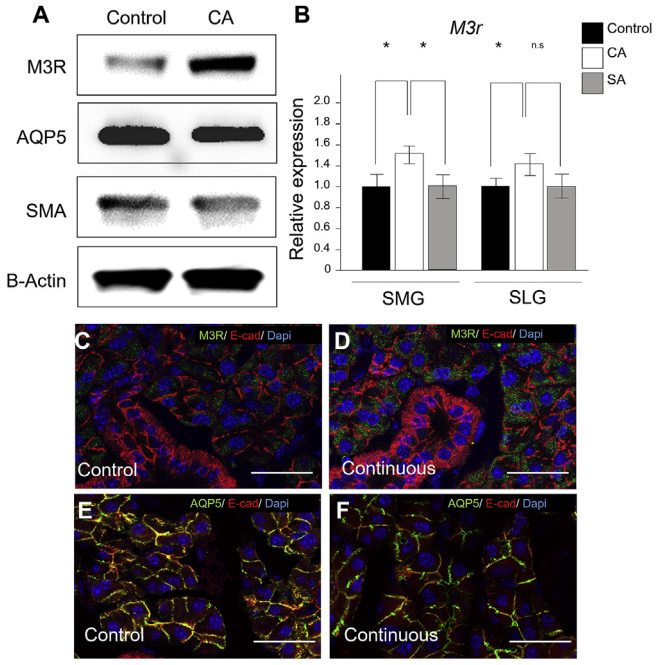


Fig. 4. Protein and gene expression throughout the salivary gland during continuous administration of pilocarpine. (A) Muscarinic acetylcholine receptor 3 (M3R), aquaporin 5 (AQP5), smooth muscle actin (SMA) and β-actin expression on Western blotting analysis for control and CA of pilocarpine. (B) *M3r* mRNA expression in the control, CA and SA groups by qPCR. $^{*}P < 0.05$ by the Bonferroni method. (C, D) The expression of M3R in the submandibular glands (SMGs) of control and CA mice on P50 by immunostaining. M3R is located inside the basement membrane. M3R (green), E-cadherin (red), DAPI (blue). Scale bars: 25 μm. (E, F) The expression of AQP5 in the SMGs of control and CA mice by immunostaining. AQP5 is located in the apical membrane of acinus cells. AQP5 (green), E-cadherin (red), DAPI (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the dose of pilocarpine should be adjusted depending on the severity of side effects, and continuous administration is effective for dry mouth. Elucidation of molecular mechanisms will provide novel therapeutic strategies for SS patients such as regenerative medicine with pilocarpine targeting acinus cells. Our study provides new insights for future research into the regeneration of organs such as salivary glands.

Acknowledgements

We thank Ms. Ryoko Hoshino and Dr. Takumi Matsushita, Department of Oral-facial Disorders, Osaka University Graduate School of Dentistry for their excellent technical supports.

This work was supported by JSPS KAKENHI Grant Number 15H02575 and 16K20573.

The authors declare no conflicts of interest associated with this manuscript.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2018.06.125.

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