Changes of salivary functions in experimental periodontitis model rats

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ABSTRACT

OBJECTIVE: This study was designed to investigate the mechanism of salivary dysfunction in an experimental periodontitis rat model and to examine the improvements in salivary secretion following treatment of the experimental periodontitis.

METHODS: In the experimental periodontitis rat model, which included a unilateral ligature for 4 weeks around the second upper molar, several salivary functions were investigated. Changes in the salivary function were evaluated 4 weeks after removal of the ligature in some rats.

RESULTS: The periodontitis model showed significant reductions in the weight of the bilateral major salivary glands and pilocarpine-induced salivary secretion. The model also showed an increase in the number of apoptotic cells in bilateral salivary glands. According to Ca^{2+} imaging and Western blotting, there were no differences in the muscarine-induced intracellular Ca^{2+} mobilization in acinar cells or in the M3 receptor and AQP5 expression levels in the salivary glands between the sham and the periodontitis model. Following removal of the ligature, differences in the weights of salivary glands and pilocarpine-induced salivary secretion between the sham and the periodontitis model animals were not found.

CONCLUSION: These results suggest that experimental periodontitis leads to hyposalivation and that relief from it improves salivary function. It is likely that lower levels of salivary secretion are caused by the decrease of functional acinar cells in salivary glands in the experimental periodontitis model, and the bilateral gland effects in the unilateral periodontitis model are caused by systemic rather than by local effects.

Introduction

Saliva plays various important roles in the maintenance of oral health and contributes to the stability of tooth enamel, oral immunity and moisture retention in the oral mucous membrane. However, saliva secretion is easily disordered, i.e. by aging, autoimmune dieases, neck radiotherapy and amounts of drugs.[1] Rapid structural and functional changes of salivary glands, such as weight, morphology and salivary activity including salivary secretion, are reported in experimental animals.[2,3] Some clinical studies have reported low salivary secretion rates in periodontitis patients.[4,5] Animal studies suggest that desalivation leads to periodontitis.[6] Based on these human and animal studies, it is thought that low salivary status is a risk factor for periodontitis.

An experimental periodontitis model in rats that employs the ligation of molars has been frequently used in dental research to investigate the pathology of periodontitis. Ligatures around the cervical area of the molars in rats allow for plaque accumulation, periodontal inflammation and alveolar bone loss in the periodontal tissues, similar to the effects observed in human periodontitis. Interestingly, recent studies have reported that the experimental periodontitis model exhibits vacuolization and apoptosis of salivary glands and a reduction of salivary secretion.[7,8,9] These animal studies suggest that periodontitis leads to hyposalivation. Combined with the above-mentioned conventional knowledge in the clinical field, periodontitis may become trapped in a negative spiral and progressively worsen.

The aims of the present study were to verify whether experimental periodontitis leads to hyposalivation and whether relief from the experimental periodontitis leads to improvement of salivation, as well as to clarify the mechanisms in detail. For this purpose, we investigated changes in salivary functions in the experimental

periodontitis model in rats. A unilateral ligation of the maxillary molar in rats was employed for the experimental periodontitis model. Changes of salivary functions in the experimental rats were compared with those of sham rats. Removal of the ligation was used to evaluate recovery from the experimental periodontitis.

Materials and Methods

Experimental animals and creation of the periodontitis model

The experiments were conducted on male Wistar rats. All rats were housed individually in plastic cages under regular light/dark conditions (lights on from 8:00 a.m. to 8:00 p.m.). The temperature was maintained at 23 ± 1°C. The rats had access to water and laboratory pellets *ad libitum* except during the experimental periods. The study design was approved by the institutional review board of Kyushu Dental University.

Following the induction of general anesthesia through intraperitoneal injection of sodium pentobarbital (50 mg/kg), the 6-week-old rats were fixed on a stereotaxic frame on their backs, and the unilateral second maxillary molar (the right side alone) was tied with a 4-0 silk ligature (Ethicon, NJ, USA) in the cervical area. In sham rats, the ligature was removed just after the ligation. The experiments for the experimental periodontitis were performed at 4 weeks after the ligation (PerioM). In another experiment, the ligature was removed 4 weeks after ligation in some rats. Four weeks later, recovery from the experimental periodontitis was evaluated (Re-PerioM).

To confirm the establishment of periodontitis in the model, alveolar bone destruction and gingival inflammation in the maxillary region were investigated 4 weeks after ligation. After being anesthetized with

sodium pentobarbital (50 mg/kg, intraperitoneally), the rats were transcardially perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS) or with saline alone, and the maxillary regions were removed. X-ray images of the maxillary regions were taken on films with a handmade metal scale (1 cm) in the buccal-lingual direction using soft X-ray equipment (Softex, Kanagawa, Japan). The X-ray films were then scanned into a computer, and the distances from the central point between the respective cemento-enamel junctions (CEJs) of neighboring molars and the top of the alveolar bone crests (ABCs) were calculated. In the analysis, a few bones that had been de-fleshed through boiling were also used. Other specimens were then decalcified for 3 weeks in a 10% EDTA-containing solution (pH 7.4, adjusted by NaOH) or for 2 weeks in a 10% formic acid-containing solution and processed for histology. Combined with the teeth and periodontal tissues, 10-μm-thick slices were created using a microtome in the rostral-caudal direction after the tissues were embedded in paraffin, and the slices were then stained with hematoxylin and eosin (HE). The histological observations were performed between the first and second maxillary molars under an optical microscope.

Measurements of salivary secretion

To evaluate the salivary secretory ability, rats anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally) were intraperitoneally injected with pilocarpine (10 μmol/kg, Kanto Chemical, Tokyo, Japan). The rats were placed in the supine position. Whole saliva was collected from the oral cavity with pre-weighed cotton balls for 1 h. One milligram of whole saliva was regarded as 1.0 μL, and the saliva output was standardized by body weight.

Measurement of gland weights and histology

After being anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally), the rats were transcardially perfused with PBS, and the parotid gland (PG) and submandibular gland (SMG)/sublingual gland (SLG) were removed and immediately weighed. After post-fixation in 4% paraformaldehyde or 10% formaldehyde for over 48 h, some of the removed salivary glands were cut into 6-μm-thick slices by a freezing cryostat or a microtome after being embedded within paraffin and stained with HE.

To detect apoptotic cells in the SMG and PG, TUNEL methods were performed. An ApopTag in situ apoptosis detection kit (Millipore, MA, USA) was used to carry out TUNEL staining as described elsewhere.[10] The sections were treated with 0.02 mg/mL proteinase K (Wako, Osaka, Japan) for 15 min at room temperature and incubated with 3% H_2O_2 for 5 min at room temperature. After rinsing, the sections were incubated in equilibration buffer for 10 min, and then terminal deoxynucleotidyl transferase and dUTP-digoxigenin were added to the sections, which were then incubated in a 37°C humidified chamber for 1 h. The reaction was then stopped, and the slices were washed and incubated with anti-digoxigenin-peroxidase solution, colorized with DAB/H_2O_2, and counterstained with hematoxylin. Two examiners, who were blinded to the treatment assignment, performed the following histometric analyses using a light microscope. The apoptotic cells in acinar cells were counted at a magnification of ×400 in 15 randomly selected fields.

Parotid cell preparation and Ca^{2+}-imaging

The Ca^{2+}-imaging technique was used to investigate whether acinar cells in salivary glands show different physiological functions in the sham and PerioM. The cell preparation has been previously described in detail.[11,12,13] Following induction of general anesthesia with intraperitoneal sodium pentobarbital (60 mg/kg), the

PG was immediately removed and placed in a cold balanced salt solution (BSS) containing 120 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 20 mM Hepes (adjusted to pH 7.4 by NaOH) and 0.5% bovine serum albumin. After mincing, the material was then digested for 30 min at 37°C with 50 U/mL collagenase (Wako, Osaka, Japan) in BSS, and the suspension was gently passed through a pipette 10 times every 10 min. After they were filtered through a 100-μm nylon mesh, the cell preparations were loaded with 2 μM fura-2AM (Dojindo, Kumamoto, Japan), which was suspended in 10 mL of BSS, and incubated for 30 min at room temperature. The preparation was rinsed twice and stored at 4°C until the time of the experiment.

After the cell preparation was dispersed on poly-*d*-lysine-coated glass-bottom dishes (MatTek, MA, USA), the dishes were mounted on the stage of an inverted fluorescence microscope (Olympus, Tokyo, Japan) and perfused with BSS at a rate of 1 mL/min at room temperature. The excitation of fura-2 was induced every second by alternate illuminations of 340 and 380 nm light, and the resultant fluorescence (510-550 nm; F340 and F380) was measured using an objective lens (Olympus, Tokyo, Japan) and a silicon-intensified target camera (Hamamatsu Photonics, Hamamatsu, Japan). Muscarine (Sigma-Aldrich, MO, USA) was applied to the cells by superfusion from separate storage bottles that contained the media to which they had been added. Acini were observed as spherical or ovoid structures that had a globular appearance, while the ducts showed obvious tubular structures.[11] In the present study, cell clusters consisting of 3-7 acinar cells were selected.[12] The ratio of 340/380 nm indicated the relative free cytosolic Ca^{2+} concentration. All Ca^{2+} responses induced by the drugs were expressed as Δ ratios by subtracting the basal ratio values before the drug applications from the peak ratio

values during the drug applications.

Western blotting

In the preparation of the cells for Ca^{2+} imaging, parts of the ipsilateral PG were removed from both the sham and PerioM for Western blotting. The tissues were homogenized using a Dounce-type glass tissue homogenizer in 20 mM PIPES/NaOH buffer (pH 7.0), which contained 1 mM EDTA and a protease inhibitor cocktail (Roche, Basel, Swiss). Crude extracts were obtained as supernatants by centrifugation (2,000 x g, 10 min, 4°C) and then subjected to a protein assay.[14] Bovine serum albumin was used as the standard. Small aliquots of crude extracts (20 μg of protein) were separated on 10% polyacrylamide gels according to the method of Laemmli,[15] and they were electrophoretically transferred to Immobilon-P membranes (Millipore, MA, USA). The membranes were blocked in Tris-buffered saline (TBS) containing 5% skim milk and 0.1% Tween 20 and then probed with anti-M3 rabbit polyclonal antibody (Biomol, PA, USA), anti-AQP5 rabbit polyclonal antibody (Alpha Diagnostic, TX, USA) and anti-ß-actin mouse monoclonal antibody (Sigma-Aldrich, MO, USA) overnight at 4°C. The membranes were subsequently washed with TBS containing 0.1% Tween 20 and incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz, CA, USA) for 1 h. Immunoreactive bands were detected using enhanced chemiluminescence (Amersham, NJ, USA).

Measurement of mean blood pressure

To examine the effect of periodontitis on cardiovascular regulation, the mean blood pressure (MBP) was recorded under conscious conditions with a tail-cuff system (Softron, Tokyo, Japan) in a cylindrical heating system that was set to 38°C, and the results were analyzed with a software (Softron, Tokyo, Japan).

Statistical analysis

Numerical values were represented as the mean ± SE in the present study. The two-tailed unpaired Student's *t*-test was used to compare all of the parameters, except for Ca^{2+}-imaging, between the sham and experimental periodontitis model rats. The two-way ANOVA was used for Ca^{2+}-imaging. Less than 5% probability was used to establish statistical significance.

Results

Experimental periodontitis model findings

Fig. 1A shows the alveolar bone appearance on X-ray images in the ligatured and contralateral sides in the sham and PerioM groups. The alveolar bone loss was calculated as the vertical distance between the CEJs and ABCs on X-ray images, and it was significantly larger in the ligatured region of PerioM (n = 11) compared with the same side in sham animals (n = 9) (Fig. 1B). Furthermore, as shown in Fig. 1C, the ligatured region exhibited obvious inflammatory features in the gingiva and destruction of the connective tissue with alveolar bone loss at 4 weeks post-ligation (n = 4) compared with the contralateral side. These results suggest that the ligation of the second upper molar induces alveolar bone loss at 4 weeks post-ligation, thus confirming the establishment of periodontitis in our laboratory model.

Fig. 1 here.

To examine the salivary secretory function, we measured saliva outputs for 1 h following pilocarpine administration in the sham and PerioM animals. There was no significant difference in body weight between the

sham and PerioM animals (Fig. 2A). At 4 weeks post-ligation, saliva output was significantly lower in the PerioM than in the sham animals (Fig. 2B). At 4 weeks post-ligation, the total weight of the three major salivary glands in the PerioM group was significantly reduced to 79% (1611 ± 44 mg, $n = 5$, $P < 0.01$), compared with that of the sham animals (2032 ± 36 mg). As shown in Figs. 2Ca and Cb, significant reductions in the weight of the bilateral glands were seen in the unilateral PerioM group.

Fig. 2 here.

The number of apoptotic cells in the PG and the SMG was compared between the sham (n = 4) and PerioM animals (n = 4) (Fig. 3). The number of TUNEL-positive acinar cells with brown nuclei was significantly increased in the PerioM group compared with the sham group in the bilateral PG (Fig. 3C) and SMG (Fig. 3F). Compared with the sham animals (Fig. 3D), vacuolization of the acini was observed in the bilateral SMG in the PerioM animals (Fig. 3E). In addition, there was no obvious infiltration of inflammatory cells in the entire salivary glands in HE stainig sections (the sham and the PerioM: n = 9 and 9, respectively).

Fig. 3 here.

Saliva secretion from the salivary glands is closely linked to increase in the intracellular Ca^{2+} concentration in salivary acini, and the Ca^{2+} response is mainly induced via muscarinic receptors following acetylcholine release from the parasympathetic nerve.[16,17] To examine the effect of periodontitis on cellular function in the salivary gland, muscarine-induced Ca^{2+} mobilization in digested parotid acini and the expression levels of M3 receptor and AQP5 were compared between the sham and PerioM animals. In the experiment, we selected acini in the PG because this gland consists of homogeneous serous acini and shows no vacuolization aspects on

histology. From 6 sham and 5 PerioM rats, 50 and 55 acini were analyzed, respectively. There was no significant difference in the basal ratio of the intracellular Ca^{2+} concentration between the sham and PerioM animals (0.78 ± 0.02 and 0.78 ± 0.03, respectively). There was no significant difference in the dose-dependent muscarine-induced Ca^{2+} responses between the sham and the PerioM animals (Fig. 4A). There was no difference in M3 receptor and AQP5 expression levels detected between the sham and PerioM animals on Western blotting (n = 7 each, Fig. 4B).

Fig. 4 here.

We tested whether salivary functions were recovered when the ligature was removed after 4 weeks of ligation. Four weeks later, recovery from the PerioM was evaluated by histological observation of maxillary regions, measurement of the distance between the CEJs and ABCs, and weights of salivary glands and salivary secretion (Fig. 5). The distance between the CEJs and ABCs on the ipsilateral side at 4 weeks ligation and 4 weeks removal (Re-PerioM in Fig. 5A, 0.70 ± 0.03 mm; n = 9) was still significantly larger than on the contralateral side (0.30 ± 0.02 mm) and at 8 weeks in the sham animals (8-week sham in Fig. 5A, 0.31 ± 0.04 mm; n=5), while it was smaller than that at 4 weeks ligation (PerioM in Fig. 1B, 1.10 ± 0.06 mm). In the histological observation of gingival and alveolar bone, the ligatured region exhibited no obvious inflammatory features in the gingival (Fig. 5B). However, a small amount of alveolar bone loss was seen in the Re-PerioM group. Figs. 5Ca and Cb show no changes in weights of the PG and SMG, respectively, on both the ipsilateral and contralateral sides of the Re-PerioM animals (n = 7), compared with those of the 8-week sham group (n = 8). Fig. 5D shows no significant difference in saliva outputs between the 8-week sham (n = 8) and the Re-PerioM

groups (n = 6). These results suggest that physiological salivary functions at least recovered after relief from experimental periodontitis, although alveolar bone did not completely recover.

Fig. 5 here.

Because atherosclerosis has been reported in the periodontitis model,[18] we measured the MBP in the sham and PerioM rats. However, there was no significant difference in the MBP between the sham (97.0 ± 4.4 mmHg, n = 5) and PerioM (98.2 ± 5.1 mmHg, n = 5) groups.

Discussion

The present study evaluated changes in salivary function using an experimental periodontitis model (PerioM) with a unilateral ligature around the second upper molar. In the PerioM group, saliva output following pilocarpine administration was lower than in the sham animals. After removal of the 4-week ligature (Re-PerioM), they were consistent with those in the sham animals (8-week sham). The results supported the hypothesis that periodontitis (or periodontal inflammation) contributes to hyposalivation[7,8,9] and suggested that periodontal treatment improves salivation. Periodontitis patients have shown low salivary status in early cross-sectional studies.[4,5] Considering the results from the present study, this is likely reflected by not only the intrinsic low salivary status before the development of periodontitis but also the salivary hypofunction secondary to periodontitis. Conversely, salivary function of periodontitis patients may progressively improve following periodontal treatment.

In the present study, there was no infiltration of inflammatory cells in the entire salivary glands in the

PerioM group, which was consistent with the results of other studies.[7,8,19,20] Nevertheless, we found a significant increase in the number of apoptotic cells in the PG and SMG in the PerioM group. These results are consistent with those of early studies, which demonstrated significant apoptosis and/or vacuolization in the salivary glands in the periodontitis model 2 or 4 weeks after ligation.[7,9] The present study also demonstrated that there were no differences between the sham and the PerioM animals in M3 receptor and AQP5 expression levels and the muscarine-induced intracellular mechanism of the acini. In addition, there were no differences in the MBP between them. Previous studies show that structural and functional changes are rapidly occurred in salivary glands.[2,3] Taken together, the low salivary secretion induced by pilocarpine in the PerioM animals was likely related to the decrease in the number of functional cells in salivary glands rather than to changes in the cellular mechanisms in the salivary glands and the dysfunction of cardiovascular regulation. In addition, recovery of salivary secretion after removal of the 4-week ligature (RE-PerioM) was likely related to the recovery in the number of functional cells in the salivary glands.

Importantly, an increase in the number of apoptotic cell deaths was observed bilaterally in the unilateral PerioM group. Ekuni et al.[7] reported vacuolization and apoptosis in salivary glands in a bilateral periodontitis model. They and other groups also reported that the rats with experimental periodontitis showed increases in the plasma levels of reactive oxygen metabolites, TNF-α[7] and corticosterone.[21] Although we need to identify them in the present PerioM group, such circulating substances in the blood stream may result in changes in the salivary glands.

An early study that used a unilateral periodontitis model reported that, at 1 week after the ligation of the

first lower molar, the reduction of methacholine-induced saliva output from the SMG was predominantly induced on the ipsilateral side,[8] which was different from our results. This early study reports that alveolar bone loss was approximately 0.5 mm. We temporally tested the alveolar bone loss at 1 week post-ligation with the same method in the present study and obtained approximately 0.3 mm on the side ipsilateral to the ligation and none on the contralateral side. Therefore, we thought that the ligature in the early study was more severe than in the present study. Such severe periodontitis in the lower molar, which is near the location of the SMG, may lead to predominant dysfunction of the ipsilateral SMG due to the direct diffusion of some inflammatory factors. In contrast to the results of the present study and the other study,[9] another research group has shown a slight enlargement of the SMG in a bilateral periodontitis model 22 days after ligation of the lower molars.[19] Because the group also reported significant decreases in the absolute mucin level in the SMG,[19] the enlargement of the SMG might have been caused by edema of the SMG rather than an acceleration of gland function. Any discrepancies among the studies that have employed experimental periodontitis models may be explained by different ligature sites, ligation periods, ligature methods and microbiological circumstances in each laboratory.

The present study showed that periodontitis or periodontal inflammation causes salivary changes in rats. However, it may be possible that any type of inflammation in the mouth or even in any region of the body could create similar effects on the salivary glands. Thus, the effects may not be specific for periodontal disease. In order to reveal the mechanism of changes of salivary functions in experimental periodontitis, it is necessary to clarify this question in the future.

In conclusion, the results obtained from the PerioM animals support the hypothesis that periodontitis (or

periodontal inflammation) leads to hyposalivation and suggest that dysfunction of the salivary glands is improved following periodontal treatment.

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Figure legends

Fig. 1 Alveolar bone loss in the periodontitis model secondary to ligation of the unilateral second maxillary molar. (A) Alveolar bone appearance (left panels) and X-ray images (right panels) in the periodontitis model at 4 weeks post-ligation. Compared with the contralateral side (a), the ligation side (b) showed obvious alveolar bone loss (scale bars = 1 mm). The black and white arrowheads indicate the cemento-enamel junctions (CEJs) and

alveolar bone crests (ABCs), respectively (right panels). (B) Distances from the CEJs to the ABCs on the ligation side of the periodontitis model at 4 weeks post-ligation (PerioM) were significantly increased, compared with those in the sham group and the contralateral sides of the PerioM group. ** represents $P < 0.01$ against the sham of the same side. Contra., contralateral. (C) Histology of alveolar bone and gingiva in the periodontitis model. Specimen was decalcified in a 10% EDTA-containing solution. Compared with the contralateral side (a), the ligation side (b) after 4 weeks showed obvious alveolar bone loss and gingival inflammation (scale bars = 0.25 mm).

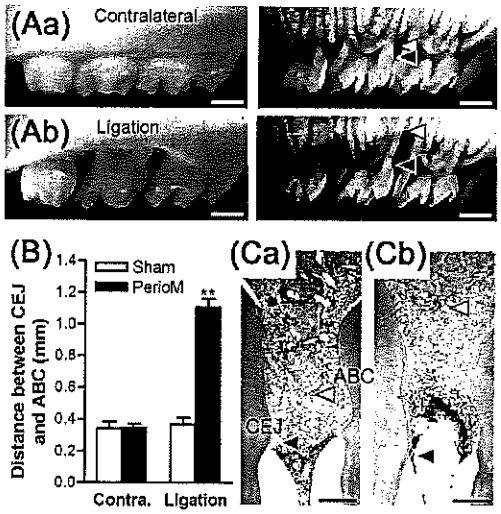
Fig. 2 Pilocarpine-induced saliva output and salivary gland weights in the experimental periodontitis model (PerioM). (A) Body weights in the sham and PerioM animals. (B) Saliva output for 1 h following pilocarpine administration (10 μmol/kg, *i.p.*) in the sham and the PerioM groups. * represents $P < 0.05$ relative to the sham group. (C) Weights of the parotid gland (PG) (Ca) and the submandibular and sublingual glands (SMG/SLG) (Cb) of the ipsilateral (Ligation) and contralateral (Contra.) sides in the sham and the PerioM groups at 4 weeks post-ligation. * and ** represent $P < 0.05$ and 0.01 relative to the same side in the sham, respectively.

Fig. 3 Comparison of apoptosis in salivary glands between the sham (Sham) and experimental periodontitis models (PerioM) using the TUNEL method. Compared with the sham group (n = 4), an increased number of TUNEL-positive acinar cells with brown nuclei was observed on both the ipsilateral and contralateral sides of the salivary glands in the PerioM group (n = 4). (A) and (B) show the TUNEL staining images with hematoxylin

of the ipsilateral sides of the parotid glands (PG) in the sham and the PerioM, respectively. (C) The apoptotic index in the ipsilateral and contralateral sides of the PG of the PerioM was significantly higher than in the sham animals. (D) and (E) show the TUNEL staining images with hematoxylin of the ipsilateral sides of the submandibular glands (SMG) in the sham and the PerioM, respectively. (F) The apoptotic index on the ipsilateral and contralateral sides of the SMG of the PerioM group was significantly higher than that in sham group. Note that the number of vacuolizations was increased in the acini of the SMG in the PerioM animals (arrow heads). Scale bars = 50 μm.

Fig. 4 Muscarine-induced increase in the Ca^{2+} concentration and expression levels of M3 receptors and AQP5 in digested parotid acini of the model 4 weeks post-ligation. (A) Change in the intracellular Ca^{2+} concentration following muscarine application in the parotid acini of the sham and periodontitis model (PerioM). The intracellular Ca^{2+} concentrations increased in a dose-dependent manner (left panel). There was no difference in the change in the intracellular Ca^{2+} concentration in the parotid acini between the sham and the PerioM animals (right panel). (B) Western blotting for the M3 receptor and AQP5 in the parotid gland (PG) of sham and PerioM animals (each n = 7). Crude extracts from the PG of sham and PerioM animals were analyzed for expression of M3 receptors and AQP5 by immunoblotting with specific antibodies as indicated. β-actin was used as a loading control. The results presented are representative of seven independent similar experiments. Note that there was no difference in the M3 receptor and AQP5 expression levels between the sham and the PerioM groups.

Fig. 5. Effects of removal of ligature after 4 weeks ligation on the distance between the cemento-enamel junctions (CEJs) and alveolar bone crests (ABCs), the weight of salivary glands and the saliva output. The ligature was removed after 4 weeks of ligation, and 4 weeks later, the parameters were evaluated. (A) Effects of the removal of the ligature on the distance between the CEJs and ABCs. The distance was calculated by using X-ray films. Compared with the contralateral side (Contra.) and the sham group, the ligation side (Ligation) still showed obvious alveolar bone loss in the model of 4 weeks ligation and 4 weeks removal (Re-PerioM) (scale bars = 1 mm). ** represents P <0.01 against the 8-week sham at the same side. (B) Histology of alveolar bone and gingiva in the Re-PerioM group. Specimen was decalcified in a 10% formic acid-containing solution. Compared with the contralateral side (a), the ligation side (b) of Re-PerioM still showed obvious alveolar bone loss (scale bars = 0.25 mm). The black and white arrowheads indicate the CEJs and ABCs, respectively (right panels). (C) Effects of removal of the ligature on the weight of the parotid glands (PG) (Ca) and the submandibular and sublingual glands (SMG/SLG) (Cb) of the ipsilateral (Ligation) and contralateral (Contra.) sides. There were no significant differences in the weights between the ligation side and the contralateral side, and between the 8-week sham and the Re-PerioM groups. (D) Effects of removal of ligature on saliva output. There were no significant differences in the saliva output between the 8-week sham and the Re-PerioM groups.



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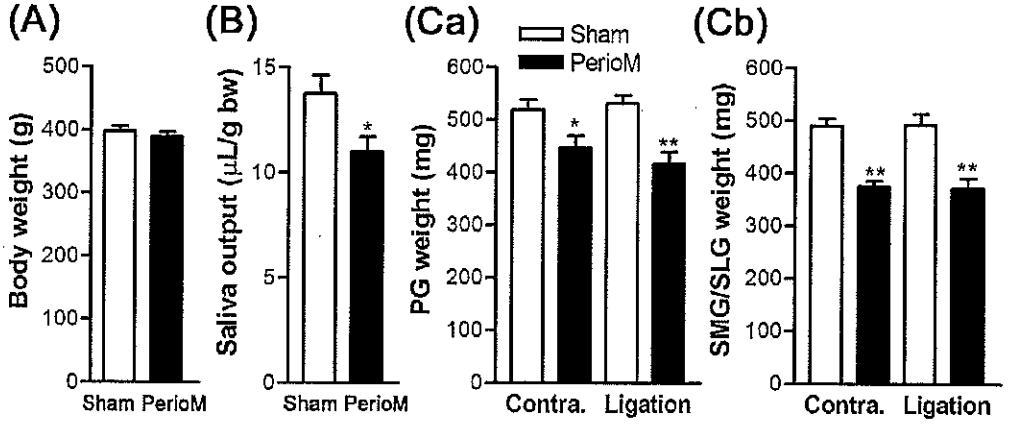

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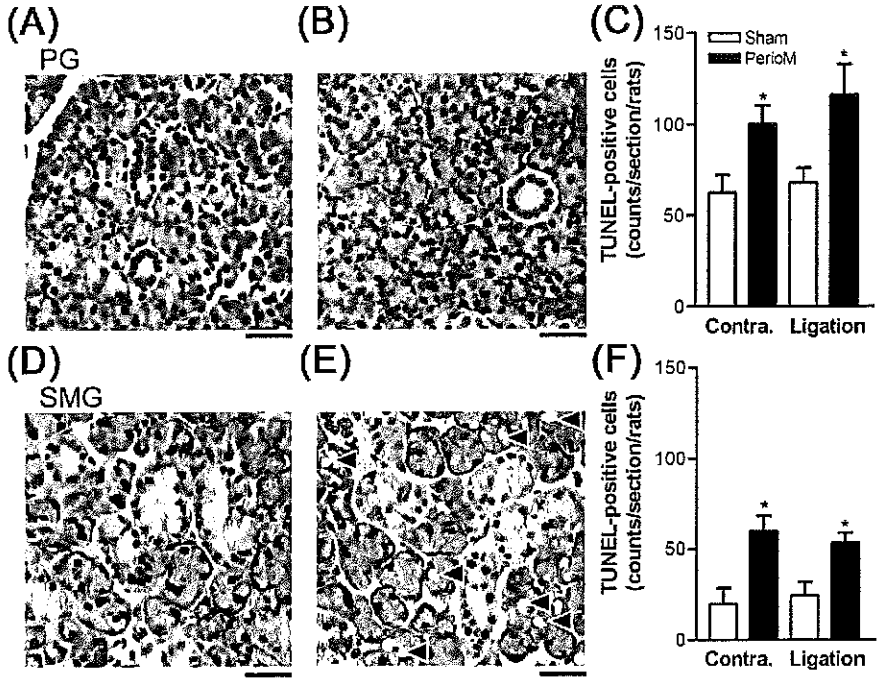

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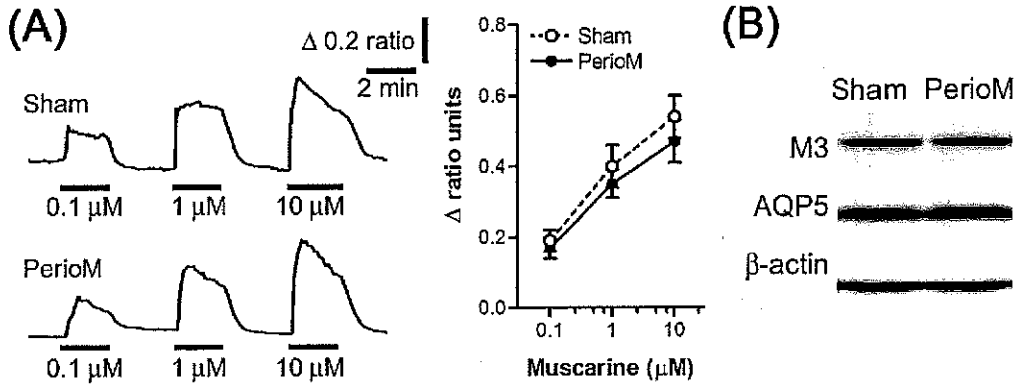

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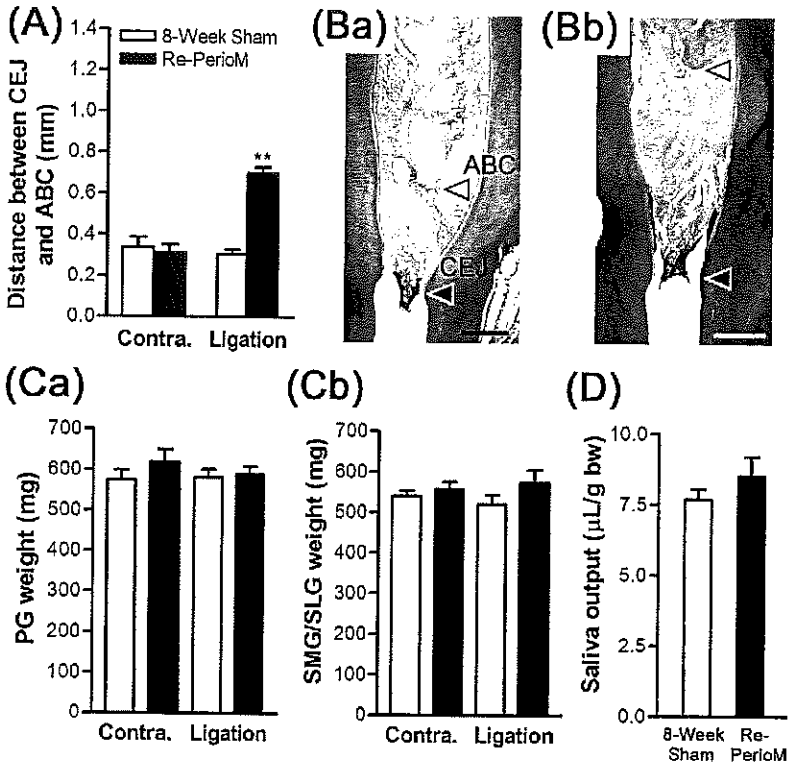

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