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Reduced methacholine-induced submandibular salivary secretion in rats with experimental periodontitis

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ARTICLE INFO

ABSTRACT

Objective: Saliva is the first barrier to the entry of bacteria and viruses into the body and is considered a necessary instrument in oral health. Intraperitoneal injection of lipopolysaccharide endotoxins results in submandibular gland (SMG) hyposalivation. The objective of present studies was to assess if periodontitis, a chronic inflammatory disease caused by oral bacteria, alters cholinergic-induced SMG salivary secretion.

Design: An experimental periodontitis model (EP) (cotton thread ligature around the neck of the first lower molars) was used. Male Wistar rats (300–380 g) were randomly divided into 3 groups: control, 7 days-bilateral EP and 7 days-unilateral EP (to study if there were different effects at the ipsilateral and contralateral side). The following determinations were performed in SMG: (1) dose–response curves to the cholinergic agonist methacholine, (2) prostaglandin E (PGE) content, (3) inducible nitric oxide synthase (iNOS) activity and (4) histology of gland sections.

Results: The molars with EP, no matter the group, exhibited significant and similar bone loss ($p < 0.001$). Bilateral EP reduced methacholine-induced salivary secretion ($p < 0.05$, dose 1 μg/kg; $p < 0.001$, dose 3–30 μg/kg), increased PGE content ($p < 0.01$), stimulated iNOS activity ($p < 0.05$). Ipsilateral glands of unilateral EP animals presented lower methacholine-induced salivary secretion ($p < 0.05$, dose 3 μg/kg; $p < 0.001$, dose 10–30 μg/kg), and higher PGE content than contralaterals ($p < 0.001$). In turn, at 3 and 10 μg/kg of methacholine, contralateral glands showed significantly lower secretion than control animals ($p < 0.001$). Histological studies of glands revealed partial loss of secretor granular material and periductal oedema in the bilateral and unilateral EP groups as compared to controls.

Conclusions: As far as we know, the present results demonstrate for the first time that EP reduces methacholine-induced SMG salivary secretion.

1. Introduction

Saliva represents the first barrier to the entry of bacteria and viruses into the body and thus changes in the secretion are important in the statement and progression of oral infectious processes.[1,2] The numerous biologic functions of saliva, e.g. lubrication, antimicrobial protection, cleansing and maintenance of mucosal integrity make it a necessary instrument in

oral health.[1,2] Salivary gland secretion is a reflex, controlled mainly by the autonomic nervous system.[3] The parasympathetic nervous system via impulses in the chorda tympani nerve releases acetylcholine and evokes a copious salivary secretion by activating muscarinic receptors. Since salivary secretion is almost completely abolished by atropine, it is clear that release of acetylcholine from parasympathetic nerves plays the main role in evoking fluid secretion.[3] In turn, the sympathetic nervous system, whose neurons are located in the superior cervical ganglia, controls salivary secretion by acting on α- and β-adrenergic receptors. Other non-adrenergic, non-cholinergic neuropeptides are released from autonomic nerves and evoke salivary secretion.[3,4] Some neuropeptides, e.g. substance P and calcitonin gene-related peptide are also found around ducts and blood vessels within the salivary glands in sensory nerve fibres that mainly come from the trigeminal ganglion.[5] Thereafter, the normal submandibular gland (SMG) secretor activity as well as the secretion from all of the salivary glands is the result of a complex and simultaneous activation of an entire repertoire of receptors and their associated intracellular pathways.[3]

We have recently demonstrated that submandibulectomy aggravates bone resorption in rats subjected to 7 days-experimental periodontitis (EP).[6] Periodontitis is a frequent oral pathology representing an infective disease of the gums. It is caused by bacteria present in dental plaque that induces the breakdown of the tooth supporting apparatus until teeth are lost and, in most cases it is also a disease with a chronic progression.[7] The destruction of periodontal tissue is mainly due to complex interactions between the pathogenic bacteria and the host-derived mediators generated during the immunoinflammatory responses.[7] As the integrity of the epithelium of a periodontal pocket is violated, especially in advanced states of the pathology, bacterial products and also whole bacteria can enter underlying host tissues and the blood stream. Moreover, certain bacteria, such as *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*, possess virulence factors that allow for direct invasion of intact tissues.[7] As a consequence, patients are exposed to transient bacteriemias, even with chewing and tooth brushing. The exposure to lipopolysaccharide and other bacterial products can trigger the sequence of events of inflammation.[7,8] Periodontitis causes oxidative stress whose consequences occur in the oral cavity[9] and in most distant organs.[10,11]

Salivary gland function is impaired by autoimmune diseases and inflammation.[12–14] Inflammatory cytokines are induced by lipopolysaccharide stimulation in the SMG of the mice.[15] In the adult male rat, an acute intraperitoneal single dose of lipopolysaccharide increases inducible nitric oxide synthase (iNOS) activity and prostaglandin E (PGE) content in the SMG and blocks stimulated salivary secretion.[14] Additionally, after 4 weeks of EP an imbalance of circulating oxidative/anti-oxidative status and apoptosis of SMG is observed.[9]

These investigations suggest that periodontitis might impair salivary gland function. Thus, the objective of present work was to analyse in the SMG of rats if 7 days-EP (1) caused changes in cholinergic-induced salivary secretion, considered un index of SMGs activity since acetylcholine released from parasympathetic nerves is the principal regulator of fluid secretion,[3] (2) was associated with increments in iNOS activity

and PGE production, index of inflammatory response, and (3) produced changes in SMG histology. In addition to the control and bilateral EP groups, a third group of animals with unilateral EP was include to assess to what extent EP produced different effects at ipsilateral and contralateral side.

2. Materials and methods

2.1. Chemicals

Chloralose and methacholine were obtained from FLUKA (Laborchemikalien, Berlin, Germany), xylazine hydrochloride from König Laboratories SA (Buenos Aires, Argentina), ketamine hydrochloride from Holliday-Scott SA (Buenos Aires, Argentina), HEPES, L-valine, $CaCl_2$, antiserum anti-PGE, standard PGE from Sigma Chemicals (St. Louis, MO), [^{3}H]PGE from New England Life Science (Boston, MA), [^{14}C]-arginine from Amersham Pharmacia Biotech (Piscataway, NJ) and AG-50W-X8 resin from Bio-Rad Laboratories (Hercules, CA). All solvents were of analytical grade.

2.2. Animals

Adult male Wistar rats from our own colony with an initial body weight of 300–380 g were randomly divided into 3 groups: (1) control, (2) 7 days-bilateral EP and (3) 7 days-unilateral EP. They were kept in group cages in an animal room with a photoperiod of 12 h of light (07.00–09.00 h), room temperature at 22–25 °C, humidity: 52–56% and maintained on standard Purina chow pellets and tap water ad libitum. All experiments were performed following the National Institute of Health guidelines for the care and use of laboratory animals (NIH 85-23, revised in 1985) and protocols were approved by the Ethical Commission of the School of Dentistry, University of Buenos Aires.

2.3. Induction of experimental periodontitis

EP was induced under general anaesthesia with a mixture of 2% xylazine hydrochloride (5 mg/kg; i.p.) and 5% ketamine hydrochloride (50 mg/kg; i.p.). A cotton thread ligature was placed around the neck of the right first lower molars (unilateral EP; the contralateral molars were used as the respective controls) or around both first lower molars (bilateral EP) and served as a retention device for oral microorganism. Ligature was pushed into the gingival sulcus and was left in place until sacrifice (7 days post surgery).[6]

2.4. Microscopic examination of periodontal bone loss: distance method

Immediately after sacrifice, hemimandibles were resected, defleshed and stained with 1% aqueous methylene blue to delineate the cement–enamel-junction and the alveolar crest. A stereoscopic microscope was used to measure the distance between the cement–enamel-junction and the alveolar crest at the level of the mandibular first molars as described previously.[6] The mean of the 6 recordings (3 lingual and 3 vestibular) on each molar was used as a measure of the total alveolar bone loss.

2.5. Salivary secretion: dose–response curves

Salivary responses were determined in anaesthetised rats (100 mg/kg of chloralose, 2 ml of 0.9% NaCl, i.v.). The right femoral vein was cannulated with a polyethylene catheter (P-40 catheter, Rivero y Cia, Argentina) to inject as a bolus the sequential doses of methacholine as previously described.[16] The trachea was intubated through a mid line incision in the neck, and the ducts of the SMG were exposed and cannulated with a fine glass cannulae. No resting (unstimulated) flow of saliva was observed. The secretion induced by the cholinergic agonist methacholine (0.3, 1, 3, 10 and 30 μg/kg in saline) during 3 min after the administration of each dose was colleted on aluminium foil and weighed. All studies were performed using 6–7 rats per group. Results were expressed as milligrammes of saliva per gland.

2.6. PGE content

PGE content was measured by specific radioimmunoassay. Individual glands were homogenised in 1.5 ml ice cold ethanol (100%), centrifuged at $10,000 \times g$ for 15 min at 4 °C, and the supernatant were collected and evaporated in a Speed-Vac. The residues were re-suspended with radioimmunoassay buffer and the Sigma antiserum was used as described.[17] The cross-reactivity of PGE_2 was 100% with PGE_1 and lower than 0.1% with other prostaglandins. The intra-assay and inter-assay coefficients of variation for PGE were 8.2% and 12%, respectively. All studies were performed using 6–7 rats per group. The PGE content was expressed as femtograms (fg) per mg of weight tissue.

2.7. iNOS activity

iNOS activity was measured by a modification of the $[^{14}C]$-citrulline method of Bredt and Snyder[18] which measures the conversion of $[^{14}C]$-arginine into $[^{14}C]$-citrulline. The method indirectly determines NO production, since the enzyme converts arginine into equimolar amounts of citrulline and NO. Because the SMG contains a very active urea cycle in the tissue, arginine is also converted to citrulline by this cycle, giving false high values for NOS activity. In order to obviate the problem, the arginase of the urea cycle was blocked by the addition of 25 mM L-valine to the HEPES buffer of homogenisation and incubation. To measure the activity of the inducible-calcium independent NOS (iNOS), the method technique was performed with the absence of Ca^{2+} in the assay buffer (2 mM EGTA). The details of the method used were previously published.[17] All studies were performed using 6–7 rats per group. The results are expressed in femtomol (fmol) of NO produced per min per mg of protein.

2.8. Histology

After extraction, glands were cut in small pieces, fixed in 4% neutral buffered formaldehyde, embedded in paraffin and 5 μm sections were stained with haematoxylin and eosin. Light microscopy was performed on an Axiolab Karl Zeiss microscope (Göttingen, Germany). All photographs were taken at 630× magnification using a Canon PowerShot G5 camera (Tokyo, Japan). The histological analysis was performed by a trained histopathologist blinded to the experimental data. The overall examination of the samples was carried out at 10× magnification, and representative areas of submandibular gland specimens were then analysed at 630× magnification. At least 10 fields were evaluated and the data are presented qualitatively by depicting the pictures of representative fields.

2.9. Protein determination

Protein concentrations were determined by the method of Bradford[19] with bovine serum albumin as standard.

2.10. Statistical analysis

Data were expressed as the means ± S.E.M ($n = 6–7$). Comparisons between groups were performed using a Student's t-test when 2 groups were compared (iNOS, PGE, bone loss determinations) or two-way analysis of variance, followed by Bonferroni's post test for multiple comparisons (salivary secretion studies). All analyses were conducted using the Graph Pad Instat software (Graph Pad Software, Inc., San Diego, CA). Differences with $p < 0.05$ were considered statistically significant.

3. Results

Table 1 shows that the placement of a cotton ligature around the lower first molar leads to periodontitis as revealed by the greater bone loss (distance between the cement–enamel junction and the alveolar bone crest) observed in EP molars. Percent of body weight gain from rats with ligature was smaller than from controls (bilateral EP: $-4.55 \pm 2.43\%$; unilateral EP: $2.51 \pm 2.03\%$; control: $7.18 \pm 2.23\%$; $p < 0.001$ and $p < 0.01$ versus control respectively).

The effects of bilateral and unilateral EP on methacholine dose–response curves are shown in Fig. 1. Methacholine-induced salivary secretion is consider as an index of SMG function capacity since the release of acetylcholine from parasympathetic nerves plays the main role in evoking fluid secretion.[3] Dose–response curves to methacholine demonstrated an inhibitory effect of EP on methacholine-evoked salivation. In bilateral EP animals, the curve not only showed a rightward shift (significant lower response at 1, 3, 10 and 30 μg/

Table 1 – Effect of 7 days-EP on bone loss (distance between the cement–enamel-junction and the alveolar crest determined as described in Section 2) at the level of lower first molar. Values are means ± SEM (7 rats per group).

	Bone loss (mm)
Control	0.73+0.06
Bilateral EP	1.20+0.04[***]
Unilateral EP: contralateral	0.63+0.06
Unilateral EP: ipsilateral	1.15+0.04[+++]

[***] $p < 0.001$ versus control.
[+++] $p < 0.001$ versus contralateral.
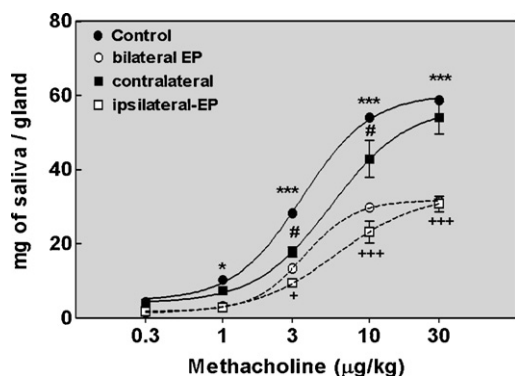
Fig. 1 – Effect of bilateral EP (○) and unilateral EP (ipsilateral (□), contralateral (■)) on methacholine-evoked salivary secretion of SMGs. Values are means ± SEM (6–7 rats per group). $^{*}p < 0.05$, $^{***}p < 0.001$ versus bilateral EP; $^{+}p < 0.05$, $^{+++}p < 0.001$ versus contralateral and $^{#}p < 0.001$ versus contralateral.

contralaterals ($p < 0.05$, dose 3 μg/kg; $p < 0.001$, dose 10–30 μg/kg), with a reduction in the maximal response of 43%. In addition, methacholine-induced saliva of contralateral glands was significantly lower than the saliva obtained from control animals at 3 and 10 μg/kg of methacholine ($p < 0.001$).

The evaluation of submandibular PGE content and iNOS activity showed significant increments in rats subjected to bilateral EP as compared to controls ($p < 0.01$ and $p < 0.05$ respectively) (Fig. 2). In the unilateral EP group, whilst glands ipsilateral to EP presented higher PGE content than contralaterals ($p < 0.001$), no change in the iNOS activity was observed (Fig. 3).

Histological analysis of SMG of animals with EP (unilateral and bilateral EP groups) (Fig. 4) revealed alteration of the epithelial architecture with marked reduction of the highness of the granular convoluted ducts, partial loss of secretor granular material and periductal oedema as well as slight anisokaryosis of acinar cells compared to control group.

kg of methacholine) but also exhibited a significant reduction (47%) in the maximum salivation compared with that of control animals ($p < 0.001$) (Fig. 1). In animals with unilateral EP, glands ipsilateral to EP presented a secretion similar to that of animals with bilateral EP and lower than
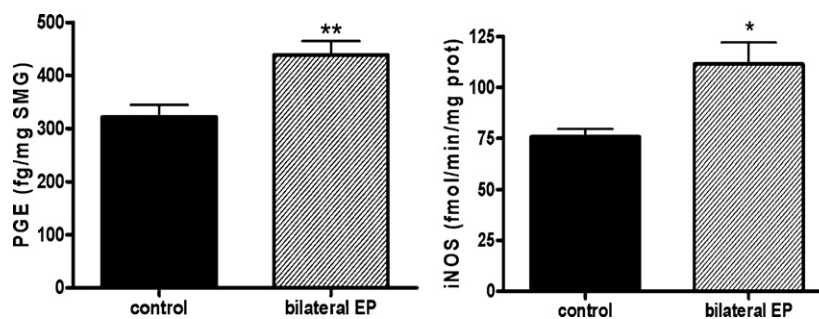
4. Discussion

In this paper we analysed the effects of unilateral and bilateral EP on SMG function. Studies with unilateral EP using the contralateral gland of the same animal as the corresponding

Fig. 2 – Effect of bilateral EP on submandibular PGE content and iNOS activity. The results are expressed in fg PGE /mg of SMG and fmol of NO produced per min per mg of protein. Each bar represents the mean ± SEM (6–7 rats per group). $^{*}p < 0.05$, $^{**}p < 0.01$ versus control.
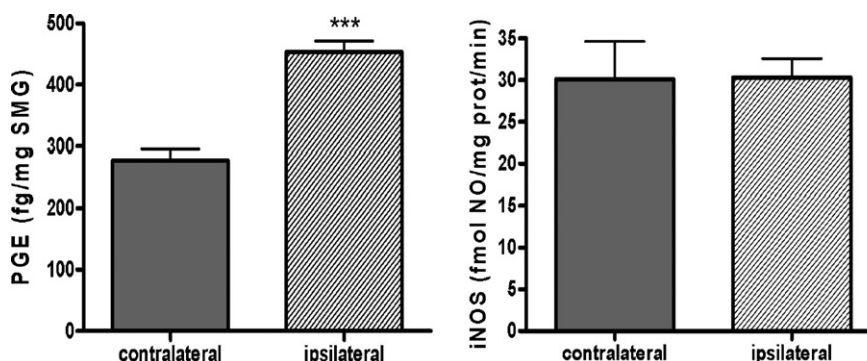
Fig. 3 – Effect of unilateral EP on submandibular PGE content and iNOS activity. The results are expressed in fg PGE /mg of SMG and fmol NO produced per min per mg of protein. Each bar represents the mean ± SEM (6–7 rats per group). $^{***}p < 0.001$ versus contralateral.
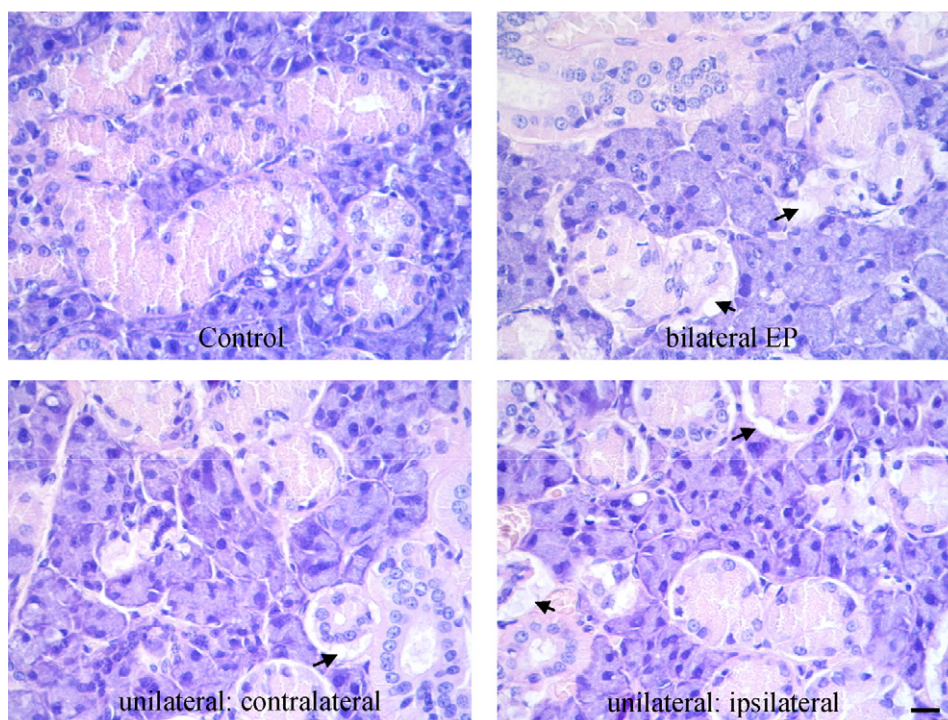
Fig. 4 – Microphotograph of SMG stained with haematoxylin and eosin. SMG of periodontitis groups (unilateral and bilateral) showed alteration of the epithelial architecture with reduction of the highness of the granular convoluted ducts with partial loss of secretor granular material and periductal oedema (arrows). Slight anisokaryosis of acinar cells compared to control group was observed in EP groups. Pictures were taken at 630× magnification. Scale bar = 20 μm.

control, were performed to rule out any effects due to circulating signals. The molars with EP, no matter the group, exhibited significant and similar bone loss calculated as the distance between the cement–enamel junction and the alveolar bone crest. In spite of the limitations of these linear measurements to determine bone loss, they are recommended for short (<15 days EP) observation periods in relation to the area method.[20]

Results presented herein show evidence that EP affected glandular activity as revealed by the changes in cholinergic-induced saliva secretion. Seven days-EP caused a drastic reduction in the capacity of SMG to secrete saliva in response to cholinergic stimulation.

Inflammatory states, as gingivitis and periodontitis, produce large amounts of NO and PGE mainly due to the activation of inducible enzymes.[21–23] In the SMG, in accordance with the existence of a strict defence system via the innate immune system, up-regulation of acute phase proteins and inflammation cytokines have been described after experimental induction of inflammation.[24] The increments of PGE and iNOS observed in the glands of animals with bilateral 7 days-EP suggest a glandular inflammatory response that was accompanied by a reduced salivary secretion.[25] In agreement, we have previously described that, the induction of an acute infection by the i.p. injection of lipopolysaccharide, causes an inhibition of SMG salivation in the rat and that this effect appears to be due to increased PGE production.[14] In unilateral EP animals, though there is a good correlation between the PGE content and the glandular secretion, iNOS

activity decreased to half of the values found in controls both in the ipsilateral and in the contralateral glands. Further experiments are needed to shed light on the nature of this systemic effect.

After 4 weeks of EP, imbalance of circulating oxidative/anti-oxidative status and apoptosis of SMG is observed.[9] PGE and NO are thought to be involved in the destruction of exocrine gland tissue observed in the Sjögren's syndrome,[26] an autoimmune disease characterised by symptoms of oral dryness, and in post-irradiation treatment.[27] Present results show that the impairment of SMG function induced by 7 days - EP was associated with the presence of ductal oedema and a reduction of the highness of granular convoluted ducts and secretory granular material. Remarkably, there was no change in acinar structure taking in consideration that fluid secretion is produced by the acini. Perhaps the modifications of one or more elements of the signal transduction pathway could lead to the down-regulation of salivary function in the absence of change of acinar structure.[29] In the mice, the activation of innate immune responses through Toll-like receptor 3 causes loss of cholinergic-induced saliva volumen without changes in SMG histology.[28]

EP might also induce some kind of alteration in food consumption, as suggest by the slight, but significant, lower body weight gain, and/or in chewing activity, and/or in grooming and thereafter in the reflex salivary secretion.[30] Taken in consideration that, the secretor activity in the rat SMG results from the complex and simultaneous activation of an entire repertoire of receptors and associated intracellular

pathway, the coupling of signals is likely to occur.[31] Sympathetic activation of SMG by inflammatory mediators during EP has been postulated.[32] In rats subjected to EP, mucin release (a β-adrenergic response) increases during resting conditions whilst the β-stimulatory action with isoproterenol decreases.[32] Accordingly, the intraperitoneal injection of lipopolysaccharide endotoxins produces a highly significant inhibition of salivary secretion stimulated by noradrenaline as well as by methacholine.[14] Within the SMG around ducts and blood vessels there are also sensory nerve fibres that mainly come from the trigeminal ganglion. Since activation of the trigeminal ganglia takes place during EP,[33] peptidergic mechanisms may also be involved in the effects observed in the SMG. Stimulation of muscarinic, α-adrenergic and substance P receptors activates the same intracellular pathway to produce salivary secretion.[31] Thereafter, our results might be reflecting an heterologous desensitisation to methacholine consequence of prolonged peptidergic and/or α_1-adrenergic stimulation.[3,34]

EP induces circulating oxidative stress[9] and direct and/or indirect effects on the secretor tissue and/or on blood flow implicating neurological pathways that cross in the trigeminal nuclear[35] and/or complex reflexive mechanisms of the so called "neuro-immune axis[36,37] could provide explanations for the reduced methacholine-induced submandibular salivary secretion observed in rats with EP as compared to the control group. It seems feasible that besides circulating signals, neural inputs originating in the EP region would affect gland function. This was suggested by the lower secretion and higher PGE content that was detected in the gland ipsilateral to EP. Further studies are needed to elucidate the mechanisms involved.

In summary, present results demonstrate, for the first time, that periodontitis inhibits the in vivo methacholine-stimulated salivary secretion in rats. As decreased saliva secretion aggravates the bone resorption in rats subjected to EP,[6] the changes in glandular activity induced by EP might affect the progression of periodontitis as well as the oral beneficial conditions due to saliva.[1,2,38]

Acknowledgements

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