Changes in PGE2 signaling after submandibulectomy alter post tooth extraction socket healing.

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Abstract

Saliva is very important to oral health, and a salivary deficit has been shown to bring serious problems to oral health. There is scant information about the mechanisms through which salivary glands participate in post-tooth extraction socket healing. Therefore, the aim of the present study was to investigate the effect of submandibulectomy (SMx), consisting of the ablation of submandibular and sublingual glands (SMG and SLG, respectively), on PGE_2 signaling and other bone regulatory molecules, such as OPG and RANKL, involved in tooth extraction socket healing. Male Wistar rats, 70g body weight, were assigned to an experimental (subjected to SMx) or a control group (sham operated). One week later, the animals in both groups underwent bilateral extraction of the first mandibular molars. The effect of SMx on different stages of socket healing after tooth extraction (7, 14 and 30 days) was studied by evaluating some parameters of inflammation, including PGE_2 and its receptors, and of bone metabolism, as well as by performing bone biomechanical studies. SMx increased TNFα and PGE_2 content as well as COX-II expression in tooth socket tissue at almost all the studied time-points. SMx also had an effect on mRNA expression of PGE_2 receptors at the different time points, but did not significantly alter osteoprotegerin (OPG) and RANKL mRNA expression at any of the studied time points. In addition, an increase in bone mass density was observed in SMx rats compared to matched controls, and the structural and mechanical bone properties of the mandibular socket bone were also affected by SMx. Our results suggest that the SMG/SLG complex regulates cellular activation and differentiation by modulating the production of molecules intervening in tooth extraction socket repair, including the PGE_2 signaling system, which would therefore account for the higher density and resistance of the newly formed bone in SMx rat.

Introduction

Wounds in the oral cavity are repaired relatively easily, likely due to the involvement of saliva in oral tissue repair processes. Nevertheless, the described endocrine function of the salivary glands should not be disregarded as a possible mediator in oral tissue repair[1].

Saliva is very important to oral health. A lack of saliva has been shown to bring serious problems to oral health, and delay the repair of mouth lesions. In a previous work we showed that in the first 72 h post tooth extraction, submandibulectomy produces a delay in the organization of granulation tissue and alters the synthesis of some inflammatory mediators involved in wound healing, such as nitric oxide (NO), tumor necrosis factor-α (TNFα), and prostaglandin E_2 (PGE_2), thus delaying tissue repair in the early stages of the healing process.[2] Post-tooth extraction socket healing involves the repair of both the mucosal and the bone tissue of the socket that held the tooth[3]. Hence, tooth extraction is a good model to study the effect of salivary gland dysfunction or absence on the repair of hard and soft tissues of the oral cavity.

Prostaglandins (PGs) are present in inflammatory processes leading to wound repair[4,5]. It has been demonstrated that PGE_2 plays a key role in bone fracture repair processes[6]. The rate-limiting step for the synthesis of PGE_2 and other prostaglandins is the conversion of arachidonic acid to prostaglandin endoperoxides by cyclooxygenase (COX) enzyme, which exists in two isoforms, expressed constitutively COX-I and inducible COX-II [7]. Expression of COX-II is regulated by different stimuli, such as TNFα[8] and shear stress[9]. The induction of COX-II is regarded as an important step in inflammatory conditions. COX-I and COX-II are expressed in inflamed bone tissue[10]. PGs are considered important local factors that modulate bone metabolism through their effects on osteoblasts and osteoclasts[11]. PGE_2 is also a major eicosanoid produced by osteoblasts. Systemic injection of PGE_2 increases both bone resorption and formation; however, formation can be greater than resorption, resulting in substantial increases in bone mass in rats, dogs, and humans[12]. The most consistent osteogenic effects of PGE_2 are seen in cultures designed to study osteoblastic

differentiation. Osteoblastic differentiation in marrow stromal cell and primary calvarial cell cultures is stimulated by PGE_2,[13,14], and osteoblastic differentiation is decreased in marrow stromal cell cultures from mice that are deficient in endogenous PGs production[15,16]. PGE_2 is induced by several factors that have diverse actions on bone. Understanding the role of PGs in skeletal metabolism is challenging because PGs act locally and transiently, are regulated at several levels, have multiple receptors, and can have opposing effects depending on the test system. It seems likely that a major function of PGE_2 is to modulate or integrate several signaling pathways by engaging additional Gas-activated signaling pathways. Whether or not the regulatory function of PGs contributes to the catabolic or anabolic actions of the factor that induces them, and ultimately to bone loss or gain, will depend both on the factor inducing PGs production and on the local cellular milieu. PGE_2, acts locally on target cells, such as osteoblast and osteoclast precursors, fibroblasts and mesenchymal cells, and mediates its tissue-specific pharmacological activity via four different G protein-coupled receptor subtypes, EP1, -2, -3, and -4. The EP1 receptor is coupled to intracellular Ca2+ mobilization, while EP2 and EP4 receptors increase intracellular cAMP accumulation. In contrast, EP3 inhibits intracellular cAMP accumulation.[17] The anabolic action of PGE_2 in bone has been linked to an elevated level of cAMP, thereby implicating the EP2 and/or EP4 receptor subtypes in bone formation[4].

Bone repair processes, in which osteoblasts are responsible for bone formation, involve the activation of bone precursor cells and the expression of bone regulatory molecules that are important for optimal repair of the bone lesion itself. It is known that the balance between osteoprotegerin (OPG) and the ligand of the nuclear factor receptor activator κB (RANKL) is key to the balance between osteoclast bone resorption and osteoblast bone formation, thus determining the quantity of bone that is formed[18]. OPG is a decoy receptor for the receptor activator RANKL. By binding to RANKL, OPG prevents RANK-mediated nuclear factor kappa B (NF-κB) activation, which is a central and rapid acting transcription factor for immune-related genes, and a key regulator of

inflammation, innate immunity, and cell survival and differentiation[19]. By binding to RANKL on osteoblast/stromal cells, OPG blocks the RANKL-RANK interaction between osteoblast/stromal cells and osteoclast precursors, thus inhibiting both differentiation of osteoclast precursors into mature osteoclasts and the resorptive activity of these mature cells[18].

Extraction socket repair involves bone formation processes that take several weeks to complete (approximately 30 days)[3]. These processes have not been studied in detail under conditions of salivary gland dysfunction. Therefore, the aim of the present study was to investigate the effect of submandibulectomy on PGE_2 signaling and other bone regulatory molecules such as OPG and RANKL, involved in tooth extraction socket healing.

Material and Methods

Male Wistar rats (School of Pharmacy and Biochemistry of the University of Buenos Aires, Buenos Aires, Argentina), aged 21 days, were used throughout. They were housed in steel cages in groups of 6, fed regular rat chow and water ad libitum, and maintained on a 12:12 hour light–dark cycle. The protocol was examined and approved by the Institutional Ethics Committee of the School of Dentistry, University of Buenos Aires, Argentina. Experimental procedure: the animals (70 g) were randomly assigned to an experimental or a control group (18 rats per group). Those in the experimental group underwent submandibulectomy (SMx). Bilateral excision of the submandibular gland (SMG) and sublingual gland (SLG) was performed[20]. The control group was subjected to a sham operation (Sham). One week later, bilateral extraction of the first mandibular molars was performed under i.m. anesthesia (8 mg of ketamine and 1.28 mg of xylazine per 100 g of body weight). Given that adult rats suffer hypercementosis in dental roots, the experiment started with animals weighing less than 100 gr in order to perform successful extractions, avoid fracturing the tooth, and ensure that the extraction socket was free of root waste that could interfere with the repair

process. There were no differences in body weight between groups at the end of the experiments, reaching approximately 240 g.

The animals were euthanized (CO_2 chamber) in groups of 12 (6 controls and 6 SMx) at 7, 14, or 30 days post–tooth extraction. The mandibles were resected and processed for histological and biochemical analyses. An additional group of control and SMx animals (n=20) was euthanized at 30 days post-tooth extraction for biomechanical studies. The experiments and their corresponding determinations were performed three times.

Biochemical studies

The socket tissue occupying the alveolus was obtained using a biopsy punch of 2mm in diameter from each hemimandible, and was processed to measure, TNF-α content and prostaglandin E (PGE_2) content, COX-II protein determination and OPG, RANKL and EP receptors mRNA expression, according to technique requirements.

Determination of TNF-α

For TNF-α preservation after extraction, the socket tissue was immediately homogenized in ice-cold PBS buffer containing protease inhibitory cocktail for mammalian tissue extracts (Sigma-Aldrich, St Louis, MO, USA). Concentrations of rat TNF-α were determined using specific rat enzyme-linked immunosorbent assays, following the manufacturer's instructions (BD Pharmingen, San Diego, CA, USA).

Determination of PGE content

PGE content of the socket tissue was determined by specific RIA as described previously [21]. Rabbit antiserum from Sigma-Aldrich (St Louis, MO, USA) was used; assay sensitivity was 12.5 pg/tube. The cross-reactivity between PGE_2 and PGE_1 was 100%, although other PGs exhibited less than

0.1%. The intra-assay and interassay coefficients of variation for PGE were 8.2 and 12%, respectively. PGE content was expressed as picograms per milligram of wet weight (pg/mg ww).

Western blot for cyclooxygenase II (COX-II) protein measurements

Socket tissue was lysed in ice-cold protein extraction buffer (150mM NaCl, 50mM Tris–HCl, pH 7.4, 1% Triton X-100, and containing protease inhibitory cocktail from Sigma-Aldrich). The homogenates were centrifuged briefly, and the supernatants were removed and stored at -70 ºC until use. After total protein determination using the Bradford assay, samples were separated by SDS/PAGE and blotted onto polyvinylidene fluoride membranes (PDVF, Immobilon-P, Millipore, Billerica, MA). The membranes were blocked in 3% BSA solution for 2 hours, and probed overnight at 4 ºC with anti-COX-II rabbit antibody (1/5,000; Cayman Chemical, Ann Arbor, MI). They were then incubated with anti-rabbit IgG alkaline phosphatase (1/2,000; Santa Cruz Biotechnology, Dallas, TX) as the secondary antibody. After extensive washing, protein bands were detected using the ECL system (GE Healthcare, Buckinghamshire, United Kingdom). Blots were also probed with anti β-actin (Sigma-Aldrich; 1/2,000) to confirm equal loading of protein. Quantification was performed using Image J 1.42 software (National Institutes of Health, Bethesda, MD). Band densities were expressed as a ratio to the reference band densities.

RNA isolation and Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from socket tissue by homogenizing it in TRI reagent® according to the manufacturer's recommendations (Sigma Aldrich, Co. St Louis, MO, USA). After DNAase digestion for the first-strand cDNA synthesis, 3 μg of total RNA were reverse-transcribed using M-MLV reverse transcriptase with random primers and ribonuclease inhibitor (Promega Corp. Madison, USA). The amplification profile consisted of 30 or 35 cycles of denaturalization of 2 μl or 4 μl of cDNA at 94ºC for 30 seconds, annealing at different temperature according to the specific primer,

which were used for 35 seconds, and extension of DNA at 72ºC for 45 seconds after a 10 minute denaturation step at 90ºC. RT-PCR was performed for EP1, EP2, EP3, EP4, OPG, RANKL and β-actin as housekeeping molecules. The specific primers for PCR amplifications were designed using the Primer 3 Software. The primers used in this study are listed in Table 1. The PCR products were analyzed by 2% agarose gel electrophoresis, visualized with GelRed™ nucleic acid gel stain (Biotium, Inc. Fremont, CA, USA) using a Gel Doc XR+ imaging system from BioRad (CA, USA), and analyzed using image Lab software. The relative mRNA was normalized to β-actin, and results were expressed as an arbitrary unit of relative optical density.

Histological studies

One hemimandible from each animal was decalcified in 5% formic acid, embedded in paraffin, and semiserially sectioned in a frontal plane (bucco-lingual direction) at the level of the mesial socket of the first mandibular molar, to obtain 10 µm thick sections. The sections were stained with Hematoxylin-Eosin.

Densitometric Studies

Bone densitometry (BMD) and bone mineral content (BMC) at the extraction site in the rat hemimandible were determined 30 days post-tooth extraction. Determinations were performed using a whole body scanner by dual energy x-ray absorptiometry (DXA) and a specifically designed software for small animals (DPX Alpha, Small Animal Software, Lunar Radiation Corp. Madison WI). Assessment of BMD by DEXA does not provide the volume but rather shows the mass of mineral in the studied skeletal region, in this case the extraction socket, regardless of the bone structure in that particular region. BMD can only be considered an indicator of the degree of mineral concentration in the extraction socket. Therefore, changes in BMD are analogous to those occurring in BMC, and for this reason they should not be directly attributed to changes in bone structure.

DEXA does not provide information on geometric properties, which are the true determinants of the structural properties of bone[22].

Biomechanical Studies

To assess mechanical properties, it was necessary to perform complete tooth extraction from one hemimandible, to carry out biomechanical tests in the alveolar bone only, without the presence of dental tissue. The right hemimandible was subjected to a three-point bending mechanical test[23] in an Instron test machine (model 4442; Instron, Canton, MA, USA). Each bone was placed on two lower supports, with the lateral aspect facing down and centered along its length. The supports were equidistant from the bone ends and separated by a constant distance L (distance between supports = 11 mm), equivalent in no case to more than two fifths of the bone length. Loads were applied transversally to the bone axis on the alveolar region at a rate of 5.00 mm/ min, which is useful to describe the static properties of the alveolar bone structure.

Statistical analysis

The results are expressed as mean and standard error. Student's t- test was used to establish comparisons between two groups using GraphPad Prism Software (GraphPad Software, San Diego, CA, USA). Comparisons among more than two groups were established using two-way ANOVA, and individual differences were identified using Tukey's post hoc test. Statistical significance was set at a value of $p<0.05$. Statistical analyses were performed using Statistic 7 software (StatSoft, Inc., Tulsa, OK).

Results

Inflammatory parameters

TNF-a production

Post-tooth extraction tissue TNFα content was higher in SMx groups at both 7 and 30 days compared to the respective control groups ($p<0.05$). A significant decrease in TNFα production was observed in both control and SMx animals as the healing process progressed from day 7 to day 30 ($p<0.05$) (Figure 1).

Prostaglandin E content and COX-II protein expression

Levels of PGE_2 were significantly higher in the SMx group than in the control group at all the studied time points (7 days: $p<0.01$; 14 and 30 days: $p<0.05$). The levels of PGE_2 were highest in both groups at 7 days post–tooth extraction; the difference was statistically significant compared to levels observed at 14 and 30 days post–tooth extraction in each group, respectively ($p<0.001$) (Figure 2). In addition to the increase in PGE_2 content, protein expression of COX-II, the enzyme that synthetizes PGE_2, was also found to increase both at 7 ($p<0.05$) and 14 ($p<0.01$) days post tooth extraction in the SMx group compared to the corresponding control group. Like PGE_2, COX-II expression in post-tooth extraction socket tissue was higher at 7 days than at 14 days in the SMx group ($p<0.05$) (Figure 3).

Messenger RNA expression

Since the bone formation process is regulated by the balance between osteoprotegerin (OPG) and RANK-L, and post-tooth extraction wound healing involves trabecular bone formation, we studied mRNA expression of both molecules in extraction-socket tissue to analyze whether SMx has an effect on the expression of these molecules. OPG mRNA expression was higher than RANK-L mRNA expression at 14 and 30 days post tooth extraction, irrespective of SMx ($p<0.001$). There were no differences in OPG and RANK-L mRNA expression between groups (control and SMx) at

any of the studied time points. Expression of RANK-L mRNA was highest at 7 days, and decreased at 14 and 30 days post tooth extraction ($p<0.05$) (Figure 4).

Prostaglandin PGE_2 acts through 4 different types of EP receptors: EP1- to 4. In order to identify the effect of submandibulectomy on PGE_2 receptor subtypes, and thus detect the signaling pathway of PGE_2 in extraction socket tissue healing, we studied the expression of mRNA for the different subtypes of PGE_2 receptors in post tooth extraction socket tissue.

Comparison of all EP subtypes, showed that EP2 mRNA expression was the highest. In fact, EP2 mRNA expression was 5-fold higher than EP4 and EP1 mRNA expression (data not shown). In addition, EP2 mRNA expression was significantly higher in the SMx group than in the control group at 14 days ($p<0.01$) (Figure 5).

The mRNA expression of prostaglandin E receptor EP4 was highest at 7 days and decreased with time in both groups (+, $p<0.5$; +++ $p<0.001$), and was found to be significantly higher in SMx rats than in their respective controls at 7 and 30 days post tooth extraction ($p<0.01$). There was no difference in EP4 mRNA expression between groups at 14 days post tooth extraction (Figure 6).

The mRNA expression of prostaglandin E_2 receptor EP1 was high at 7 days post-tooth extraction and decreased with time in control groups ($p<0.05$). However, EP1 mRNA expression was higher after 14 days ($p<0.001$) than after 7 days, and was barely detectable at 30 days in the SMx groups. In addition, EP1 mRNA expression was significantly higher in the SMx group than in the control group at 14 days ($p<0.001$), and significantly lower in the SMx than in the control group at 30 ($p<0.05$) days post tooth extraction (Figure 7).

Although EP3 mRNA expression was not detected in socket tissue, EP3 mRNA expression was found in positive control samples such as liver and spleen (data not shown).

Histological examination

Histological examination of socket sections at 7 days post tooth extraction showed woven bone formation (deep in the hole) and intense osteoblast activity on the developing trabeculae, evidenced

by the presence of cuboidal osteoblasts lining the trabeculae (Figure 8A). At 14 days, the sockets were virtually completely filled with woven bone trabeculae, also lined by cuboidal osteoblasts showing that osteoblast activity and bone formation still continued to occur (Figure 8B). At both 7 and 14 days, the sockets were completely covered by epithelium, and both experimental and control sockets exhibited similar histological features.

At 30 days post tooth extraction, woven bone formation was complete and the presence of lamellar bone trabeculae could be observed. SMx rat sections showed wider trabeculae and smaller medullary spaces than controls (Figure 8C).

Biomechanical studies

The biomechanical structural properties of the right hemimandible, calculated from the analysis of the load/deformation curve obtained in the mechanical bending test [23], were significantly increased at the end of the study period (30 days) in the SMx group as compared to the control group. The structural properties of the alveolar bone are shown in Figure 9. Load at yielding (Wy), load at fracture (Wf), and structural stiffness (Wydy) were significantly higher in SMx than in control rats ($p<0.05$; $p<0.001$).

Bone mineral density measured in the mandibular bone of the tooth socket was also significantly higher at the end of the study period in the SMx than in the control group ($p<0.05$) (Figure 10).

Discussion

Wound healing is a highly dynamic process, and involves complex interactions of extracellular matrix molecules, soluble mediators, various resident cells, and infiltrating leukocyte subtypes. The immediate goal of repair is to achieve tissue integrity and homeostasis[24]. The wound healing process is the same in all tissues, and it involves four phases that overlap in time and space: hemostasis, inflammation, tissue formation, and tissue remodeling. All these phases exert specific contributions,

and the well-defined chronology of the wound healing phases is crucial to optimal tissue repair and restoration. Each tissue involves specific cells according to the organ to be repaired. Tooth extraction is a good model to study the healing process in soft and hard tissues. Since the teeth are inside the dental socket in the jaw bone, a wound is created in the socket when they are extracted. Wound healing involves the healing of the mucosa and the formation of new bone in the extraction socket; this is trabecular bone, which has bone marrow. Thus, bone healing involves osteogenic cell differentiation to restore bone function in the socket. Since the tooth extraction wound is inside the mouth, it is constantly bathed by saliva, which participates in wound healing. In the present work, we studied the effect of submandibulectomy (SMx) on different stages of socket healing after tooth extraction, by evaluating some inflammatory parameters and protein molecular expression and by performing biomechanical studies. The observed increased TNFα at 7 and 30 days and increased PGE_2 content at 7, 14 and 30 days in SMx rat post tooth extraction socket tissue compared with controls, suggests the involvement of the SMG/SLG complex in wound repair processes. There is evidence that TNFα can induce transcription of genes via stimulation of transcription factor kB, and thus regulate the synthesis of various proteins[25]. Hence, TNFα could be an inducing factor of COX-II synthesis and therefore of PGE_2 production, which together with TNFα have been shown to be involved in bone repair[26]. The present results showed that PGE_2 production was highest at 7 days post extraction, when mesenchymal cells abound, gradually declining at 14 and 30 days, when bone cells involved in the repair processes differentiate. These results are in agreement with those of Arikawa et al. (2004), who demonstrated a decrease in osteoblast production of PGE_2 compared to their progenitor cells, the mesenchymal stem cells derived from bone marrow.[27] Nevertheless, PGE_2 levels were always higher in SMx rats than in controls (Fig. 2). It is known that PGE_2 causes significant increases in bone mass and bone strength when administered systemically or locally[4,6]; this could explain the increase in bone mass density in SMx rats 30 days post-tooth extraction compared to matched controls. In addition, the structural and mechanical bone properties of the

mandibular socket bone were also affected by SMx. The increased bone resistance to both deformation (stiffness) and fracture (strength), and the increased yielding point suggest that alveolar bone of SMx rats is harder and contains more mineral elements compared to that of controls. Therefore, salivary gland dysfunction could alter bone response and behavior in affected individuals when undergoing bone repair therapies such as bone regeneration treatments for implant-supported prosthetics, which are increasingly frequent in the dental practice.

Based on these results, it could be suggested that the SMG and SLG regulate cellular activation and differentiation, and modulate the production of molecules intervening in tooth extraction socket repair. The increase in PGE_2 levels and production induced by the lack of SMG/SLG saliva could be partly responsible for these changes observed in the healed extraction socket. Nevertheless, the presence of alterations in bone repair caused by the increased masticatory activity due to hyposalivation cannot be ruled out[28].

Histological examination showed marked bone formation activity in extraction socket tissue between days 7 and 14 post-tooth extraction. This period coincides with the stage in which there is a predominance of bone trabeculae neoformation, with marked osteoblastic activity on the new trabeculae. At these stages, our results showed higher COX-II expression and PGE_2 levels in SMx rats than in controls, suggesting that PGE_2 is involved in osteoblast activity and bone formation. At 30 days post tooth extraction, when socket healing with new bone formation was complete, the observation of wider lamellar bone trabeculae and smaller medullary spaces in rats with SMx as compared to controls suggests the presence of higher bone density, as confirmed by densitometry. Since the bone formation process is regulated by the balance between OPG and RANK-L, and wound healing in the post tooth socket involves trabecular bone formation, we studied mRNA expression in socket tissue of SMx rats at 7, 14 and 30 days post-tooth extraction. The observed pattern of RANK-L mRNA expression, showing highest expression at 7 days followed by a decrease at 14 and 30 days, together with the consistently higher levels of OPG mRNA at all the studied time

points suggest a preponderance of bone formation in the extraction socket, as is expected in a bone repair process. OPG can reduce the production of osteoclasts by inhibiting the differentiation of osteoclast precursors, and has also been shown to regulate osteoclast resorption in vitro and in vivo[18]. The major effect of PGE_2 on bone resorption is generally considered to occur indirectly, via upregulation of RANKL expression and inhibition of OPG expression in osteoblastic cells. However, this was not observed in the present study.

Some authors have suggested that the EP2 receptor subtype is a major contributor to the local bone anabolic activity of PGE_2[4]. Other authors suggest an important role for EP4 receptor subtype[17]. Consistent with the anabolic effects of EP2R and EP4R agonists in vivo, both EP2R and EP4R have been implicated in the osteogenic effects of PGE_2 in vitro[29,30]. We evaluated the different subtypes of EP receptor mRNA expression at the different studied time points. Our results showed far higher expression of EP2 receptor mRNA as compared with EP1 and EP4 mRNA, and no EP3 receptor mRNA expression in post-tooth extraction tissue. It is important to mention that PGE_2 retains agonist activity in the nanomolar range at all four EP receptor subtypes. Although they only share 20–30% of their structural homology, the EP subtypes bind to PGE_2 in the following rank order of affinities: EP3> EP4>>EP2 > EP1 with Kd values ranging 100-fold from 0.33 to 25 nM.[31]

EP2 mRNA expression was higher in SMx rats at 14 days post tooth extraction compared to control rats, but was similar in both groups at the remaining time points. EP4 mRNA expression was high at 7 days post tooth extraction in both groups. EP4 receptor expression decreased at later stages of wound repair in the control group, as the bone forming activity stopped. Conversely to EP2, EP4 mRNA expression was significantly higher in SMx rats than in controls at 7 and 30 days post tooth extraction. Again, SMx was found to alter mediators of wound repair, and the increased expression of EP2 and EP4 may explain why PGE_2 causes the bone changes observed in socket healing in SMx rats. Both EP4 and EP2 respond to PGE_2, and are coupled to activation of adenylate cyclase and activate osteogenesis and osteoblast proliferation, and thereby stimulate de novo bone formation[17].

Also, both receptors are implicated in PGE_2-induced osteoclastogenesis[32,33]. It is interesting in this regard that we did not observe any involvement of PGE_2 production, or EP2 and EP4 mRNA expression associated with regulation of OPG or RANKL mRNA expression in this type of wound healing, where newly bone formation predominates. It is possible that, unlike what occurs in bone fracture healing, in this type of wound healing involving formation of new bone in the extraction socket, PGE_2 may play a role in promoting osteoblast differentiation and activation at the expense of osteoclast activation.

SMx also had an effect on EP1 mRNA expression in the socket tissue at the different time points studied here. EP1 mRNA expression was significantly higher at 14 days and significantly lower at 30 days post tooth extraction in SMx than in the respective control groups. In control rats, EP1 mRNA expression was highest at 7 days and then decreased in a time-dependent manner. The role of EP1 in osteoblastic differentiation and bone metabolism is not as well established. Selective EP1 agonists have been shown to stimulate the proliferation of osteoblast progenitors, but to impair osteoblastic differentiation[13], and the loss of EP1 receptors accelerates osteoblastic differentiation and fracture repair[34]. It has been hypothesized that EP1 acts as a negative regulator of bone formation and skeletal growth, while loss of EP1 promotes maintenance of bone during aging[15]. In contrast to EP2/4, which promote osteoblast differentiation, EP1 negatively regulates osteoblast differentiation, which may in turn, maintain the stem cell population and act as a 'brake' to slow osteogenic differentiation of stem cells. Therefore, EP1 may be a key negative regulator in progression of stem cell differentiation, acting to maintain stem cells in a less differentiated state as a mechanism to balance the physiological and pathological aspects of PGE_2 signaling. Hence, we postulate that the expression of EP1 observed at 7 days in both groups would favor the production of osteoblast progenitors. Furthermore, as the repair process progresses over time and EP1 expression decreases, as observed in the control group, the differentiation and activation of osteoblasts led by the EP2/4 signals would be

favored until bone-healing is complete. Thus, consolidation of the osteoid matrix and mineralization of the bone would be favored.

We posit that the changes caused by the lack of the GSM/GSL complex on PGE_2 levels and on the expression of its receptors in the tooth-extraction wound, lead to the formation of a bone with greater bone mass density and resistance in the tooth extraction socket of SMx rats. Thus,

acting via EP1, PGE_2 would keep the osteoblastic population more undifferentiated, and at the same time it would stimulate osteoblast differentiation and activation through EP2/4. As a result, these changes in the expression of PGE receptors observed in SMx rats may prolong bone-forming activity, maintaining the population of progenitor cells over time and favoring bone formation and synthesis. The abrupt fall in EP1 expression in the final stage of the repair process (30 days) in SMx rats counteracts its action favoring rapid closure and consolidation of socket bone mineralization, and would therefore account for the higher density and resistance of the newly formed bone observed in the experimental group.

In conclusion, taking into account the key endocrine and exocrine functions of submandibular and sublingual glands in the oral cavity, our results suggest that the alterations found in PGE_2 signaling in the post tooth extraction socket repair process, as well as the changes in bone mineral density and biomechanical properties of the newly formed bone in animals subjected to submandibulectomy, could be due to homeostatic changes in the bone in response to the absence of these glands. Nevertheless, further studies need to be conducted to better determine the properties of the newly formed bone, whether it is biologically and functionally adequate and suitable for implant placement.

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Legend to Figure

Figure 1: TNFα content in extraction socket tissue, *p<0.05 vs. corresponding control, +p<0.05 vs. 7 days

Figure 2: PGE2 content in extraction socket tissue. *p<0.05, **p<0.01 vs. corresponding controls. +++p<0.001 vs. other time points.

Figure 3: COX-II expression in extraction socket tissue by western blot. *p<0.05, **p<0.01 vs. corresponding control. +p<0.05 vs. other time points.

Figure 4: (A) OPG and (**B**) RANK-L mRNA expression in tooth extraction socket tissue at the different study time points. RANK-L +p<0.05 vs. RANK-L at 14 days.

Figure 5: EP2 mRNA expression in extraction socket tissue at the different time points. **p<0.01 vs. corresponding control.

Figure 6: EP4 mRNA expression in extraction socket tissue at the different time points. **p<0.01 vs. corresponding control, +p<0.05, +++p<0.001 vs. corresponding group at 7 days.

Figure 7: EP1 mRNA expression in extraction socket tissue at the different time points. *p<0.05,***p<0.001 vs. corresponding controls, +++p<0.001 +p<0.05 vs. corresponding group at 7 days.

Figure 8: Microphotographs of buccolingual sections of the mesial alveolus of the first lower molar obtained at 7 (A), 14 (B), and 30 (C) days post tooth extraction. At 7 and 14 days, the sockets were filled progressively by trabeculae as the healing process advanced. Osteoblasts lining the trabeculae (arrowhead in A and B) could also be observed. At 30 days, the socket was completely healed and filled with lamellar bone trabeculae. Note the smaller lacunar area (*) among the trabeculae in SMx sections than in controls. H&E stain, 50 x magnification

Figure 9: Structural properties of alveolar bone. Load at yielding (A), load at fracture (B), and structural stiffness (C) were significantly higher in SMx than in control rats (*$p<0.05$; *** $p<0.001$). (N) Newton, as measure of force.

Figure 10: Bone mineral density (BMD) of alveolar bone post-tooth extraction after 30 days.*$p<0.05$ vs. control.

mRNA	Primer sequence	Product (bp)	Annealing T^{o}
EP1	5'CCCTGCTGGTATTGGTGGT3' 5'AGGTGGGACGTGAATCCAGA3'	592	59ºC
EP2	5'TCCCTGCCTTTCACAATCTT3' 5'TGAGCGCATTAGTCTCAGGAC3'	156	59ºC
EP3	5'TGTGTGTACTGTCCGTCTGC3' 5'TCAGGTTGTTCATCATCTGGCA3'	231	62ºC
EP4	5'GTCACTGACCTACTGGGCAC3' 5'CCGGGTTTCTGCTGATGTCT3'	754	63ºC
OPG	5'GTTCTTGCACAGCTTCACCA3' 5'AAACAGCCCAGTGACCATTC3	121	59ºC
RANKL	5'ACCAGCATCAAAATCCCAAG3' 5'TTTGAAAGCCCCAAAGTACG3'	204	60ºC
β-Actin	5'ATTGAACACGGCATTGTCACC3' 5'GGTCATCTTTTCACGGTTGGC3'	156	56.7ºC

Table 1