Effects of saliva on early post–tooth extraction tissue repair in rats

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ABSTRACT

The aim of the present study was to perform a biochemical, histological, and histomorphometrical evaluation of the mechanisms involved in tissue repair in rats subjected to submandibulectomy-induced hyposialia, 24, 48, and 72 hours of post–tooth extraction. We studied the correlation between the lack of submandibular saliva and the modulation of inflammatory mediators involved in tissue repair, such as prostaglandin E_2, nitric oxide (NO), and tumor necrosis factor alpha (TNF-α). Rats with hyposialia showed a delay in socket healing, slow replacement of the clot with granulation tissue, and fewer cells and collagen fibers, concomitant with a longer inflammatory process, as compared to controls. The lack of saliva induced by submandibulectomy modified the levels of prostaglandin E_2, NO, and TNF-α, and tissue response in the early stages of wound healing compared to controls, and could thus determine alterations in later osteogenic response. Our results allow concluding that hyposialia modulates the parameters of inflammation studied here, and that it is essential for optimal healing. Therefore, these findings provide evidence for the importance of submandibular saliva to final bone socket healing.

Traumatic and surgical wounds are frequent in the oral cavity. One of the most common oral wounds is the extraction socket wound after tooth removal.[1] Saliva may have wound healing properties as it contains several immunobiologically active substances, such as immunoglobulins, antibacterial factors, and growth promoting mediators. Most saliva (90%) is produced by the major salivary glands, with the parotid gland contributing 20% and the submandibular (SMG) and sublingual (SLG) glands secreting the remaining 70% of the daily output of saliva. The SMG and SLG are of great interest due the type and content of the secretion they produce (electrolytes, water, growth factors, such as epidermal growth factor, transforming growth factor-α, and nerve growth factor, immunoglobulins, and enzymes, among others components), and also because they are responsible for basal secretion of saliva during rest, in humans.

A decrease in salivary secretion, termed hyposialia, leads to alterations in the physiologic functions of saliva, such as reduced cleansing and lubrication of the oral mucosa,[2] reduced antibacterial activity,[3] and delayed wound healing.[4]

The healing process consists of four phases that overlap in time and space: hemostasis, inflammation, tissue formation, and tissue remodeling. Each phase contributes specifically to wound healing. In addition, the temporal sequence of the healing stages is crucial to optimal tissue repair and restoration of tissue integrity. Wound healing in the socket follows similar principles as soft tissue healing, except that it also involves bone healing in the socket at later stages.[1]

The first stages of wound healing involve fibrin clot formation as a result of hemostasis. The clot provides a matrix for the influx of inflammatory cells including peripheral monocytes, which give rise to macrophages and neutrophils, which in turn remove contaminating bacteria.[5] Macrophages play a pivotal role in wound healing mainly by contributing to wound sterilization, by increasing the inflammatory response, and by performing tissue debridement. In addition, macrophages initiate the development of granulation tissue (GT) and release a variety of mediators such as nitric oxide (NO), proinflammatory cytokines, tumor necrosis factor-alpha (TNF-α), interleukin 1 and 6, and growth factors.[6,7] NO is a signal molecule involved in immune responses, angiogenesis, epithelialization, and formation of GT.[8] TNF-α is a proinflammatory and immunoregulatory cytokine, and as such plays a role in the recruitment of inflammatory cells and in bone resorption,[9] inhibits bone collagen synthesis, induces collagenases, and stimulates osteoclast differentiation.[10] The alveolar bone repair process is initiated immediately after tooth extraction and can be derailed by various local and / or systemic factors, such as salivary alterations, radiation, and deficient diets, among others.[11–14] Nevertheless, there is scant information on the effect of saliva particularly on the mediators of inflammation and on tissue response in socket wound healing.

Hyposialia is an increasingly frequent clinical problem in view of its multifactorial etiology: polymedication, oncological treatment, autoimmune diseases, stress, and advanced age, among other causes.[15] The quality of life of patients is affected to a great extent, since they are bound to suffer functional and organic alterations. The

decreased salivary flow produces dental caries, oral candidiasis, gingivitis, and periodontitis.[16] In view of the association between hyposialia and its possible deleterious systemic effects and the increased risk of oral disease, gaining further knowledge about this disease is essential. The process of wound healing after tooth extraction has been extensively studied in humans and experimental animals.[17,18] However, there is little evidence on the effect of hyposialia on the mechanisms involved in tissue repair and on inflammatory mediators[19] such as NO, prostaglandins (PGs) and TNF-α. Therefore, the aim of the present work was to study the effect of saliva on wound repair using an experimental model of "pure" hyposialia induced by submandibulectomy, in the absence of systemic factors that could affect the wound healing process. For this purpose, we used tooth extraction as a model of oral wound healing to perform biochemical, histological, and histomorphometrical studies of the effects of submandibulectomy on the early stages of tissue repair post–tooth extraction in rats.

MATERIALS AND METHODS

Animals

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 and maintained on a 14:10 hours 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

Animals (70 g) were randomly assigned to an experimental or a control group. Those in the experimental group underwent submandibulectomy (SMx). Bilateral excision of the SMG and SLG was performed ($n = 30$) (16). The control group was subjected to a sham operation (Sham) ($n = 30$). 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).[20] All the animals were euthanized (CO_2 chamber) in groups of 10 at 24, 48, or 72 hours post–tooth extraction. The mandibles were resected and processed for histological and biochemical analyses.

Radiographic study

After fixation in 10% buffered formalin solution, the hemimandibles were radiographed using standard equipment and intraoral periapical radiographs (Kodak, Eastman Kodak Company, Rochester, NY). The radiographs were developed using a standard technique.

Histological processing

One hemimandible from each animal was decalcified in 5% formic acid, embedded in paraffin, and semiserially sectioned in a frontal plane (buccolingual direction) at the level of the mesial socket of the first mandibular molar to obtain 10 μm thick sections, which were stained with hematoxylin & eosin, Picrosirius red, and Masson's trichrome.

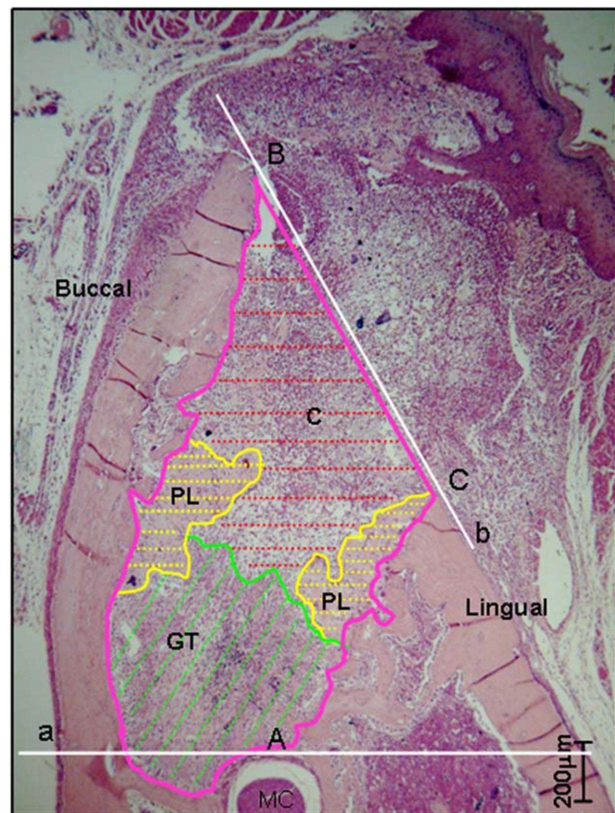

Figure 1. Microphotograph of a buccolingual section of the mesial alveolus of the first lower molar obtained 72 hours post–tooth extraction corresponding to the Sham group. Note that the socket (outlined in pink) is completely filled with clot (C-hatched area in red), granulation tissue (GT-hatched area in green) and periodontal ligament (PL-hatched area in yellow). Total area of the alveolus: Line **a** is drawn tangential to the upper cortical border of the mandibular canal (A) and perpendicular to the external surface of the buccal plate. Line **b** is drawn tangential to the highest point of the bone crest on the buccal aspect of the alveolus (B) and the highest point of the bone crest on the lingual aspect of the alveolus (C). MC Mandibular canal, B Buccal plate, L Lingual plate, H&E stain.

Histomorphometric evaluation

Digital microphotographs of the histological sections were obtained using a digital camera (Canon Power Shot® A640) fitted with a light microscope (Axioscop 2 MOT, Carl Zeiss, Jen, Germany). Histomorphometric parameters were evaluated using Image-Pro Plus Software for Windows, Version 3.0 (Media Cybernetics Inc., Rockville, MD), and were studied in two-dimensional.[21] The following histomorphometric determinations were made (1) total area of the alveolus, considering the tissues situated above line (a), drawn tangential to the upper cortical border of the mandibular canal (A) and perpendicular to the external surface of the buccal plate,[20] and delimited by the periodontal alveolar cortical bone and by line (b) drawn tangential to the highest point of the bone crest on the buccal

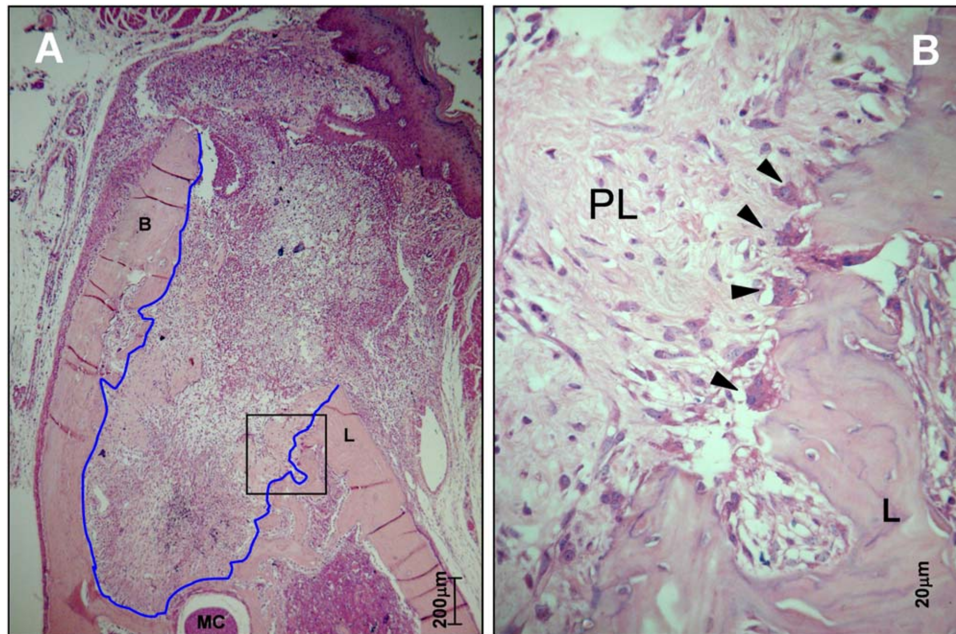

Figure 2. (A) Microphotograph of a buccolingual section of the mesial alveolus of the first lower molar obtained 72 hours post–tooth extraction, corresponding to the Sham group. The total perimeter (Pm) of the periodontal alveolar cortical bone (marked with a line) was measured to calculate the percentage of osteoblast (Ob. Pm), quiescent (Q. Pm), and eroded (E. Pm) perimeters. The number of osteoclasts on the eroded perimeter was determined. MC Mandibular canal, B Buccal plate, L Lingual plate, H&E stain. (B) This microphotograph of the square outlined in A at higher magnification allows visualization of the eroded surface on the periodontal alveolar cortical bone lined by osteoclasts (Oc +) (arrowhead). PL Periodontal ligament, L Lingual plate, H&E stain. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

aspect of the alveolus (B) and the highest point of the bone crest on the lingual aspect of the alveolus (C).

The following parameters were determined at 72 hours post–tooth extraction: the area of the alveolus occupied by the clot (C) (C. Ar), by GT (GT. Ar), and by periodontal ligament (PL) (PL. Ar); the results are expressed as a percentage (Figure 1). (2) The total perimeter of the periodontal alveolar cortical bone was measured (Figure 2A), and the percentage corresponding to osteoblast perimeter (Ob. Pm), quiescent perimeter (Q. Pm), and eroded perimeter (E. Pm) was calculated. The eroded perimeter with (Oc+) and without (Oc−) osteoclasts was also determined. The number of osteoclasts (N. Oc) on the periodontal alveolar cortical bone was determined (Figure 2B).

Biochemical studies

The socket tissue occupying the alveolus and the ridge mucosa were obtained from one hemimandible, and were processed to measure inducible nitric oxide synthesis (iNOS) activity. The socket tissue from the contralateral hemimandible was processed to assess prostaglandin E (PGE2) and TNF-α content and to perform COX-2 protein determination, according to the technique requirements.

Measurement of iNOS activity

NOS activity was measured using a modification of the method described by Bredt and Snyder,[22] measuring the conversion of [^{14}C]arginine into [^{14}C]citrulline. Because there are active urea cycles in the tissue, arginine will also be converted to citrulline by this cycle, thereby biasing results and giving higher values for NOS activity. This problem was avoided by adding L-valine (50 mM) to the HEPES buffer for homogenization and incubation to block the arginase of the urea cycle. The method indirectly measures NO production, an index of iNOS activity. The details of the method used here were published previously.[23]

Determination of PGE content

PGE content of the socket tissue was determined by specific RIA as a determinant of COX activity. Socket tissues were processed as described previously.[23] Rabbit antiserum from Sigma-Aldrich (St Louis, MO) 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 tissue weight.

Western blot for cyclooxygenase 2 (COX-2) protein measurement

Socket tissue was lysed in ice-cold protein extraction buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 1% Triton X-100, and containing protease inhibitory cocktail

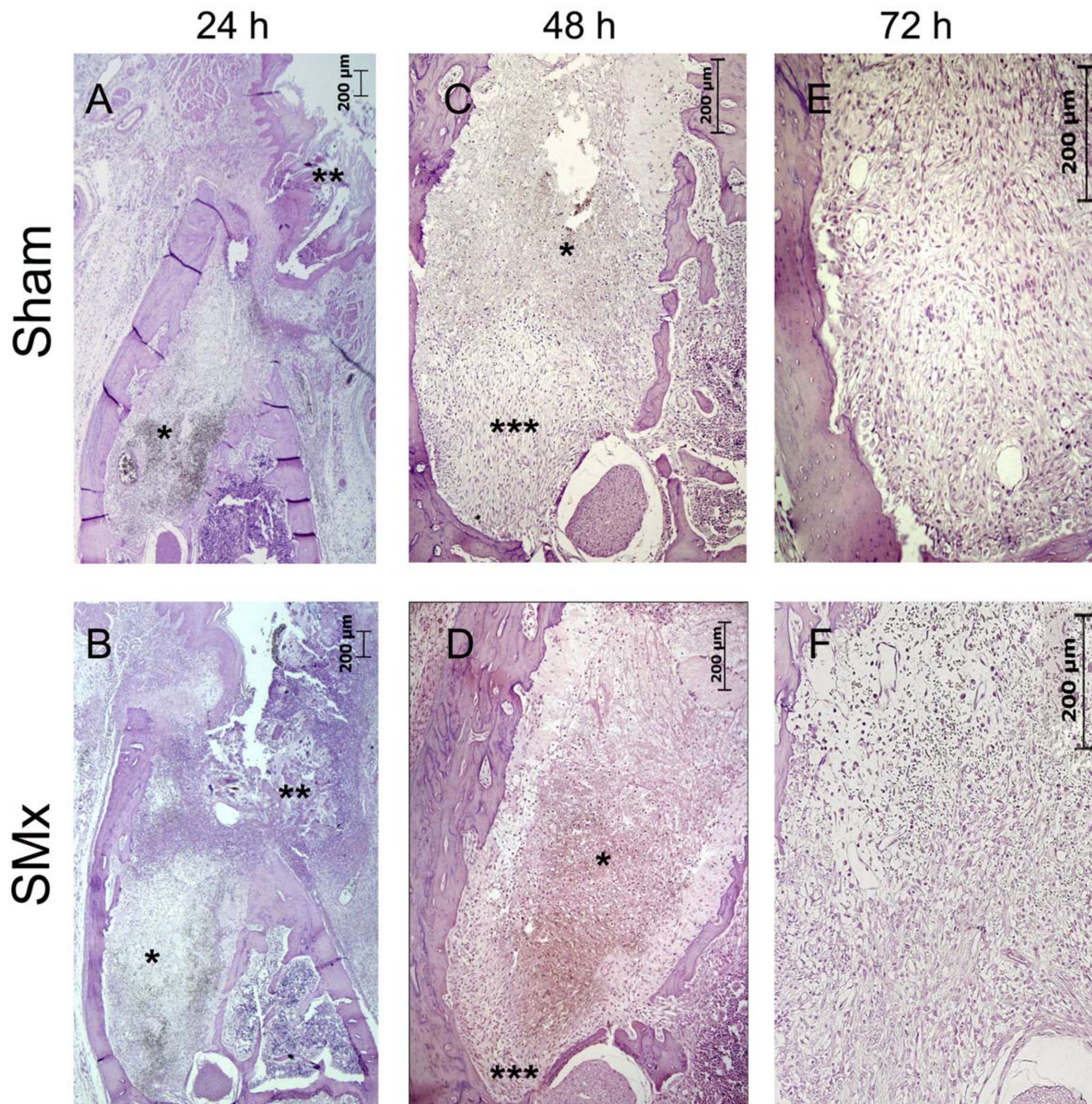

Figure 3. Microphotographs of buccolingual sections of the mesial alveolus of the first lower molar obtained 24, 48, and 72 hours post–tooth extraction. (A) (Sham) and (B) (SMx) post–tooth extraction sockets at 24 hours were filled with clot (*). Note the pseudomembrane (**) covering a larger area of SMx extraction sockets as compared to Sham sockets. H&E stain. (C) (Sham) and (D) (SMx) at 48 hours. It can be clearly observed that the apical third of tooth-extraction sockets corresponding to the Sham group (C) was filled with GT (***), whereas that of the SMx group (D) exhibited clot (*) and little GT (***). (E) (Sham) and (F) (SMx) at 72 hours. The GT in the apical third of SMx sockets (F) was less cellular, had more edema and a greater number of erythrocytes compared to the GT observed in Sham sockets (E). H&E stain. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

from Sigma-Aldrich). The homogenates were briefly centrifuged, and the supernatants were removed and stored at −70 °C 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 °C with anti-COX-2 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.

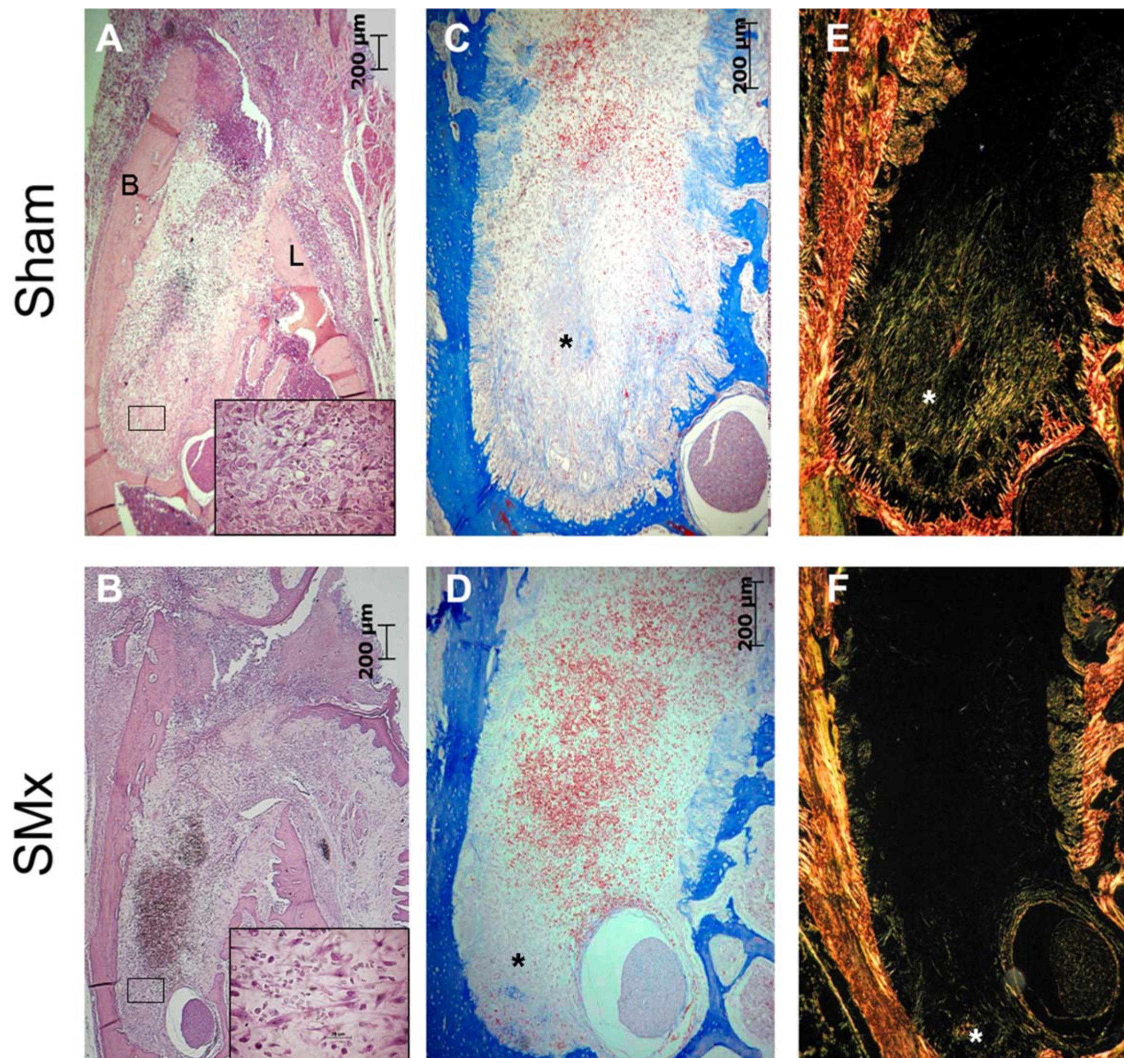

Figure 4. Microphotographs of buccolingual sections of the mesial alveolus of the first lower molar obtained 72 hours post–tooth extraction. (A) (Sham) and (B) (SMx). The images clearly show the differences between both groups as regards the characteristics of the GT in the apical third of the socket. The square outlined in the zone corresponding to the apical area of the socket shows: A (Sham) GT with fibroblasts, neoangiogenesis, scarce inflammatory cells. B (SMx) loose GT with edema, inflammatory cells and an evident decrease in fibroblasts and collagen fibers. B Buccal plate, L Lingual plate, H&E stain. (C) (Sham) and (D) (SMx); Masson's Trichrome. (E) (Sham) and (F) (SMx); polarized light microscopy of Picrosirius Red stained sections. (*) Note the evident presence of a higher proportion of collagen fibers in Sham sections as compared SMx sections.

Determination of TNF-α

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

Statistical analysis

The results are expressed as mean and standard error. Student's *t* test was used to establish comparisons between two groups. 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

No immediate or long-term postoperative complications were observed.

Histological study

Histologic examination at 24 hours post extraction showed the presence of a clot filling the socket, PL remnants

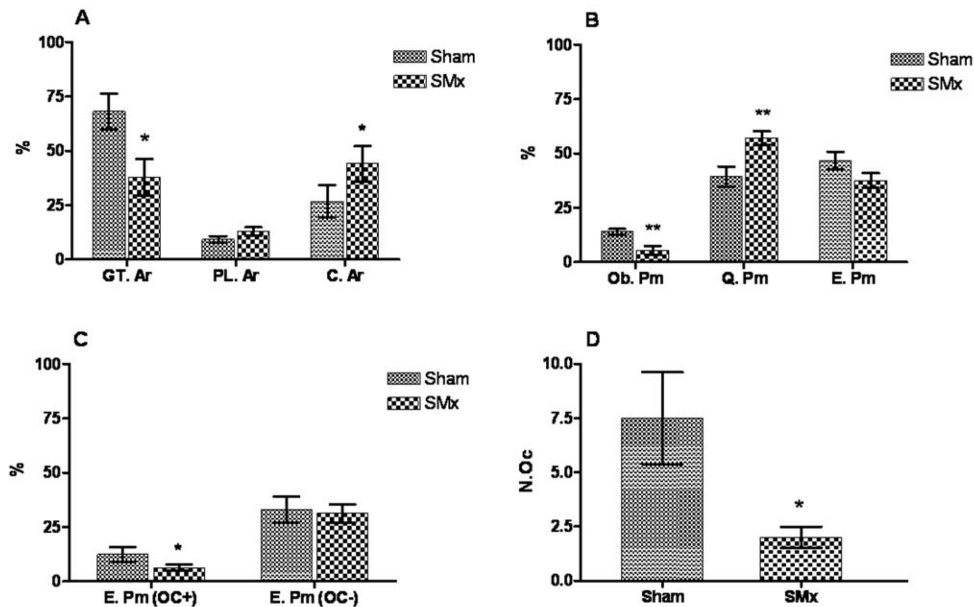

Figure 5. Histomorphometric determinations at 72 hours post–tooth extraction. (A) Percentage of tissue in the alveolus: granulation tissue (GT. Ar); periodontal ligament (PL. Ar) and clot (C. Ar). (B) Bone activity: percentage of osteoblast (Ob. Pm), quiescent (Q. Pm), and eroded (E. Pm) perimeter on the periodontal alveolar cortical bone. (C) Percentage of eroded perimeter with osteoclasts (E.Pm (OC+)) and percentage of eroded perimeter without osteoclasts (E.Pm (OC−)) on the periodontal alveolar cortical bone. (D) Number of osteoclasts on the periodontal alveolar cortical bone. Data are expressed as mean ± standard error ($n = 8$–10). Statistical significance was set at a value of $*p < 0.05$; $** p < 0.01$. Student's *t* test.

inserted in the periodontal alveolar cortical bone, and a pseudomembrane on the surface exhibiting polymorphonuclear neutrophils, cellular detritus, microorganisms, and food remains. The pseudomembrane was wider and the presence of food remains was more marked in SMx compared to Sham animals (Figure 3A and B). The differences between both groups regarding the features of the pseudomembrane were also observed at 48 hours. At the latter time point (48 hours), all the animals exhibited GT replacing the clot in the area between the clot and the periodontal alveolar cortical bone. The apical third of Sham sockets was filled with GT with neovascularization, and containing many fibroblasts and collagen fibers and scarce polymorphonuclear neutrophils, whereas that of SMx animals still contained clot and had less GT (Figure 3C and D). The proportion of GT observed at 72 hours was larger than that observed at 48 hours; however, the difference between the proportion and the characteristics of GT in SMx and Sham at 72 hours post–tooth extraction sockets remained unchanged (Figure 3E and F). The GT found in SMx was different from that observed in Sham (Figure 4A and B), exhibiting more edema, fewer fibroblasts, less neovascularization, fewer collagen fibers (as visualized by polarized light microscopy of picrosirius red stained sections), and more red blood cells and polymorphonuclear neutrophils (Figure 4B, D, F).

Histomorphometrical evaluation

Area of the alveolus occupied by the clot, by granulation tissue and periodontal ligament. Bone remodeling at the periodontal alveolar cortical bone.

SMx sockets exhibited a significantly ($p < 0.01$) smaller proportion of GT (27.79% ± 2.75) as compared to Sham (39.14% ± 2.45) 48 hours post tooth extraction.

Although the proportion of GT found at 72 hours was significantly larger compared to that observed at 48 hours (10%) $p < 0.05$, the difference in the proportion of GT between SMx and Sham extraction sockets remained unchanged ($p < 0.05$). As regards the percentage of PL 72 hours after tooth extraction, no statistically significant differences were observed between the experimental groups. Conversely, the percentage of GT and clot differed significantly ($p < 0.05$) between both groups (Figure 5A).

Analysis of remodeling on the periodontal alveolar cortical bone evidenced that the percentage of quiescent perimeter increased ($p < 0.01$) in the SMx group. Conversely the percentage of osteoblast perimeter ($p < 0.01$) and the eroded perimeter decreased in the SMx group (Figure 5B).

Whereas the percentage of eroded perimeter with osteoclasts (Oc Pm (Oc+)) was significantly ($p < 0.05$) lower in the SMx than in the Sham group, no significant differences in the percentage of eroded perimeter without osteoclasts (Oc Pm (Oc–)) were observed between groups

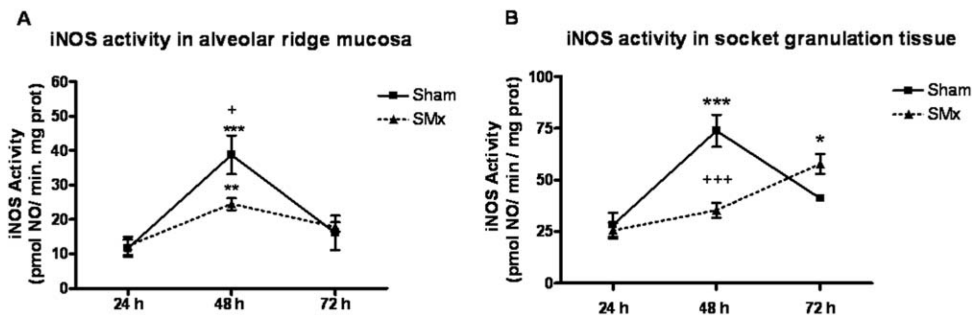

Figure 6. Determination of inducible nitric oxide synthase activity. iNOS activity in (A) alveolar ridge mucosa and (B) socket GT of Sham and SMx rats at 24, 48, and 72 hours post–tooth extraction. Data are expressed as mean ± standard error (A and B) ($n = 8$–10). Statistically significant differences within groups at the different time points (Sham vs. Sham and SMx vs. SMx) (***$p < 0.001$; **$p < 0.01$, *$p < 0.05$) and between groups at the different time points (Sham vs. SMx) $^{+++}p < 0.001$, $^{+}p < 0.05$, are shown. Two-way ANOVA and Tukey's posttest.

(Figure 5C). The number of osteoclasts (N.Oc) adjacent to the periodontal alveolar cortical bone was significantly ($p < 0.05$) lower in the SMx than in the Sham group (Figure 5D).

Inflammatory parameters

Inducible nitric oxide production

The activity of iNOS in the alveolar ridge mucosa was significantly higher in both the Sham group ($p < 0.001$) and the SMx group ($p < 0.01$) at 48 hours compared to their respective groups at 24 and 72 hours. The increase in iNOS in the SMx group at 48 hours post extraction was lower than that observed in the Sham group ($p < 0.05$). There were no differences in iNOS activity between groups at 24 or 72 hours (Figure 6A).

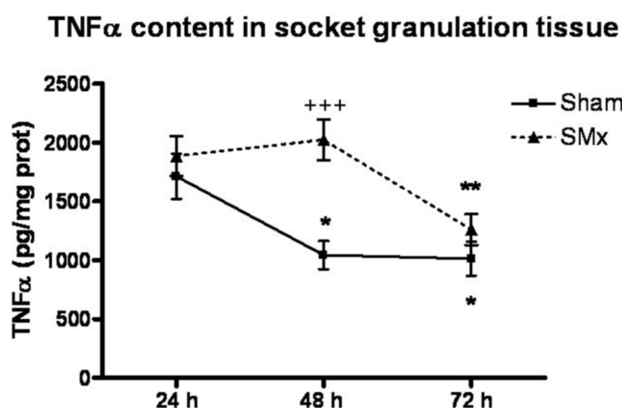

Figure 7. Determination of TNF-α. TNF-α content in GT of Sham and SMx socket at the studied experimental time points. Data are expressed as mean ± standard error ($n = 10$). Statistically significant differences within groups (Sham vs. Sham and SMx vs. SMx) (*$p < 0.05$, **$p < 0.01$) and between groups (Sham vs. SMx) at the different time points (Sham and SMx), $^{+++}p < 0.001$ are shown. Two-way ANOVA and Tukey's posttest.

Granulation tissue iNOS activity in the Sham group was significantly higher ($p < 0.001$) at 48 hours than at 24 and 72 hours post–tooth extraction. However, iNOS activity in SMx remained unchanged at the first experimental time points (24 and 48 hours), and was significantly higher 72 hours ($p < 0.05$) post–tooth extraction. iNOS activity at 48 hours post–tooth extraction was significantly lower ($p < 0.001$) in the SMx than in the Sham group (Figure 6B).

TNF-α production

TNF-α content in GT was markedly elevated 24 hours post–tooth extraction in both Sham and SMx, but decreased significantly ($p < 0.05$) at 48 and 72 hours in the Sham group. Interestingly TNF-α levels in SMx rats remained significantly higher ($p < 0.001$) at 48 hours as compared to the Sham group, and then decreased, with values at 72 hours being significantly lower ($p < 0.01$) than SMx levels at 48 hours and approaching Sham values at 72 hours post–tooth extraction (Figure 7).

Prostaglandin E content and COX-2 protein expression

No differences in GT PGE levels were observed between Sham and SMx groups at the studied time points. The highest PGE levels were observed 24 hours post–tooth extraction in both groups; the difference was statistically significant ($p < 0.05$) compared to levels observed 48 and 72 hours post–tooth extraction in each group respectively. At 72 hours post–tooth extraction, the SMx group showed higher PGE content than the Sham group, but the difference did not reach statistical significance (Figure 8A). However, PGE2 synthase expression (COX-2) increased significantly ($p < 0.01$) in the GT of the SMx group at 72 hours post–tooth extraction as compared to that of the Sham group (Figure 8B).

DISCUSSION

Although many mediators produced by the salivary glands may have a direct effect on wound healing, little is known

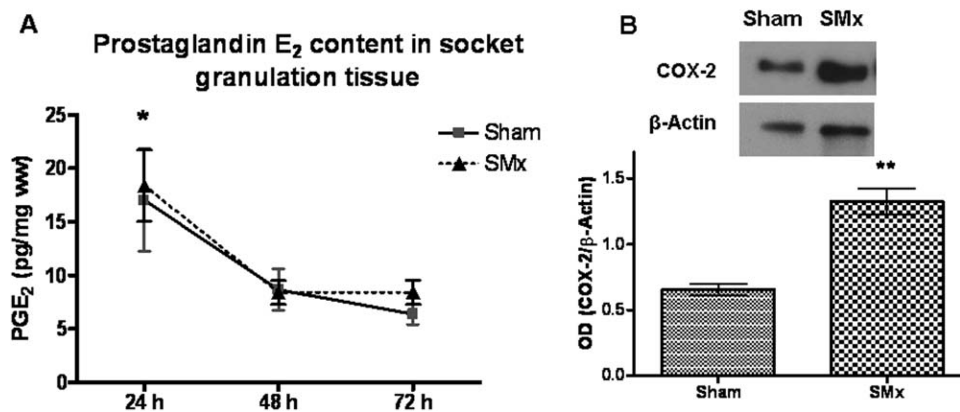

Figure 8. Determination of Prostaglandin E2 and expression of COX-2 enzyme. (A) Prostaglandin E_2 content in GT in Sham and SMx sockets at the different experimental time points. Data are expressed as mean ± standard error ($n = 8$-10). Statistically significant differences within groups at the different time points (Sham vs. Sham and SMx vs. SMx) are shown $*p < 0.05$. Two-way ANOVA and Tukey's post hoc test. (B) Cyclooxygenase-2 isoform (COX-2) in socket GT at 72 hours, as revealed by Western blot. Each band represents a pool of three animals; one of three runs is shown. The bars depict the ratio between COX-2 expression and the standardized expression of *β*-actin, both measured as the optical density (OD) of their bands. Data are expressed as mean ± standard error ($n = 3$ runs). Statistical significance was set at a value of $**p < 0.01$; Student's *t* test.

about how saliva is involved in the different stages of tissue repair.

In the present study, we assessed the effect of submandibulectomy-induced experimental hyposialia on the early stages of tissue repair, in a model of tooth extraction. In agreement with Bodner et al.,[4] the present results evidenced a delay in wound healing in rats with hyposialia, resulting from slow replacement of the clot by GT in addition to a delay in epithelialization, concomitant with a longer inflammatory process with polymorphonuclear neutrophils. Although the inflammatory component is important and plays a key role in tissue repair, the exacerbation of inflammation may delay or disrupt the repair process.[24] Clearly, the lack of self-cleaning, as well as of bactericidal compounds, growth factors, and other mediators present in saliva, that occurs in animals with hyposialia, favors the alterations observed in the early stages of socket wound healing studied herein. All these factors contribute to increased polymorphic cell recruitment in response, at least in part, to food debris lodged in the pseudomembrane that covers the surface of the wound.

The main contribution of this work is the discovery that saliva modulates inflammatory factors that correlate with histological observations. PGE_2 levels in GT were high at 24 hours post tooth extraction and diminished gradually towards 72 hours, showing no differences between groups at that time point. At 72 hours, however, COX-2 expression was found to increase in the SMx group, and a small not significant increase in PGE_2 content was observed. Our results are in agreement with experimental studies reporting high levels of PGE_2 in the first hours of cell activation caused by injury.[25] This high production of PGE_2 is consistent with the inflammatory process that always takes place during the first hours of the tissue repair process.[26–28] Based on the above, the increase in COX-2 expression observed in SMx rats 72 hours post–tooth extraction may partly be caused by the persistence of the inflammatory process associated with hyposialia, or may be a response that will be expressed as an increase in the production of PGE_2 at later stages of the post extraction socket repair process in rats with hyposialia.

In addition, it is noteworthy that we found that submandibulectomy modifies the production of another key mediator in wound healing, as is NO. In keeping with studies demonstrating that iNOS is expressed during wound repair,[8,29–31] our results showed that both GT and alveolar ridge mucosa iNOS activity in the Sham group increased significantly at 48 hours post extraction and then decreased at 72 hours, approaching tissue iNOS levels observed 24 hours post extraction. The increase in GT iNOS activity in SMx rats was delayed, occurring at 72 hours post tooth extraction. This did not occur in the alveolar ridge mucosa of SMx rats, where NO production decreased with no delay in iNOS activation. These data provide the first evidence of a correlation between the lack of submandibular saliva and the modulation of inflammatory mediators of tissue repair. Furthermore, our results are consistent with other studies showing that NOS activity is highest during the early phase of wound healing.[8,29]

It is conceivable that the majority of NO synthesis is due to the inflammatory cells present during the early phase of healing, especially macrophages.[30,32] However, and though to a lesser degree, fibroblasts, keratinocytes, and endothelial cells contribute to ongoing NO synthesis.[33,34] The decrease in iNOS activity observed in our experimental model could be due to the presence of fewer macrophages and/or differential activation of the macrophage phenotype.[7] Macrophages play a key role in removing clots, and release a number of growth factors that induce the development of GT.

Bone remodeling, with the subsequent loss of periodontal alveolar cortical bone, is a physiological process that follows tooth extraction. This process results from the absence of biomechanical demands exerted by the PL

inserted in the bone and the tooth. In this regard, we found decreased osteoclast counts concomitant with increased quiescent surfaces in the periodontal alveolar cortical bone of SMx animals. Since osteoclasts belong to the monocyte-macrophage lineage, macrophage activation and recruitment may be decreased in the alveoli of SMx rats. Macrophages are necessary for collagen synthesis, cell proliferation, fibrosis and other tissue remodeling functions.[35]

The loose newly formed GT with an evident decrease in fibroblasts, neovascularization, and collagen fiber formation observed in the SMx groups could partially be due to the lower production of NO. It has been demonstrated that collagen synthesis correlates with NO synthesis during wound healing, where, like low NO levels, excessive NO production impairs collagen synthesis.[34,36,37]

In addition, our results showed high levels of TNF-α in GT; however, the cytokine was not detected in plasma using the method employed herein (data not shown). These results are consistent with other studies showing that TNF-α levels are elevated in the very early stages of wound repair.[38] Interestingly, TNF-α levels remained elevated in the SMx group at 48 hours as compared to the Sham group and decreased significantly at 72 hours post extraction.

TNF-α secreted by inflammatory cells has been reported to both inhibit extracellular matrix synthesis and activate matrix metalloproteinases.[24,38] We suggest that NO, as well as TNF-α, could be responsible for the delay in clot organization by generating changes in certain structural proteins that are essential to wound healing, such as α-smooth muscle actin fibers, collagen, and fibronectin. Such changes result in decreased contraction and stiffness of collagen matrices[39] and also modify enzymes involved in the process, including collagenases and metalloproteinases. These possible effects suggest a time dependent role of NO and TNF-α in normal wound healing. Therefore, the balance of cytokines in a wound defines the mechanical properties of the extracellular matrix and optimal wound healing.[40]

In this work, we provide elements to elucidate the mechanism by which hyposialia induces an excessive inflammatory response, which delays tissue repair in the early stage of extraction socket healing. Clearly, NO, TNF-α, and PGE levels are altered in hyposialia. However, the complete mechanism that is triggered under these conditions remains to be identified, as it is probably a result of a set of integrated and overlapping mediators. Thus, it remains unclear whether TNF-α is a necessary mediator or whether it detracts from optimal tissue repair. Nevertheless, the inflammatory phase of wound healing must progress to a proliferative phase to advance in the wound healing process. Our results allow concluding that saliva is necessary in the early stages of post–tooth extraction wound healing for optimal wound healing in the rat, since it was found to modulate the expression and activity of inflammatory mediators in alveolar bone tissue repair at the studied time points. The hyposialia induced using the experimental model described herein exacerbated the inflammatory process that occurs in wound healing processes. It inhibited NO production, and increased GT TNF levels, leading to a delay in GT formation and also affecting the quality of the GT formed in the early stages of post–tooth extraction socket healing.

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