J Periodontal Implant Sci. 2021 Dec;51(6):374-385
https://doi.org/10.5051/jpis.2100700035
pISSN 2093-2278·eISSN 2093-2286

JPIS
Journal of Periodontal & Implant Science

Research Article
Periodontal Science

Check for updates

Adjuvant therapy with 1% alendronate gel for experimental periodontitis treatment in rats

Natália de Campos Kajimoto [1,*], **Yvonne de Paiva Buischi** [1,2], **Peter Michael Loomer** [1], **Timothy G. Bromage** [3], **Edilson Ervolino** [4], **Stephen Enrico Fucini** [5], **Natália Marcumini Pola** [6], **Beatriz Ommati Pirovani** [7], **Maria Juliana Sismeiro Morábito** [7], **Juliano Milanezi de Almeida** [7], **Flávia Aparecida Chaves Furlaneto** [8], **Maria José Hitomi Nagata** [7]

[1]Department of Periodontics, The University of Texas Health Science Center at San Antonio, San Antonio, TX, USA
[2]Department of Periodontology and Implant Dentistry, New York University College of Dentistry, New York, NY, USA
[3]Department of Biomaterials & Biomimetics, New York University College of Dentistry, New York, NY, USA
[4]Division of Histology, Department of Basic Sciences, São Paulo State University (UNESP), School of Dentistry, Araçatuba, SP, Brazil
[5]Private Practice, Hanover, NH, USA
[6]Division of Periodontics, Department of Semiology and Clinic, Federal University of Pelotas (UFPEL), School of Dentistry, Pelotas, RS, Brazil
[7]Division of Periodontology, Department of Surgery and Integrated Clinic, São Paulo State University (UNESP), School of Dentistry, Araçatuba, SP, Brazil
[8]Division of Periodontology, Departament of Oral Maxillofacial Surgery and Periodontology, University of São Paulo (USP), School of Dentistry, Ribeirão Preto, SP, Brazil

OPEN ACCESS

Received: Feb 3, 2021
Revised: Apr 12, 2021
Accepted: May 10, 2021

***Correspondence:**
Natália de Campos Kajimoto
Department of Periodontics, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78229, USA.
E-mail: periodontia.natalia@gmail.com
Tel: +55-18-98129-8519

ORCID iDs
Natália de Campos Kajimoto
https://orcid.org/0000-0001-7245-5213
Yvonne de Paiva Buischi
https://orcid.org/0000-0001-6716-8957
Peter Michael Loomer
https://orcid.org/0000-0002-4676-0895
Timothy G. Bromage
https://orcid.org/0000-0002-9843-7993
Edilson Ervolino
https://orcid.org/0000-0003-4859-0583
Stephen Enrico Fucini
https://orcid.org/0000-0002-7711-7907
Natália Marcumini Pola
https://orcid.org/0000-0001-9188-7337
Beatriz Ommati Pirovani
https://orcid.org/0000-0003-4536-6674
Maria Juliana Sismeiro Morábito
https://orcid.org/0000-0002-8913-0914
Juliano Milanezi de Almeida
https://orcid.org/0000-0002-5995-5747

ABSTRACT

Purpose: The aim of this study was to evaluate the effects of locally delivered 1% alendronate (ALN) gel used as an adjunct to non-invasive periodontal therapy.
Methods: Ligature-induced periodontitis was performed in 96 rats. The ligature was tied in the cervical area of the mandibular left first molar. The animals were randomly divided into 4 groups: 1) NT, no treatment; 2) SRP, scaling and root planning; 3) SRP/PLA, SRP followed by filling the periodontal pocket with placebo gel (PLA); and 4) SRP/ALN, SRP followed by filling the periodontal pockets with 1% ALN gel. Histomorphometric (percentage of bone in the furcation region [PBF]) and immunohistochemical (receptor activator of nuclear factor-κB ligand, osteoprotegerin, and tartrate-resistant acid phosphatase) analyses were performed. Data were statistically analyzed, with the threshold of statistical significance set at $P \leq 0.05$.
Results: The SRP, SRP/PLA, and SRP/ALN groups presented a higher PBF than the NT group ($P \leq 0.01$) at 7, 15, and 30 days. The SRP/ALN group presented a higher PBF than the SRP/PLA group in all experimental periods, as well as a higher PBF than the SRP group at 15 and 30 days. No differences were observed in the immunohistochemical analyses ($P > 0.05$ for all).
Conclusions: Locally delivered 1% ALN gel used as an adjunct to SRP enhanced bone regeneration in the furcation region in a rat model of experimental periodontitis.

Keywords: Alendronate; Dental scaling; Periodontal disease; Periodontitis; Rats

JPIS Journal of Periodontal & Implant Science

Flávia Aparecida Chaves Furlaneto
https://orcid.org/0000-0002-8072-3983
Maria José Hitomi Nagata
https://orcid.org/0000-0001-5903-5652

Funding
Maria Juliana S. D. Morábito received a scholarship from the São Paulo Research Foundation (FAPESP, São Paulo, SP, Brazil, Grant #18/03331-1). Dr. Natália de Campos received a scholarship from the Coordination for the Improvement of Higher Education Personnel (CAPES, Brasília, DF, Brazil).

Author Contributions
Conceptualization: Natália de Campos Kajimoto, Edilson Ervolino, Stephen Enrico Fucini, Juliano Milanezi de Almeida, Flávia Aparecida Chaves Furlaneto, Maria José Hitomi Nagata; Formal analysis: Natália de Campos Kajimoto, Timothy G. Bromage, Edilson Ervolino, Juliano Milanezi de Almeida, Maria José Hitomi Nagata; Investigation: Natália de Campos Kajimoto, Natália Marcumini Pola, Edilson Ervolino, Beatriz Ommati Pirovani, Maria Juliana Sismeiro Morábito; Methodology: Natália de Campos Kajimoto, Edilson Ervolino, Natália Marcumini Pola, Yvonne de Paiva Buischi, Peter Michael Loomer, Juliano Milanezi de Almeida, Maria José Hitomi Nagata; Project administration: Natália de Campos Kajimoto, Maria José Hitomi Nagata; Writing - original draft: Natália de Campos Kajimoto, Yvonne de Paiva Buischi, Peter Michael Loomer, Timothy G. Bromage, Edilson Ervolino, Stephen Enrico Fucini, Natália Marcumini Pola, Beatriz Ommati Pirovani, Maria Juliana Sismeiro Morábito, Juliano Milanezi de Almeida, Flávia Aparecida Chaves Furlaneto, Maria José Hitomi Nagata; Writing - review & editing: Natália de Campos Kajimoto, Yvonne de Paiva Buischi, Peter Michael Loomer, Maria José Hitomi Nagata, Timothy G. Bromage, Edilson Ervolino, Stephen Enrico Fucini, Natália Marcumini Pola, Beatriz Ommati Pirovani, Maria Juliana Sismeiro Morábito, Juliano Milanezi de Almeida, Flávia Aparecida Chaves Furlaneto.

Conflict of Interest
No potential conflict of interest relevant to this article was reported.

INTRODUCTION

Recent advances in knowledge regarding the etiology and pathobiology of periodontitis have led to a better understanding of this disease. Periodontitis is now recognized as a complex inflammatory disease that involves subgingival microorganisms, the host immune system and inflammatory response, and predisposing conditions. Thus, there has been a shift from treatment modalities focusing on dental plaque and periodontal pathogens to emerging treatment strategies based on plaque removal combined with host modulation. New treatment modalities combining mechanical debridement with several approaches to either control or resolve inflammation are being explored for the management of periodontitis [1].

Examples of these approaches include the systemic and localized delivery of antibiotics [2], laser photodynamic therapy [3], and bisphosphonates (BPs) [4,5]. These drugs attach to bone minerals and, after being taken up by clastic cells where bone resorption is occurring, they inhibit osteoclast differentiation [6]. In addition, BPs also promote early bone formation [7] and may act as an antagonist of many matrix metalloproteinases related to the connective tissue (CT) destruction of the periodontium [8]. Collectively, these properties make BPs a promising adjunct therapy in the non-surgical treatment of periodontal diseases [9].

Alendronate (ALN) is an aminobisphosphonate that inhibits resorption of bone tissue [10]. It has been demonstrated, in human [4] as well as in animal models [11], that the systemic use of ALN reduced alveolar bone resorption and improved its density. Previous reports have described associations between jaw osteonecrosis and systemic ALN use [12], indicating that systemic ALN for the treatment of periodontitis should be carefully considered. Therefore, the local use of ALN has been proposed as an alternative to avoid those side effects.

Local administration of 1% ALN gel as an adjunct therapy to scaling and root planning (SRP) for treating chronic [10,13-15] and aggressive periodontitis [16], as well as grade II furcation defects [5,17], has been found to decrease probing depth (PD), improve bone fill, and lead to clinical attachment level (CAL) gain. These favorable outcomes suggest that this approach may represent a novel direction in the periodontal regeneration field. However, as Kanoriya et al. [18] pointed out, histological evaluations are still lacking to support the findings of clinical trials.

To the best of the authors' knowledge, there are no histological studies evaluating the delivery of 1% ALN gel in periodontal pockets as an adjunct periodontal therapy. Therefore, the purpose of this study was to analyze, histomorphometrically and immunohistochemically, the efficacy of local administration of 1% ALN gel as an adjunct to SRP for the treatment of experimental periodontitis (EP) in rats.

MATERIALS AND METHODS

Sample size calculation

The sample size was calculated considering each animal as the study unit and the percentage of bone in the furcation region (PBF) as the primary outcome variable. The secondary outcome was immunolabeling patterns in the furcation area (FA) of each experimental group. A significant difference of 20% among groups was taken into consideration with a standard deviation (SD) of 15%, with α and β errors of 0.05 and 0.2, respectively. A number of 8 animals was estimated for each group.

https://doi.org/10.5051/jpis.2100700035

Experimental model

The study protocol was conducted and the manuscript was written according to the NC3Rs "ARRIVE Guidelines, Animal Research: Reporting In Vivo Experiments." The Ethics Committee on Animal Experimentation at São Paulo State University (UNESP) (Araçatuba, SP, Brazil) approved the study protocol (#02412/2011).

Ninety-six 3-month-old adult male rats (*Rattus norvegicus albinus*, Wistar, Philadelphia, PA, USA), weighing 250 to 300 g, were used (UNESP, School of Dentistry, Animal Care Unit). Solid food and water *ad libitum* were provided to the animals. They were randomly assigned to the following groups (n=8): 1) no treatment (NT), EP induction (control); 2) SRP, EP induction followed by SRP; 3) SRP/PLA, EP induction and SRP followed by filling the periodontal pockets with 0.1 mL of placebo gel (PLA) (Aphoticário, Araçatuba, SP, Brazil); and 4) SRP/ALN, EP induction and SRP followed by filling the periodontal pockets with 0.1 mL of 1% ALN gel (Aphoticário, Araçatuba, SP, Brazil). The PLA and ALN gel were inserted slowly into the periodontal pocket using a 1-mL syringe and insulin needle without a bevel.

Induction of periodontitis

In all procedures, animals received general anesthesia by intramuscular administration of xylazine (6 mg/kg of body weight; Coopazine, Coopers, São Paulo, SP, Brazil) and ketamine hydrochloride (70 mg/kg of body weight; Vetaset, Zoetis, Florham Park, NJ, USA). Each animal received a cotton ligature (Corrente Algodão No. 24, Coats Corrente, São Paulo, SP, Brazil) that was tied around the left mandibular first molar, which was maintained for 7 days (Johnson, 1975). In all groups other than the NT group, the same trained operator (NC) performed SRP with a curette (1-2 Mini Five curette, Hu-Friedy, Chicago, IL, USA), according to the protocol reported by Fernandes et al. [19].

Tissue processing

An overdose of thiopental (150 mg/kg of body weight; Cristalia, Itapira, SP, Brazil) was administered to euthanize the animals (7, 15, or 30 days after removing the ligature). The left mandibles were excised, fixed in 4% formaldehyde, and decalcified in 10% ethylenediaminetetraacetic acid solution. After tissue processing, the specimens were embedded in paraffin. Semi-serial sections cut in a disto-mesial direction, 5 μm in thickness, were obtained in a buccal-lingual sequence. The most central buccal-lingual sections of the FA of left mandibular first molars were used for histological, histomorphometric, and immunohistochemical analyses. They were either stained with hematoxylin, eosin, and phloxine for histological and histomorphometric analyses or subjected to an indirect immunoperoxidase method with the following primary antibodies: anti-tartrate-resistant acid phosphatase (TRAP) (SC30833, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-receptor activator of nuclear factor-κB ligand (RANKL) (SC7628, Santa Cruz Biotechnology) and anti-osteoprotegerin (OPG) (SC8468, Santa Cruz Biotechnology). The immunohistochemical protocol was previously described by Nunes et al. [20].

Microscopy procedures

Four histological sections from the center of the original furcation defect in a buccal-lingual direction were selected for the histological/histomorphometric (1 section) and immunohistochemical (3 sections) analyses. The analyses were performed by calibrated examiners (EE, NC) who were blinded regarding the treatment performed.

https://doi.org/10.5051/jpis.2100700035

The histological analysis was performed by a certified histologist (EE) considering the following parameters: 1) the degree and extent of the local inflammatory response; 2) the pattern of cellularity and CT extracellular matrix structure; and 3) the extracellular matrix structure and cellularity pattern of alveolar bone tissue.

The histomorphometric analysis was performed by another examiner (NC), as described by Garcia et al. [21]. Briefly, the total FA and the bone area (BA) were measured in square millimeters using an image analysis system (AxioVision 4.8.2, Carl Zeiss MicroImaging GmbH, Jena, Germany). To establish the boundary of the FA, a straight line was drawn from the apex of the mesial root until the apex of the distal root. This line then followed the whole root cementum outer surface in the furcation region. The BA was demarcated inside the FA. The same FA apical limit was drawn, and the entire outer surface of the alveolar bone inside the FA was delimitated. PBF was calculated using the following formula: PBF=(BA×100)/FA.

A semiquantitative analysis of the immunolabeling of RANKL and OPG in the FA was performed at ×400 magnification (performed by NC) [22]. The immunolabeling was scored as:

- 0: no immunolabeling (0% of cells per area)
- 1: low immunolabeling (<25% of cells per area)
- 2: moderate immunolabeling (<50% of cells per area)
- 3: high immunolabeling (<75% of cells per area)

A quantitative analysis was performed for TRAP, which was enumerated in an area of 2,048 × 1,536 pixels at 0.254 μm/pixel in the center of the interradicular septum using a Leica DM 5000B microscope and Leica HC PL FLUOTAR 20×/0.30 lens. The coronal limit of the area was the alveolar bone crest in the FA, which extended apically for a distance of 1,000 μm.

Statistical analyses

Data were shown as mean and SD (continuous variables) or median, interquartile range, and maximum and minimum values (ordinal variables). Data normality and the homogeneity of variance were analyzed. Each parameter (PBF, TRAP-positive cell number, RANKL, and OPG scores) was evaluated individually. The significance level was considered to be 5% in all analyses. For PBF, the significance of differences among groups was determined by 2-way analysis of variance (ANOVA), followed by the *post hoc* Tukey test when ANOVA suggested a significant difference between groups ($P \leq 0.05$). The significance of differences among groups in relation to immunolabeling pattern of RANKL, OPG, and TRAP was determined by the Kruskal-Wallis test.

RESULTS

Qualitative histological analysis

At 7 days, the NT group presented CT with an intense inflammatory infiltrate in the furcation region and around the margins of the interradicular septum. Most specimens showed extensive extracellular matrix destruction. A few specimens showed necrotic bone spicules surrounded by a large number of inflammatory cells. The interradicular septum, made of very thin bone trabeculae, had a very irregular contour (Figure 1A) and was surrounded by many active osteoclasts. At 15 and 30 days, the histological patterns were similar to 7 days specimens, except for the magnitude of the inflammatory infiltrate, which gradually decreased over time. The CT around the interradicular septum was still very inflamed and not

https://doi.org/10.5051/jpis.2100700035

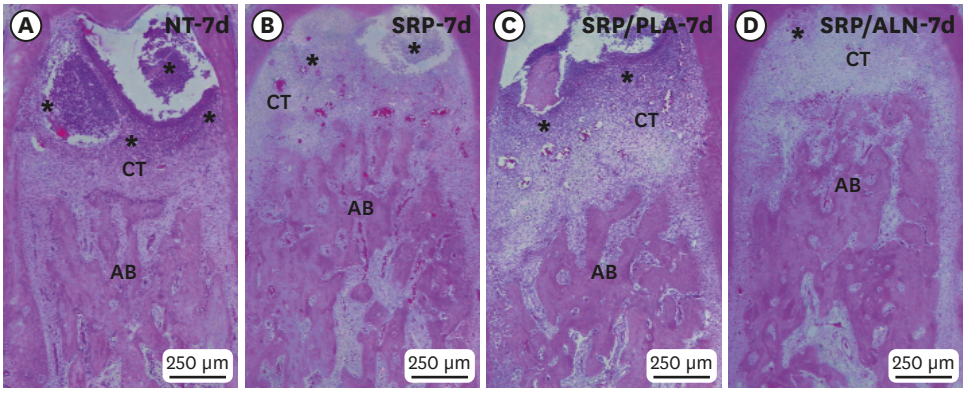

Figure 1. Periodontal tissues in the furcation area of the mandibular left first molar in all groups at 7 days, in the NT (A), SRP (B), SRP/PLA (C), and SRP/ALN (D) groups (hematoxylin, eosin, and phloxine staining). Black asterisks indicate inflammatory infiltrate.
NT: no treatment, SRP: scaling and root planning, SRP/PLA: SRP followed by filling the periodontal pocket with placebo gel, SRP/ALN: SRP followed by filling the periodontal pockets with 1% alendronate gel, AB: alveolar bone, CT: connective tissue.

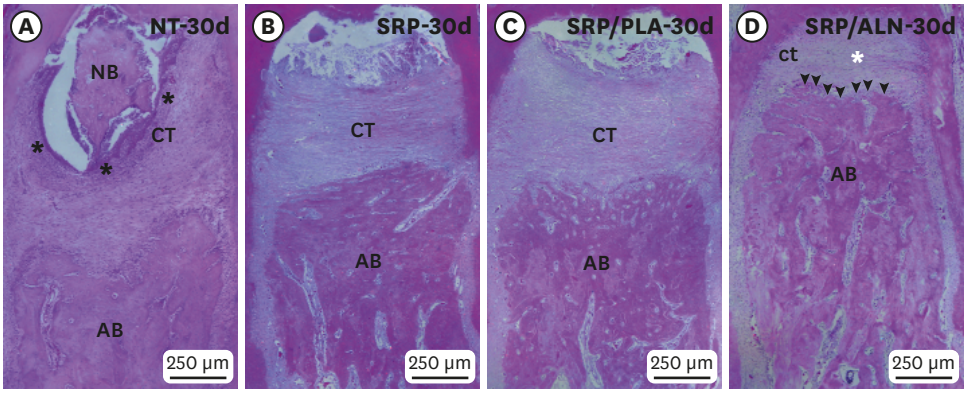

Figure 2. Periodontal tissues in the furcation area of mandibular left first molar of all groups at 30 days, in the NT (A), SRP (B), SRP/PLA (C), and SRP/ALN (D) groups (hematoxylin, eosin, and phloxine staining). Data are presented as below: black asterisks, inflammatory infiltrate; white asterisk, region with a large amount of collagen fibers and fibroblasts; black arrowheads, region with many active osteoblasts.
NT: no treatment, SRP: scaling and root planning, SRP/PLA: SRP followed by filling the periodontal pocket with placebo gel, SRP/ALN: SRP followed by filling the periodontal pockets with 1% alendronate gel, AB: alveolar bone, CT: connective tissue, NB: necrotic bone.

structured. Very thin and irregular bone trabeculae were observed (Figure 2A), surrounded by many active osteoclasts.

At 7 days, the SRP and SRP/PLA groups had a moderate amount of inflammatory cells extending throughout the entire CT of the furcation region. Inflammatory cells surrounding necrotic bone spicules were observed in a few specimens of these groups. The CT near the interradicular septum constituted a small amount of collagen fibers and fibroblasts. The interradicular septum showed irregular bone trabeculae (Figure 1B and C) and was surrounded by active osteoclasts. At 15 and 30 days, the histological findings were similar to those described for specimens with 7 days, except for the magnitude of the inflammatory infiltrate, which significantly and gradually decreased over time (Figure 2B and C).

At 7 days, the SRP/ALN group presented a moderate number of inflammatory cells, collagen fibers, and fibroblasts in the CT. The inflammatory infiltrate was restricted to the CT. The

https://doi.org/10.5051/jpis.2100700035

JPIS Journal of Periodontal & Implant Science

Table 1. Means and standard deviations of percentage of bone in the furcation according to group and time point (intergroup comparisons)

Group	7 days	15 days	30 days
NT	21.07±6.02	35.88±6.88	28.7±10.39
SRP	52.55±11.27[a)]	49.44±7.7[b,c)]	48.17±5.38[d)]
SRP/PLA	50.12±10.79[a,e)]	46.03±10.72[c)]	50.89±7.82[d)]
SRP/ALN	60.43±6.15[a)]	59.2±8.65[b)]	65.01±15.47[d,f)]

NT: no treatment, SRP: scaling and root planning, SRP/PLA: SRP followed by filling the periodontal pocket with placebo gel, SRP/ALN: SRP followed by filling the periodontal pockets with 1% alendronate gel.
[a)]Significant difference when compared to the NT group at 7 days ($P \leq 0.01$); [b)]Significant difference when compared to the NT group at 15 days ($P \leq 0.01$); [c)]Significant difference when compared to the SRP/ALN group at 15 days ($P \leq 0.05$); [d)]Significant difference when compared to the NT group at 30 days ($P \leq 0.01$); [e)]Significant difference when compared to the SRP/ALN group at 7 days ($P \leq 0.05$); [f)]Significant difference when compared to the SRP and SRP/PLA groups at 30 days ($P \leq 0.05$).

bone margins had few inflammatory cells and a similar quantity of collagen fibers and fibroblasts. The interradicular septum was made of thin bone trabeculae (Figure 1D), with a few active osteoclasts. At 15 and 30 days, a moderate amount of fibroblasts, a large quantity of collagen fibers, and small isolated foci of inflammatory cells were observed in the CT of the furcation region. The bone trabeculae were thicker, with a slightly irregular contour (Figure 2D), with few clastic cells and many active osteoblasts. At all evaluation time points, some round osteoclasts were observed away from the bone margins. This event was rarely seen in the other groups.

Histomorphometric and statistical analyses

Statistically significant differences were not detected in the FA among the NT, SRP, SRP/PLA, and SRP/ALN groups. The mean values and SDs of PBF (in %) for each group at 7, 15, and 30 days after removing the ligatures, along with intergroup comparisons, are presented in Table 1.

PBF at 15 days was significantly higher than its values at 7 days in the NT group. PBF at 30 days was higher than at 7 days in the SRP/ALN group (in an intragroup comparison).

Immunohistochemical analyses

A highly specific pattern was observed for detecting RANKL, OPG, and TRAP, as evidenced by the absence of immunolabeling in negative controls for the immunohistochemical reactions. A brownish color was observed in the immunolabeled cells, confined to the cytoplasm and, at a small scale, to the extracellular matrix (RANKL and OPG) or exclusively the cytoplasm (TRAP).

All groups showed positive immunolabeling for RANKL, OPG, and TRAP at 7, 15, and 30 days. RANKL (Figure 3A-D) and OPG (Figure 4A-D) immunolabeling was predominantly detected in fibroblasts and osteoblasts and, at a smaller scale, in the extracellular matrix. TRAP (Figure 5A-D) immunolabeling was observed predominantly in multinucleated osteoclasts.

No differences were found in the pattern of immunolabeling for RANKL ($P > 0.05$; Figure 3E) and OPG ($P > 0.05$; Figure 4E) or in the number of TRAP-positive cells ($P > 0.05$; Figure 5E) among groups. Although a similar quantity of TRAP-positive cells was observed in all groups, a larger number of osteoclasts were rounded and non-attached to the bone surface in the SRP/ALN group than in the NT, SRP, and SRP/PLA groups.

https://doi.org/10.5051/jpis.2100700035

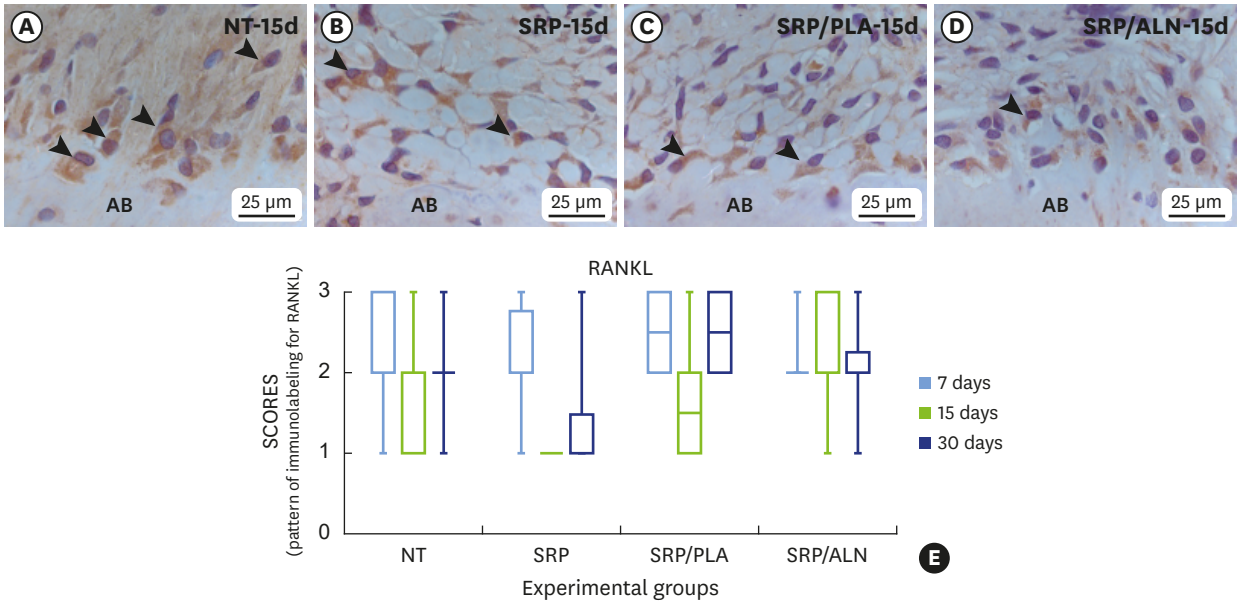

Figure 3. RANKL immunolabeling in the furcation region of the mandibular left first molar. RANKL immunolabeling patterns in the NT (A), SRP (B), SRP/PLA (C), and SRP/ALN (D) groups at 15 days. Median and interquartile range of the scores for RANKL for each group at 7, 15, and 30 days (P>0.05) (E). Black arrowheads indicate immunolabeled cells. Counterstaining: Harry's hematoxylin.
NT: no treatment, SRP: scaling and root planning, SRP/PLA: SRP followed by filling the periodontal pocket with placebo gel, SRP/ALN: SRP followed by filling the periodontal pockets with 1% alendronate gel, AB: alveolar bone, RANKL: receptor activator of nuclear factor-κB ligand.

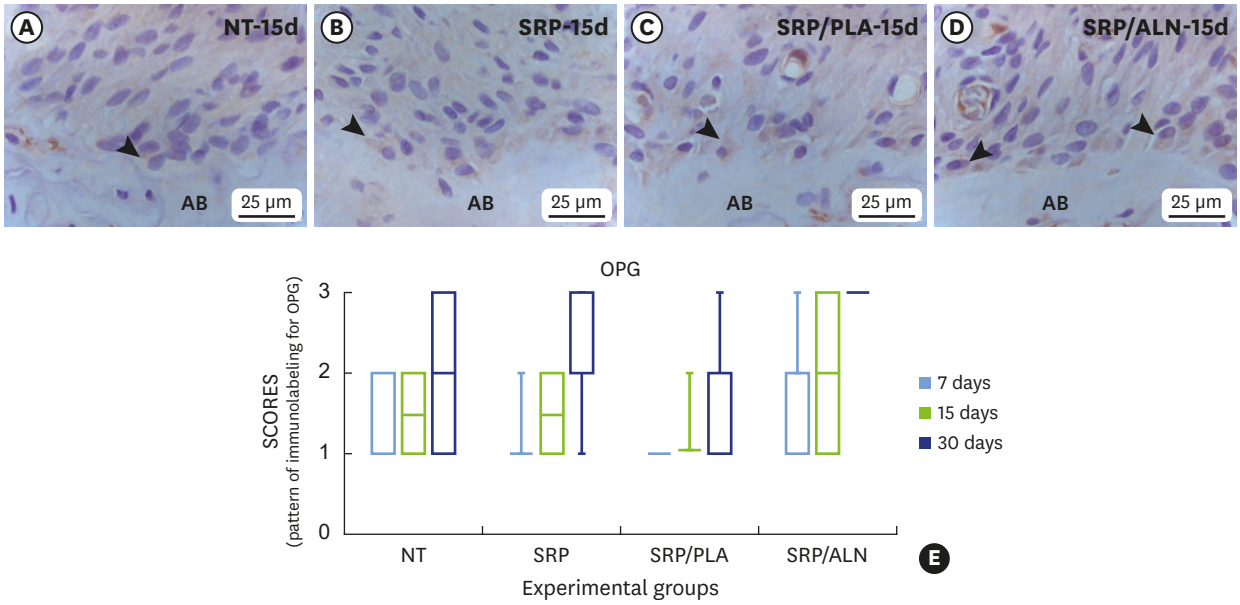

Figure 4. OPG immunolabeling in the furcation area of the mandibular left first molar. OPG immunolabeling patterns in the NT (A), SRP (B), SRP/PLA (C), and SRP/ALN (D) at 15 days. Median and interquartile range of the scores for OPG for each group at 7, 15, and 30 days (P>0.05) (E). Black arrowheads indicate immunolabeled cells. Counterstaining: Harry's hematoxylin.
NT: no treatment, SRP: scaling and root planning, SRP/PLA: SRP followed by filling the periodontal pocket with placebo gel, SRP/ALN: SRP followed by filling the periodontal pockets with 1% alendronate gel, AB: alveolar bone, OPG: osteoprotegerin.

DISCUSSION

Even though human cell cultures have been thought to be excellent models for replicating some features of periodontitis activity at a cellular level, knowledge regarding the complex

https://doi.org/10.5051/jpis.2100700035

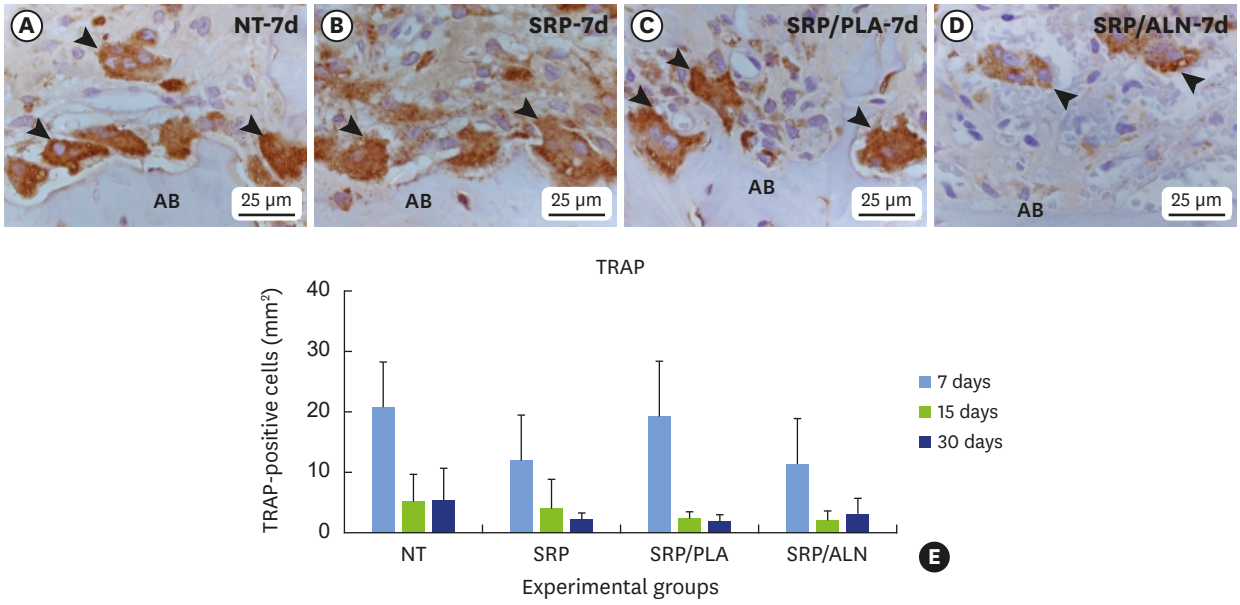

Figure 5. TRAP-positive cells in the NT (A), SRP (B), SRP/PLA (C), and SRP/ALN (D) groups at 7 days. Means and standard deviations of TRAP-positive cells in the alveolar bone of the furcation area of mandibular left first molar for each group at 7, 15, and 30 days, along with intergroup comparisons (P>0.05) (E). Black arrowheads indicate immunolabeled cells. Counterstaining: Harry's hematoxylin.
NT: no treatment, SRP: scaling and root planning, SRP/PLA: SRP followed by filling the periodontal pocket with placebo gel, SRP/ALN: SRP followed by filling the periodontal pockets with 1% alendronate gel, TRAP: tartrate-resistant acid phosphatase, AB: alveolar bone.

defense mechanisms of the host is still lacking. Therefore, further investigation of the host defense mechanism using animals is of the utmost importance for the establishment of improved therapies to treat periodontitis [23].

To our knowledge, this is the first study to histologically evaluate the local administration of 1% ALN gel as an adjunct to SRP for treating EP in rats. In this study, all groups that received SRP treatment presented greater PBF than the control group (NT), demonstrating the effectiveness of this approach to treat periodontitis [24]. Previous clinical studies have demonstrated that filling periodontal pockets with 1% ALN gel as an adjunct therapy to SRP led to increased PD reduction, CAL gain, and bone fill [5,10,13-18,25]. Our results support these findings. The histomorphometric analysis showed a significantly higher PBF, as well as more collagen fibers and fibroblasts in the furcation region in the SRP/ALN group than in the other groups at all experimental time points.

The capacity to modulate alveolar bone metabolism may explain the efficacy of the local use of 1% ALN gel in adjunct to SRP to treat periodontitis [26]. ALN is a powerful inhibitor of bone resorption, acting by binding to the bone mineral component and interfering with the action of clast cells [27]. According to Nakagawa et al. [6], nitrogen-containing BPs (N-BPs), including ALN, affect osteoclast performance and are resilient against inhibition by mevalonate metabolism. They interfere with several cellular functions that are necessary for bone resorption and osteoclast survival that lead to apoptosis, thereby preventing abnormal bone resorption. However, Halasy-Nagy et al. [28] demonstrated in an *in vitro* study that the mechanism of ALN and risedronate for inhibiting bone resorption does not involve apoptosis of clast cells. In fact, animal studies have demonstrated that N-BPs can interrupt bone resorption without reducing the amount of clast cells and might even increase their quantity [29,30].
In addition to the number of osteoclasts, their shape and proximity to the bone should be

https://doi.org/10.5051/jpis.2100700035

considered when evaluating the bone resorption process. In the present study, even though a similar quantity of TRAP-positive cells was noticed in all groups, the histological analysis showed a larger amount of osteoclasts that were rounded and non-attached to the bone surface in the SRP/ALN group than in the NT, SRP, and SRP/PLA groups. Osteoclasts need to adhere effectively to the bone surface to be able to perform bone resorption, meaning that it is possible that the non-attached osteoclasts in the present study were not useful as "bone resorbers." Despite methodological differences, this hypothesis is partially supported by the findings of a previous animal study of our group [30], which evaluated the effect of long-term therapy with another N-BP (zoledronate) on the healing of extraction sockets. It was concluded that long-term therapy with zoledronate stimulated non-attached osteoclast development in extraction sockets, thereby decreasing local bone resorption. Kaynak et al. [31], applied ALN on the bone surface of the mandibular molars in rats and also observed a higher number of rounded osteoclasts when ALN was used in comparison to the control group.

During normal bone remodeling, the RANKL/OPG ratio may be a key factor associated with bone mass [32]. In the present study, even though the SRP/ALN group showed a significantly higher PBF than the other groups at all experimental time points, similar patterns of RANKL and OPG immunolabeling were observed in all groups. These results are contradictory to the findings of previous studies [33,34]. However, they corroborate the findings of an *in vitro* study performed by Kim et al. [35]. These authors investigated the expression level of both RANKL and OPG in mouse osteoblastic cells that received ALN or pamidronate alone or combined with 1,25-$(OH)_2$ vitamin D3. Diverse concentrations of ALN and pamidronate did not consistently alter RANKL and OPG messenger RNA (mRNA) expression, irrespective of the presence of 1,25-$(OH)_2$ vitamin D3. However, when inserted into cocultures of mouse bone marrow and osteoblastic cells, both ALN and pamidronate interfered with osteoclast formation and bone resorption, but did not modify RANKL and OPG mRNA expression. The authors reasoned that regulation of RANKL and OPG expression does not mediate bone resorption by BPs [35]. Therefore, it could be hypothesized that the bone resorption inhibition promoted by the use of locally delivered 1% ALN gel in this study was not essentially mediated by the regulation of RANKL/OPG expression. The precise mechanism of the inhibitory action of 1% ALN gel on bone resorption has yet to be completely understood.

It has been previously observed that BPs not only induced the secretion of inhibitors of osteoclast-mediated resorption by osteoclasts, but also stimulated the establishment of osteoblast precursor cells and mineralized nodules, thus leading to the early production of osteoblasts [36]. These results are supported by the histological findings of the present study, since many active osteoblasts were noticed in the SRP/ALN group at 15 and 30 days.

In this study, 1% ALN gel was subgingivally delivered into the periodontal pockets. The advantages of using this technique include the delivery of a substantial concentration at the target site with a diminished dose, fewer applications, and excellent patient compliance [37]. Local delivery may present major advantages regarding adverse effects and patient acceptability when compared to systemic regimens [38]. Gels are easy to prepare and administer, and also present higher biocompatibility, reduced allergic host reactions, and rapid elimination through the regular catabolic pathway and at the delivered site [25]. In the present study, we found ALN gel preparation to be reliable and effortless in terms of clinical manipulation. During the experiment, no animals presented any adverse effects regarding its use.

https://doi.org/10.5051/jpis.2100700035

In contrast to the findings of the present study, others found that ALN had no inhibiting effect on bone resorption [39,40]. These contradictory findings could be attributed to many factors such as dosage of the medicament, number of applications, administration route, and type of formulation (gel or liquid). It remains unclear which medication dose and frequency will lead to the best clinical outcomes [26].

Although caution should be exercised when extrapolating results from animal studies to a clinical setting, the rat EP model used in the present study provides important data on the biological basis of periodontal healing [21,22,30,34].

To summarize, it can be concluded that 1% ALN gel used as an adjunct to SRP enhanced bone regeneration in the furcation region in EP in rats.

REFERENCES

1. Bartold PM, Van Dyke TE. Host modulation: controlling the inflammation to control the infection. Periodontol 2000 2017;75:317-29.
PUBMED | CROSSREF
2. Jepsen K, Jepsen S. Antibiotics/antimicrobials: systemic and local administration in the therapy of mild to moderately advanced periodontitis. Periodontol 2000 2016;71:82-112.
PUBMED | CROSSREF
3. Akram Z, Abduljabbar T, Sauro S, Daood U. Effect of photodynamic therapy and laser alone as adjunct to scaling and root planing on gingival crevicular fluid inflammatory proteins in periodontal disease: A systematic review. Photodiagnosis Photodyn Ther 2016;16:142-53.
PUBMED | CROSSREF
4. Lane N, Armitage GC, Loomer P, Hsieh S, Majumdar S, Wang HY, et al. Bisphosphonate therapy improves the outcome of conventional periodontal treatment: results of a 12-month, randomized, placebo-controlled study. J Periodontol 2005;76:1113-22.
PUBMED | CROSSREF
5. Pradeep AR, Kumari M, Rao NS, Naik SB. 1% alendronate gel as local drug delivery in the treatment of Class II furcation defects: a randomized controlled clinical trial. J Periodontol 2013;84:307-15.
PUBMED | CROSSREF
6. Nakagawa T, Ohta K, Kubozono K, Ishida Y, Naruse T, Takechi M, et al. Zoledronate inhibits receptor activator of nuclear factor kappa-B ligand-induced osteoclast differentiation via suppression of expression of nuclear factor of activated T-cell c1 and carbonic anhydrase 2. Arch Oral Biol 2015;60:557-65.
PUBMED | CROSSREF
7. Reszka AA, Rodan GA. Mechanism of action of bisphosphonates. Curr Osteoporos Rep 2003;1:45-52.
PUBMED | CROSSREF
8. Teronen O, Konttinen YT, Lindqvist C, Salo T, Ingman T, Lauhio A, et al. Human neutrophil collagenase MMP-8 in peri-implant sulcus fluid and its inhibition by clodronate. J Dent Res 1997;76:1529-37.
PUBMED | CROSSREF
9. Giannobile WV. Host-response therapeutics for periodontal diseases. J Periodontol 2008;79:1592-600.
PUBMED | CROSSREF
10. Sharma A, Pradeep AR. Clinical efficacy of 1% alendronate gel as a local drug delivery system in the treatment of chronic periodontitis: a randomized, controlled clinical trial. J Periodontol 2012;83:11-8.
PUBMED | CROSSREF
11. Weinreb M, Quartuccio H, Seedor JG, Aufdemorte TB, Brunsvold M, Chaves E, et al. Histomorphometrical analysis of the effects of the bisphosphonate alendronate on bone loss caused by experimental periodontitis in monkeys. J Periodontal Res 1994;29:35-40.
PUBMED | CROSSREF
12. Malden N, Lopes V. An epidemiological study of alendronate-related osteonecrosis of the jaws. A case series from the south-east of Scotland with attention given to case definition and prevalence. J Bone Miner Metab 2012;30:171-82.
PUBMED | CROSSREF

13. Pradeep AR, Sharma A, Rao NS, Bajaj P, Naik SB, Kumari M. Local drug delivery of alendronate gel for the treatment of patients with chronic periodontitis with diabetes mellitus: a double-masked controlled clinical trial. J Periodontol 2012a;83:1322-8.
PUBMED | CROSSREF

14. Sharma A, Raman A, Pradeep AR. Role of 1% alendronate gel as adjunct to mechanical therapy in the treatment of chronic periodontitis among smokers. J Appl Oral Sci 2017;25:243-9.
PUBMED | CROSSREF

15. Dutra BC, Oliveira AM, Oliveira PA, Manzi FR, Cortelli SC, Cota LO, et al. Effect of 1% sodium alendronate in the non-surgical treatment of periodontal intraosseous defects: a 6-month clinical trial. J Appl Oral Sci 2017;25:310-7.
PUBMED | CROSSREF

16. Sharma A, Pradeep AR. Clinical efficacy of 1% alendronate gel in adjunct to mechanotherapy in the treatment of aggressive periodontitis: a randomized controlled clinical trial. J Periodontol 2012b;83:19-26.
PUBMED | CROSSREF

17. Wanikar I, Rathod S, Kolte AP. Clinico-radiographic evaluation of 1% alendronate gel as an adjunct and smart blood derivative platelet rich fibrin in grade II furcation defects. J Periodontol 2019;90:52-60.
PUBMED | CROSSREF

18. Kanoriya D, Pradeep AR, Garg V, Singhal S. Mandibular degree II furcation defects treatment with platelet-rich fibrin and 1% alendronate gel combination: a randomized controlled clinical trial. J Periodontol 2017;88:250-8.
PUBMED | CROSSREF

19. Fernandes LA, Martins TM, Almeida JM, Nagata MJ, Theodoro LH, Garcia VG, et al. Experimental periodontal disease treatment by subgingival irrigation with tetracycline hydrochloride in rats. J Appl Oral Sci 2010;18:635-40.
PUBMED | CROSSREF

20. Nunes NL, Messora MR, Oliveira LF, Lisboa M, Garcia MC, Rêgo RO, et al. Effects of local administration of tiludronic acid on experimental periodontitis in diabetic rats. J Periodontol 2018;89:105-16.
PUBMED

21. Garcia VG, Novaes VC, de Almeida JM, Longo M, Ervolino E, Bomfim SR, et al. Evaluation of the progression and treatment of experimental periodontitis in rats subjected to chemotherapy with 5-fluorouracil. Support Care Cancer 2015;23:2007-17.
PUBMED | CROSSREF

22. Garcia VG, Longo M, Gualberto Júnior EC, Bosco AF, Nagata MJ, Ervolino E, et al. Effect of the concentration of phenothiazine photosensitizers in antimicrobial photodynamic therapy on bone loss and the immune inflammatory response of induced periodontitis in rats. J Periodontal Res 2014;49:584-94.
PUBMED | CROSSREF

23. Oz HS, Puleo DA. Animal models for periodontal disease. J Biomed Biotechnol 2011;2011:754857.
PUBMED | CROSSREF

24. Sanz M, Teughels WGroup A of European Workshop on Periodontology. Innovations in non-surgical periodontal therapy: consensus report of the Sixth European Workshop on Periodontology. J Clin Periodontol 2008;35:3-7.
PUBMED | CROSSREF

25. Reddy GT, Kumar TM, Veena KM. Formulation and evaluation of alendronate sodium gel for the treatment of bone resorptive lesions in periodontitis. Drug Deliv 2005;12:217-22.
PUBMED | CROSSREF

26. Akram Z, Abduljabbar T, Kellesarian SV, Abu Hassan MI, Javed F, Vohra F. Efficacy of bisphosphonate as an adjunct to nonsurgical periodontal therapy in the management of periodontal disease: a systematic review. Br J Clin Pharmacol 2017;83:444-54.
PUBMED | CROSSREF

27. Baron R, Ferrari S, Russell RG. Denosumab and bisphosphonates: different mechanisms of action and effects. Bone 2011;48:677-92.
PUBMED | CROSSREF

28. Halasy-Nagy JM, Rodan GA, Reszka AA. Inhibition of bone resorption by alendronate and risedronate does not require osteoclast apoptosis. Bone 2001;29:553-9.
PUBMED | CROSSREF

29. Kuroshima S, Go VA, Yamashita J. Increased numbers of nonattached osteoclasts after long-term zoledronic acid therapy in mice. Endocrinology 2012;153:17-28.
PUBMED | CROSSREF

30. Nagata MJ, Messora MR, Antoniali C, Fucini SE, de Campos N, Pola NM, et al. Long-term therapy with intravenous zoledronate increases the number of nonattached osteoclasts. J Craniomaxillofac Surg 2017;45:1860-7.
PUBMED | CROSSREF

31. Kaynak D, Meffert R, Günhan M, Günhan O, Ozkaya O. A histopathological investigation on the effects of the bisphosphonate alendronate on resorptive phase following mucoperiosteal flap surgery in the mandible of rats. J Periodontol 2000;71:790-6.
PUBMED | CROSSREF

32. Gong X, Yu W, Zhao H, Su J, Sheng Q. Skeletal site-specific effects of zoledronate on *in vivo* bone remodeling and *in vitro* BMSCs osteogenic activity. Sci Rep 2017;7:36129.
PUBMED | CROSSREF

33. Dobnig H, Hofbauer LC, Viereck V, Obermayer-Pietsch B, Fahrleitner-Pammer A. Changes in the RANK ligand/osteoprotegerin system are correlated to changes in bone mineral density in bisphosphonate-treated osteoporotic patients. Osteoporos Int 2006;17:693-703.
PUBMED | CROSSREF

34. De Almeida J, Ervolino E, Bonfietti LH, Novaes VC, Theodoro LH, Fernandes LA, et al. Adjuvant therapy with sodium alendronate for the treatment of experimental periodontitis in rats. J Periodontol 2015;86:1166-75.
PUBMED | CROSSREF

35. Kim YH, Kim GS, Baek JH. Inhibitory action of bisphosphonates on bone resorption does not involve the regulation of RANKL and OPG expression. Exp Mol Med 2002;34:145-51.
PUBMED | CROSSREF

36. Brunsvold MA, Chaves ES, Kornman KS, Aufdemorte TB, Wood R. Effects of a bisphosphonate on experimental periodontitis in monkeys. J Periodontol 1992;63:825-30.
PUBMED | CROSSREF

37. Kinane DF. Local antimicrobial therapies in periodontal disease. Ann R Australas Coll Dent Surg 2000;15:57-60.
PUBMED

38. Needleman IG, Pandya NV, Smith SR, Foyle DM. The role of antibiotics in the treatment of periodontitis (Part 2--Controlled drug delivery). Eur J Prosthodont Restor Dent 1995;3:111-7.
PUBMED

39. Yaffe A, Iztkovich M, Earon Y, Alt I, Lilov R, Binderman I. Local delivery of an amino bisphosphonate prevents the resorptive phase of alveolar bone following mucoperiosteal flap surgery in rats. J Periodontol 1997;68:884-9.
PUBMED | CROSSREF

40. Jeffcoat MK, Cizza G, Shih WJ, Genco R, Lombardi A. Efficacy of bisphosphonates for the control of alveolar bone loss in periodontitis. J Int Acad Periodontol 2007;9:70-6.
PUBMED

https://doi.org/10.5051/jpis.2100700035