Experimental osteonecrosis: development of a model in rodents administered alendronate

Abstract: The main objective of this study was to cause bisphosphonate-related osteonecrosis of the jaws to develop in a rodent model. Adult male Holtzman rats were assigned to one of two experimental groups to receive alendronate (AL; 1 mg/kg/week; n = 6) or saline solution (CTL; n = 6). After 60 days of drug therapy, all animals were subjected to first lower molar extraction, and 28 days later, animals were euthanized. All rats treated with alendronate developed osteonecrosis, presenting as ulcers and necrotic bone, associated with a significant infection process, especially at the inter-alveolar septum area and crestal regions. The degree of vascularization, the levels of C-telopeptide cross-linked collagen type I and bone-specific alkaline phosphatase, as well as the bone volume were significantly reduced in these animals. Furthermore, on radiographic analysis, animals treated with alendronate presented evident sclerosis of the lamina dura of the lower first molar alveolar socket associated with decreased radiographic density in this area. These findings indicate that the protocol developed in the present study opens new perspectives and could be a good starting model for future property design.

Keywords: Osteonecrosis; Alendronate; Infection.

Introduction
In recent years, public health statistics have included the bisphosphonates (Bps) among the most common drugs prescribed in the world, since they are highly effective in the treatment of several bone diseases. However, since 2003, concerns have been raised regarding the increasing side-effects of Bps. In this context, Bps-related osteonecrosis of the jaws (BRONJ) represents a challenge in science. Even with the numerous efforts to develop experimental models of this disease, none of the BRONJ pathogenesis hypotheses has yet been completely accepted. Conversely, strong correlations have been made between this bone necrosis and possible co-factors, including Bps type and treatment length, as well as trigger agents, highlighting surgical procedures with bone manipulation, such as dental implants and tooth extractions.

Analysis of data retrieved from clinical studies indicates a strong association between tooth extractions and BRONJ. Thus, the effects of Bps therapy on the healing of alveolar sockets have been the focus of several in vivo studies. Recently, we demonstrated the development of BRONJ-like lesions in rodents by associating tooth extractions with daily high dosages of alendronate (ALN) in a long-term study. However,
studies using lower dosages of Bps showed only a transient impairment of alveolar socket healing after tooth extractions.\textsuperscript{10,11}

Within this context, the aim of this study was to develop BRONJ lesions in rodents by means of lower doses of ALN, which, in rats, corresponded with the cumulative dosages for the management of rheumatic diseases.\textsuperscript{12} Furthermore, we undertook a critical discussion of the reasons behind the strong association between tooth extractions and this disease.

**Methodology**

**Animals and reagents**

Twelve male adult Holtzman rats (\textit{Rattus norvegicus albinus}), weighing approximately 200 g each, were housed under similar conditions in cages with access to food and water \textit{ad libitum}. All experimental protocols were approved by the institution's Ethics Committee for the Use of Experimental Animals (no. 18/2009) and performed in accordance with the guidelines of the Brazilian National Council for the Control of Animal Experimentation.

Alendronate (ALN) was purchased from ALCON Laboratory (São Paulo, SP, Brazil). The drug was dissolved in sterile physiological saline (0.9\% NaCl) and diluted to the specified concentration.

**Experimental design**

After a 3-day acclimatization period, 6 rats were treated with weekly doses of alendronate (1 mg/kg body weight),\textsuperscript{12} and 6 rats were treated with weekly doses of vehicle alone (0.9\% saline), both subcutaneously.

After 60 days of the pharmacologic therapy, all animals were subjected to left lower first molar (M1) extraction under general anesthesia by a combination of ketamine chlorhydrate (Ketamina Agener, Agener União Ltda, São Paulo, SP, Brazil; 0.08 mL/100 g body weight) and 2\% xylazine (Rompum, Bayer S.A., São Paulo, SP, Brazil; 0.04 mL/100 g body weight).

The same professional performed the tooth extractions using the same technique in all animals. Initially, the rats were placed in a dorsal position and fixed in a special device. The surrounding gingivae were carefully detached from the lower first molars with a dental explorer. Then, with a Hollenback carver, the tooth was luxated and separated into 2 segments that were removed with a forceps adapted around the cervical lines of the segments.

After the surgical procedure, all animals received an intramuscular dose of antibiotic (Pentabiótico\textsuperscript{®}, Wyeth-Whitehall Ltda, São Paulo, Brazil, 0.1 mg/Kg) and anti-inflammatory Ketoflex (Ketoprofen 1.0\%, MERAL, São Paulo, Brazil, 0.03 mL/rat). Animals were euthanized by anesthesia overdose at 28 days after tooth extractions, and the ALN or sterile physiological saline administration protocol was maintained until the animals’ death.

**Specimen processing**

All tissue blocks were immersed directly in 10\% buffered formalin fixative solution for 48 h. After that, 6 specimens of each group/period were subjected to routine histological processing for descriptive and stereometric evaluation. All specimens were decalcified in tetrasodium-EDTA aqueous solution (0.5 M, pH 7.4) for 2–3 months, under agitation at room temperature. All specimens were then processed and included in paraffin blocks. Serial 4-µm sections were obtained in the bucco-lingual direction, stained with hematoxylin and eosin, and referred for evaluation by light microscopy (Leica DMI200M; Leica Microsystems, Wetzlar Hesse, Germany).

**Histological and stereometric analyses**

One Board-certified oral pathologist blinded to the group assignments performed these analyses at three distinct times to minimize discrepancy in the scores (kappa index = 0.76). The histological endpoints were evaluated at 4 fields in each section (cervical and apical thirds of the mesial and distal sockets) at magnifications of 100x, 200x, 400x, and 1000x. Evaluation included the degrees of bone necrosis (areas of empty osteocytic lacunae and heavily eroded surfaces) and infection (bacteria proportion and extension of biofilm organization). The inflammation was evaluated according to quantity and quality based on the density, type, and distribution of inflammatory cell infiltrate.\textsuperscript{4} Qualitatively, the inflammation was classified as acute (predominance of neutrophil infiltration), chronic (predominance of mononuclear
cell infiltration, such as macrophages and lymphocytes), and mixed (presence of cells of both acute and chronic processes in similar proportions). Furthermore, the degree of vascularization (number of vessels) was also evaluated. These parameters were scored on a four-point scale: 0 (absent; 0%), 1 (mild; ≤ 10%), 2 (moderate; > 10 and ≤ 50%), and 3 (increased; > 50%).

Stereometric analysis was performed with Leica Application software Suite 3.8.0 (Leica Microsystems LTD, Heerbrugg, Switzerland). The measurements were performed at the distal root of the left M1 at the regions of interest (ROIs) that included 3 different areas inside the socket. Initially, a standard ROI was determined by identification of a quadrangular area extending from the level of the cemento-enamel junction (CEJ) of the left second mandible (M2) to the apical end of the M2 and between the mesial and distal alveolar bone surfaces. This quadrangular area was divided into 3 equal ROIs (1,2,3).9

The variables analyzed included the percentage of the root socket filled with bone tissue (BV) in each ROI, following the nomenclature and abbreviations recommended by the American Society for Bone and Mineral Research.13 Bone volume (%) represented bone volume (mm$^3$) per total tissue volume (mm$^3$). All analyses were performed at a magnification of 100x, and 3 measurements were taken for each specimen, to complete the stereometric analysis. The distance between the selected sections was 50 μm.

**Image acquisition**

For radiographic evaluation, a digital radiograph of the left alveolar socket was taken after euthanasia. The left mandible was fixed in a holding device with the vertical long axis of alveolar socket perpendicular to the central x-ray beam and parallel to the sensor at a 40-cm focus-object distance. The x-ray unit was operated at 70 KVp, 10 mA, and 0.2 s (Exspectro 70x, Dabi Atlante, Ribeirão Preto, SP, Brazil).

**Radiographic bone density**

A single blinded calibrated examiner evaluated mandibular radiographs. The radiographic bone density in the mandible was determined by analysis of the gray scale in an area of 15 x 15 pixels at the ROIs, including 3 different regions within the distal root of the left M1, as described previously. Furthermore, we also evaluated one region immediately after the mesial socket of left M1, which corresponded to the body’s mandibular bone density (ROI 4).

This analysis was done with the image-analysis software Image Tool 2.03 (UTHSCA, San Antonio, Texas, USA), which provided the gray scale average and standard deviation in these pre-determined regions by means of a histogram. The bone density calculations were performed based on the average gray levels of the evaluated ROIs, which were divided by the gray level of the implant, to compensate for minimal differences among radiographs, since the density of the metallic standard was the same in all specimens.14

**Assessment of bone turnover biochemical markers**

Blood samples were collected on the day of euthanasia by cardiac puncture and centrifuged for plasma separation. The levels of serum collagen type 1 cross-linked C-telopeptide (CTX) and bone-specific alkaline phosphatase (BALP) were determined by enzyme-linked immunosorbent assay kits (CUSABIO BIOTECH CO., Ltd, Wuhan, P.R. China).9

**Statistical analysis**

Data were initially subjected to the Kolmogorov-Smirnov normality test. We then performed comparisons among groups and periods for non-parametric data, using the Kruskal-Wallis test, followed by Dunn’s multiple-comparison test or analysis of variance (ANOVA), then Tukey tests for parametric data. The data were evaluated by means of GraphPad Prism 5.0 (San Diego, CA, USA), and statistical significance was set at p < 0.05 with 95% confidence intervals.

**Results**

**Clinical and histological features**

All animals of the AL group exhibited different degrees of bone exposure (Figure 1a), while control animals presented with complete epithelial healing without signs of inflammation (Figure 1b).

A marked bone necrosis associated with an infection process was observed in animals treated
Experimental osteonecrosis: development of a model in rodents administered alendronate

with ALN, especially at the inter-alveolar septum area (Figure 2b), while control animals displayed none of these features \((p < 0.0001)\) (Figures 2a, 3a, 3b). Furthermore, animals in the AL group presented a lower degree of vascularization \((p < 0.01)\) when compared with those in the control group (Figure 4a), while no statistically significant differences were found concerning the inflammation quality and degree between these groups of animals (Figures 3c, 3d).

Regarding BV count, we observed that, at ROI 1 and ROI 2, animals of the AL group presented rates significantly less than those of CTL animals \((p < 0.01)\), while no statistically significant differences were found in ROI 3 (Figure 4b).

Figure 1. Clinical aspects of a lower first molar (M1) alveolar socket 28 days after tooth extraction. Animals treated with ALN (A) exhibited areas of exposed bone with a marked impairment of alveolar socket re-epithelialization. In contrast, animals treated with sterile physiological saline (B) presented a full epithelial lining on the alveolar socket, with no signs of inflammation or infection (asterisk).

Figure 2. Histologic sections of tooth sockets 28 days after left lower first molar extraction and stained with hematoxylin and eosin. The animals in CTL group (A) demonstrated alveolar socket area completely remodeled, with no signs of bone necrosis (asterisk), while in the ALN group (B) it was observed the inter-radicular septum retention, which was associated with bone necrosis (asterisk) and infection (arrow; original magnification x100).
Radiographic aspects

In the radiographic analysis, it could be observed that animals treated with ALN showed a degree of bone radiographic density (BRD) significantly less at ROI 1 (p < 0.01) and ROI 2 (p < 0.001) when compared with that of control animals (Figure 5). Furthermore, in the AL group, the linings of the alveolar socket were more evident when compared with those of control animals and also presented sclerosis on the M1 alveolar lamina dura (Figures 6a, 6b), as well as the highest values of BRD at the ROI 4 area (p < 0.001) (Figure 5).

Bone metabolism markers

Animals treated with ALN presented the lowest values of BALP (p < 0.01) and CTX (p < 0.001) when compared with control animals (Figures 7a, 7b).
Discussion

Given the large number of patients developing BRONJ worldwide, several animal models of BRONJ-like lesions have been recently published in an effort to further explore the disease’s pathophysiology. However, there is no standard BRONJ experimental model, since most models fail to reproduce clinical reality and do not associate several co-factors that undertake a reliable cause-effect relationship between Bps and disease pathogenesis.\textsuperscript{5,7,8,9,15,16,17,18,19,20,21,22,23,24} To the best of our knowledge, this is the first study to demonstrate 100% of BRONJ-like lesions by associating tooth extractions with alendronate in cumulative dosages for the management of rheumatic diseases.\textsuperscript{12}

The first advantage of our experimental model is the high percentage of bone necrosis achieved. The rate of bone lesions in previously published experimental models varies from 0 to 100%,\textsuperscript{6,7,8,9,15,16,17,18,19,20,21,22,23,24} and only four papers have reported 100% of BRONJ-like lesions.\textsuperscript{8,9,17,18} However, models that reported a bone lesion rate of 100% were developed by exposing animals to supra-therapeutic dose regimens\textsuperscript{9,17} or to a high-intensity trauma,\textsuperscript{8,18} which represents an artificial scenario. In contrast, we proposed an experimental model that not only achieved 100% of BRONJ-like lesions in rodents, but that also resembled human disease from clinical, radiological, and histological aspects. Additionally, our model simulated a more clinical reality of benign diseases than previous models, since we associated a less aggressive bisphosphonate schedule (type and dose) with minimal surgical trauma.

In our experimental model, several aspects had to be considered, specifically regarding the Bps regimen, which seems to be a key point in the disease’s development. Our study is the first to reproduce BRONJ-like lesions using ALN alone without the association of other

![Figure 5](image1.png)

**Figure 5.** Percentage of radiographic bone density (%) in experimental groups 28 days after tooth extractions. ROIs 1 and 2 of CTL animals exhibited a statistically significant increase in radiographic bone density compared with ROIs 1 and 2 of animals treated with ALN (**p < 0.01 in relation to ROI 1 of AL; ***p < 0.001 in relation to ROI 2 of AL). Furthermore, ROI 4 of ALN-treated animals exhibited a statistically significant increase in radiographic bone density compared with ROI 4 of matched controls (# ##p < 0.01 in relation to ROI 4 of AL).

![Figure 6](image2.png)

**Figure 6.** Radiographic features of the alveolar socket healing after 28 days of tooth extraction in animals treated with ALN (A) and sterile physiological saline (B). In the AL group (a), the linings of the alveolar socket (arrow) are more evident compared with those of the control animals (asterisk) and also presented sclerosis on the M1 alveolar lamina dura.

While some studies that used alendronate did not demonstrate BRONJ-like lesions, others did not adopt supra-therapeutic dose regimens, as aforementioned, or steroids. Furthermore, most experimental models reproduced oncologic schedules with high dosages of zolendronate. However, although zolendronate is undeniably important, it is essential to note that ALN is among the most common Bps prescribed worldwide and is specifically indicated for the treatment of benign diseases, such as osteoporosis, osteopenia, and rheumatic diseases.

In this context, to compensate for the differences in potency between alendronate and zolendronate, as well as for the lower plasma concentrations when the subcutaneous route rather than the intravenous route is used, we conclude that the total Bps dose administered over a long period of time is important for the magnitude of the reduction in bone turnover. Indeed, animals in the AL group presented the lowest levels of CTX and BALP, which are markers of bone metabolism, thereby supporting the effectiveness of our Bps treatment schedule in suppressing bone turnover at a key point in disease development.

In the scenario of clinical reality, some studies selected experimental animals with systemic or metabolic diseases to simulate the physiologic disparity between these animals and healthy animals. We do not rule out the relevance of these studies, but rather believe that healthy animals provide some relevant advantages. First, the more variables included in the experimental model, the more difficult it is to determine the true role of Bps in disease pathogenesis. Second, Bps have several applications even in healthy patients, including therapeutic options for improving the bone formation process and reducing resorption in different bone-grafting procedures, implant surface treatments, and as antibiofilm agents.

Although some authors have demonstrated spontaneous BRONJ lesions, a local trigger factor seems highly relevant to the induction of this disease in animal models. The main local agents include periodontal disease, bacterial injections in addition to bone defects, and tooth extractions. The last is undoubtedly the most relevant, since most patients develop BRONJ lesions after tooth extractions, as described in clinical studies. This finding could be related to an urgent requirement for wound healing after this surgical procedure, especially during initial phases, and, in the field of Bps, this is worsened since these drugs impair the angiogenesis and resorption process after tooth extractions. Indeed, these features are in agreement with our findings of lower CTX levels and degrees of vascularization in animals treated with ALN. Therefore, the association of a high demand requirement in an already compromised bone-remodeling environment could result in the collapse of the repaired wound.

In contrast, some authors believe that BRONJ may be associated with changes in oral bacterial behaviors due to increased bacterial activity. Indeed, as in this study, other studies have demonstrated areas of active infection and necrotic bone at the inter-alveolar bone region that have been attributed to the prolonged retention of this area.

Figure 7. Levels of BALP (A) and CTX (B) in experimental groups at 28 days after tooth extractions. In the CTX analysis, animals in the CTL group presented levels statistically significantly higher than those of animals in the AL group (**p < 0.001). In the BALP analysis, CTL animals exhibited increased levels of BALP compared with those in the AL group (**p < 0.01).
In this discussion, it is important to state that even in the presence of a relevant infection process in animals treated with ALN, no differences in the inflammatory responses were found between the groups. This is particularly interesting since a microbial infection typically triggers host immune responses and therefore enhances the degree of inflammation. A possible explanation for this finding is the dose-dependent anti-inflammatory effect of Bps that could, over the long term, lead to immunosuppressive conditions favoring the development of infections.

Our research into infection issues also demonstrated various degrees of impairment in soft tissue coverage in animals treated with ALN, which is relevant due to the essential role of epithelial coverage protection from oral bacterial infection. Indeed, it has been demonstrated that experimental BRONJ lesions in rodents can be prevented by immediate mucoperiosteal coverage. This could be of concern, since Bps inhibits the migration of oral epithelial cells and, after tooth extraction, there is a high demand for such migration, considering that the degree of reepithelialization depends on the depth and width of the wound.

One of the BRONJ diagnostic criteria is the persistence of bone exposure in the oral cavity for at least 8 weeks. In our opinion, this concept cannot be strictly extended to animal experimental models, due to inherent metabolic differences between these species. Although both rodents and humans share similar sequences of alveolar healing, it occurs much more rapidly in rats than in humans. Furthermore, when transient, the negative effects of Bps on socket healing have not been extended beyond 14 days.

In this study, we found lower BV values at the medial and apical regions in the AL group, compatible with the lowest levels of BALP in this group. These features are in agreement with those reported by authors who found inhibitory effects of ALN on osteogenesis. In contrast, we also observed no statistically significant differences in bone volume at the apical socket area between the groups, which reinforces, at least in part, the theories of other authors who found no direct effects of Bps on the ability of osteoblasts to produce bone matrix in vivo.

**Conclusion**

Within the limitations of this study, our results clearly demonstrated that the association of ALN therapy and tooth extraction was able to induce BRONJ-like lesions in rodents. This study provides an experimental model that achieved 100% of jaw lesions resembling human disease in clinical, radiological, and histological aspects, which could be useful in the development of preventive and therapeutic approaches to the treatment of this disease, as well as to further studies addressing other issues related to BRONJ pathways, including other trigger agents.

**Acknowledgments**

This study was supported by the State of São Paulo Research Foundation and the National Council for Scientific and Technological Development.

**References**


