Dental extraction following zoledronate, induces osteonecrosis in rat’s jaw

Ximena Vidal-Gutiérrez 1, José-Francisco Gómez-Clavel 2, Luis-Alberto Gaitán-Cepeda 1

1 Laboratory of Clinical and Experimental Pathology, Graduate and Research Division, Dental School, National Autonomous University of Mexico. Circuito Institutos s/n, Ciudad Universitaria, 04510 Coyoacán, D. F. Mexico city, México
2 Laboratory of Dental and Education Research, Faculty of Superior Studies-Iztacala, National Autonomous University of Mexico. Av. De Los Barrios No. 1, Col. Los Reyes Iztacala, C.P. 54090, Tlalnepantla, Estado de México

Correspondence:
Laboratorio de Patología Clínica y Experimental
Universidad Nacional Autónoma de México Circuito Institutos s/n
Ciudad Universitaria
04510 Coyoacán, D. F. México
ximena_vidal_g@comunidad.unam.mx

Received: 23/08/2016
Accepted: 08/01/2017

Abstract
Background: Bisphosphonate-Related Osteonecrosis of the Jaw (BRONJ) is clinically characterized by the presence of exposed bone in the oral cavity that persists for more than eight weeks. Previous attempts to establish an animal model have not sufficiently considered disease features. Our aim was to establish an inexpensive and replicable animal model that develops BRONJ in a short time.

Material and Methods: Thirty-two male Wistar rats were randomly divided into two groups: control and experimental. In the experimental group, we administered 0.06mg/kg intraperitoneal dose of zoledronic acid (ZA) 7 and 14 days prior to maxillary second molar extraction. At two, four and six weeks after tooth extraction, the animals were euthanized, and we dissected the maxilla following histological procedures. We stained serial slides with hematoxylin and eosin and Masson’s trichrome. The samples were harvested for macroscopic, radiologic and histological evaluation of bone changes.

Results: At two weeks postextraction, we observed exposed necrotic bone in dental socket areas in experimental groups. Radiological analysis revealed osteolytic lesions accompanied by extensive destruction and sequestrum formation in the same group. Histological examination confirmed the absence of necrotic bone in control groups in contrast with the experimental groups. The percentage of empty lacunae and the number of osteoclasts and the necrotic bone area were significantly increased \((p<0.05)\) in the experimental groups.

Conclusions: The animal model using ZA administration to prior dental extraction successfully mimicked human BRONJ lesions. Also, the model was easily replicated, inexpensive and showed different features than other previous BRONJ models.

Key words: Bisphosphonates, osteonecrosis, dental extractions, animal model, BRONJ.
Introduction
Bisphosphonates (BPs) represent a major class of antiresorptive drugs used in the prevention and treatment of osteoporosis, hypercalcemia of malignancy, multiple myeloma, Paget’s disease and bone metastases associated with breast, prostate, and lung, as well as osteogenesis imperfecta, fibrous dysplasia, and Gaucher’s disease. In afflicted patients, it effectively returns bone mineral density, reduces the incidence of bone fracture, and improves their quality of life. Despite these beneficial effects, a growing number of patients on long-term and high-dose BP therapy develop a potentially serious complication: osteonecrosis of the jaw. Our goal was to develop a clinically relevant, inexpensive, and easily replicable animal model of Wistar rats treated with zoledronic acid and subjected to oral surgery in the form of dental extraction.

Material and Methods
- Animals
We used thirty-two male Wistar rats (Rattus norvegicus, albinus) weighing from 200-250g which were obtained from the bioterium of the National Autonomous University’s Faculty of Higher Studies in Iztacala, Mexico. The rats were housed in cages at an ambient temperature (21°C-27°C) with a 12h light/dark cycle. Animals were fed a standard diet of rat chow and drink tap water ad libitum. The research protocol was conducted following the strict federal guidelines for the use and care of laboratory animals (NOM-062-ZOO-1999). The research protocol was submitted and approved by the Ethical Committee from Faculty of Superior Studies-Iztacala, National Autonomous University of Mexico, Mexico (PE209312).

- Study Design
We randomly divided the rats using a random number table into two groups (n=16 per group): the control and the experimental group. We divided the both study groups in three subgroups regarding the sacrificed moment: two weeks (n=6), four weeks (n=6) and six weeks (n=4) after dental extraction. An allocation concealment was performed.

- Study Procedures
We established the dose of zoledronic acid (Zometa®, Novartis Pharma©, Basel, Switzerland) at 0.06mg/kg, according to previous studies (18) and the experimental group was administered intramuscularly to the seventh and fourteenth day before dental extraction. At the same time, the control rats received the same volume of saline solution.

- Dental extractions and post-surgical care
We extracted the second maxillary molar, as follows: We administrated intramuscularly animals previously anesthetized with ketamine (60mg/kg) and xylazine (7mg/kg), once verified sedation, we performed the oral disinfection and then we detached the gingiva around the second maxillary molar using a sharpened dental instrument. Later the luxation and extraction of the second maxillary tooth were done using a modified veterinary dental instrument for rodent animals (iM3® Cat. No D2001). We continuously monitored the cardiac frequency and body temperature during all surgical procedures, following the ethical standards of care for experimental animals. Surgical events as surgery’s length, dental fracture, residual roots and alveolar lamina fracture, were recorded. All of pre-surgical, surgical and post-surgical cares were done according to NOM-062-ZOO-1999 federal guidelines for the use and care of laboratory animals. Animals from each study group were euthanized by over exposure to CO₂ at two, four and six weeks posterior to dental extraction (Fig. 1).
Immediately after their sacrifice, samples of all animals was dissected, photographed, and radiographed using a standard dental X-ray. All samples were fixed in 10% buffer formaldehyde by 72 h minimum. Later all samples to posteriorly decalcify in 2% HNO$_3$ for 48h. After decalcification period, all samples were embedded in paraffin to be cut at 5μm thick. Samples were sagittally sectioned. Three histological slides per sample were obtained and stained with hematoxylin and eosin technique to be used for histological analysis. In three random samples per group, additionally, three central sections were stained with Masson’s trichrome for cross-reference. We used a light microscopy (Leica DM750) for histological examination, and photomicrographs were taken using the Leica LAS-EZ software.

- Macorposcopic Analysis
We obtained the follow data from the macroscopic evaluation of dissected maxilla: open socket, exposed bone, loss of adjacent teeth, and bone fracture. We used these data to determine the clinical presence of BRONJ as long as present at least three of the four conditions.

- Radiological Analysis
The X-ray images were obtained from the maxilla dissected immediately sacrifice to all animals. The standardized radiological parameters for all study groups were 60-70kV with an exposure time of 0.24 seconds. X-ray images were analyzed according to Pacheco using the IMAGE J software® (9). All images were converted into an 8-bit data (256 grey levels) generating a scale (0-255) for each image. The value zero represented the lowest and 255, the highest attenuation of X-ray beams. Image density analyses were conducted using grey levels of the animals alveolar bone tissue.

- Histological Analysis
We considered that the distance between distal first molar root to mesial third molar root was the region of interest (ROI), and then we assessed on the hematoxylin-eosin & Masson’s trichrome stained sections. For each animal/socket, histological slide sections from the lingual, medial and vestibular area were evaluated; we considered four zones of ROI by histological features, and they were photographed at 40x using a binocular light microscope Leica DM3000 (Fig. 2). We superposed a grid image with 36 points on histological fields per histological section. The grid image comprised of all tooth sockets from the coronal limits adjacent to the

![Image](image-url)
gingival epithelium until the lower apical limit. Histological changes were evaluated using the following qualitative criteria: the presence of ulcerative lesion with exposed and necrotic bone, osteolysis, presence of hyperplasia of the epithelium accompanied by inflammatory cell infiltration, and the presence of sequestrum and bacterial colonies. (6,7,12,13).

Also, we obtained quantitative data, included gingival tissue repair area (Tissue, mm²), empty lacunae (#/mm²), presence and quantity of osteoclasts (#/mm²). We defined osteonecrosis as a loss of more than five contiguous osteocytes with confluent areas of empty lacunae. The analysis was performed in 4 zones of ROI, stained with Masson’s trichrome to visualize fibrous network in bone marrow areas and evaluate the organization of bone matrix.

All measurements were performed by a blinded observer previously calibrated.

- Statistical Evaluation
Data were expressed as mean ± standard derivation (SD). We determinate the normal distribution and homogeneity for data before statistical analyses. Data were then analyzed using ANOVA and Student’s t-test. For data that did not fit in the distribution of normality, we used the Mann-Whitney and Kruskal-Wallis tests. Values of \( p < 0.05 \) were considered statistically significant. To establish possible associations between each group we used bivariate \( \chi^2 \) tests with a 95% significance level (\( p < 0.05 \)). We used STATA 12 for Windows (StataCorp LP., Lakeway, Texas, USA) to analyze the data.

**Results**

In general, study animals tolerated the procedure well. Furthermore, animals showed suitable hemostasis and rapid recovery and no associated lesions were observed post anesthesia. (Fig 3a,b)

Surgical procedure in the experimental group was longer than in the control group. Furthermore, dental fractures occurred during the extractions, resulting in the presence of residual root tips and fracture of the alveolar lamina or teeth extracted along with interradicular bone (Table 1).

- Macroscopic Analysis
In the experimental groups, rats showed ONJ, while in the control groups, the dental socket, was epithelialized although some areas exhibited accumulation of detritus. In the experimental groups, we observed dental opened socket; large areas of exposed bone as well as fractures. Furthermore, at the sixth week, we observed open sockets with large areas of exposed bone in the three experimental animals, while the control groups showed a healing dental socket cover it by epithelium. (Fig 3c,d).

Analysis of the alveolar characteristics was performed using \( \chi^2 \) tests and showed that the differences between the experimental and control groups were statistically significant (\( p < 0.05 \)).

- Radiographic Analysis
This analysis revealed the presence of osteolytic lesions accompanied by extensive destruction and formation of sequestrum in experimental groups. In the control group, the sockets were nearly filled with mineralized connective tissue exhibiting normal trabecular patterns. In contrast, radiographs from animals treated with ZA displayed a lack of bone formation in alveoli (Fig 3f-g).

Statistically, significant differences were found in bone density between groups (\( p < 0.05 \)) (Table 2).

- Histological Analysis
Histologically, the control groups showed the normal course of healing in extraction sockets with increase mineralized connective tissue. Numerous differentiated osteoblasts and developing a bone matrix with a high percentage of osteocytes appeared on the lining of the socket walls and expanding to the central region of the socket with a centripetal pattern, and complete mucosal coverage. Experimental groups showed extensive ulcerative lesion accompanied by exposed and necrotic bone with sequestrum and bacterial colonies.
Fig. 3. Observation of specimens at different times. Immediately after dental extraction, the control group shows a complete extracted molar (a); in contrast, the experimental group shows a fractured root (b). The rats sacrificed at six weeks after extraction in control group shows complete healing of oral mucosa (c) the experimental group shows incomplete healing of oral mucosa, showing exposed alveolar bone (d). Radiographically the control group (f) shows a linear opaque radio-density around extraction socket in contrast with the experimental group (g) which shows mottled trabecular pattern around extraction socket. Histological examination of ROI at 6 weeks after tooth extraction. Black arrows point to areas of inflammation (i), yellow arrows to osteonecrosis area (k). The experimental group exhibit areas with fewer bone lacunae and areas of dead bone, the control group shows bone deposition and presence of osteocytes in an organized bone matrix (h,j).

Table 1. Surgery features * time, minutes.

| Surgery Study Groups | features observed | Surgery Length * Mean (SD) | Tooth Fractures (%) | Residual Root (%) | Alveolar lamina Fracture (%) |
|----------------------|-------------------|-----------------------------|---------------------|------------------|-----------------------------|
| Control Groups       |                   |                             |                     |                  |                             |
| Group 1 (n=6)        |                   | 7.83 (±0.75)                | ---                 | ---              | ---                         |
| Group 2 (n=6)        |                   | 7.83 (±0.75)                | ---                 | 16%              | 16%                         |
| Group 3 (n=4)        |                   | 7.75 (±0.5)                 | ---                 | ---              | ---                         |
| Experimental Groups  |                   |                             |                     |                  |                             |
| Group 4 (n=6)        |                   | 17.16 (±1.94)               | 16%                 | 33%              | 33%                         |
| Group 5 (n=6)        |                   | 18 (±1.89)                  | 16%                 | 33%              | 33%                         |
| Group 6 (n=4)        |                   | 18 (±0.81)                  | ---                 | 25%              | 25%                         |

SD: Standar Deviation-zero.

Table 2. Gray level (GL) values in ROI analyzed with Student’s t-test between groups with 5% significance level (P<0.05).

| Groups | Mean (±standard deviation) | P value |
|--------|-----------------------------|---------|
| Second week (n=6) | | |
| Experimental | 155.83 (±2.31) | 0.000* |
| Control | 160.83 (±5.03) |
| Fourth week (n=6) | | |
| Experimental | 144.16 (±6.73) | 0.000* |
| Control | 171.5 (±7.12) |
| Sixth week (n=4) | | |
| Experimental | 138 (±2.94) | 0.000* |
| Control | 194.5 (±6.35) |

* significant 5%
Inflammatory cell infiltration in the connective tissue was equally observed at first weeks. The morphometric analysis showed that the experimental groups had lower osteoclast number than each control group ($p<0.05$), also showed higher necrotic bone fractions and demonstrated significantly higher empty lacunae ($p<0.05$). The control groups showed bone matrix with highly eosinophilic and organized (Fig. 3). In contrast, the experimental group exhibited scant, poorly organized bone matrix (Table 3).

The control groups showed bone matrix with highly organized architecture. In contrast, the experimental groups showed bone matrix with highly organized architecture. The rats receiving bisphosphonates showed more surface osteoclasts. By extracting only the second maxillary molar we were able to produce BRONJ in the experimental group (See Fig. 3) (17,15,20-22), while previous studies had extracted all hemimandible molars to obtain the same result (20,22).

The rats receiving bisphosphonates showed more surgical complications than the control group (Table 1). Our results demonstrated that the usage of zoledronic acid increases the alveolar bone density. This condition makes the surgical procedures difficult and they require a longer time than usual. We also observed that the experimental group exhibit evident delayed reepithelization at the fourth week, in contrast with other BRONJ models which showed the same condition but during a longer period of time. (2,9,22,23) The X-Ray results showed no overlapping structures, radiolucent areas with a lack of bone formation (EG). These data were analyzed considering the grey-level density in the alveolar area. Several other studies have used X-Ray and micro CT, but this work did quantitative analyses and not only descriptive ones (11,14,17).

To assess the specific effects of bisphosphonate on the alveolar bone tissue, we treated our animals in the ab -

Discussion
Currently, it is necessary to obtain an animal model for a better understanding of pathophysiology and to develop standards for the prevention and treatment of BRONJ. The aim of our study was to develop a clinically relevant, inexpensive, and easily replicable animal model affected by BRONJ.

We found that a short lapse dose of BP before the surgical procedure develops BRONJ while other studies employed high doses and over longer periods of time (14,18,19). To assess the specific effects of bisphosphonate on the alveolar bone tissue, we treated our animals in the absence of other diseases or medications. In contrast, other studies concerning BRONJ have changed the metabolic conditions of rats by using additional bisphosphonate’s therapies with immunosuppressant drugs (Table 4).

The dental extraction is the strongest risk factor for the development of BRONJ in patients receiving bisphosphonates. By extracting only the second maxillary molar we were able to produce BRONJ in the experimental group (See Fig. 3) (17,15,20-22), while previous studies had extracted all hemimandible molars to obtain the same result (20,22).

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Table 3. Histological Examination.

| Study Groups | Histological features observed | Osteocyte Lacunae (%) | Bone Matrix (%) | Inflammatory Infiltrate (%) | Contaminated Tissue (%) | Osteonecrosis (%) |
|--------------|--------------------------------|-----------------------|-----------------|----------------------------|------------------------|------------------|
| Control Groups | Group 1 (n=6) | 100% | --- | 67% | --- | 50% | 50% | 17% | 83% | --- | 100% |
|               | Group 2 (n=6) | 100% | --- | 100% | --- | 17% | 83% | 17% | 83% | --- | 100% |
|               | Group 3 (n=4) | 100% | --- | 100% | --- | --- | 100% | 17% | 83% | --- | 100% |
| Experimental Groups | Group 5 (n=6) | --- | 100% | --- | 100% | 100% | --- | 100% | --- | 100% |
|               | Group 6 (n=6) | --- | 100% | --- | 100% | 100% | --- | 100% | --- | 100% |
|               | Group 7 (n=4) | 25% | 75% | --- | 100% | 100% | --- | 100% | --- | 100% |

- zero.
factors, etiology, prevention and treatment of BRONJ. Additionally, this animal model provides an additional useful, practical, and cost-effective model to develop new therapeutic strategies to cure this particular side effects in human.

Recently, a new side effect has been described, Medication-Related Osteonecrosis in Jaws (MROJ). We appreciate that our study is limited to one type of antiresorptive medication, acid zoledronic. However, it is enough to develop BRONJ. Thus, our research group considers that is necessary to develop animal models using other antiangiogenic and antiresorptive agents such as denosumab and bevacizumab.

Finally, in the model involving the extraction of the second maxillary molar of rats treated with two zoledronic acid doses of 0.06mg/kg per two weeks, osteonecrosis lesions were found in all cases. The model has to be used to analyze the changes occurring in the tissues involved in the extraction at around six weeks; it is no necessary the extension of the study beyond this time. BRONJ studies have been based on case reviews and animal studies.

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Conflict of Interest
The authors have declared that no conflict of interest exist.