Development of a Rat Model of Bisphosphonate-Related Osteonecrosis of the Jaw (BRONJ)

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The purpose of this study was to develop a rat model predictive of bisphosphonate-related osteonecrosis of the jaw (BRONJ) after exodontias. Thirty female rats were randomized into 2 groups, control and experimental. The experimental group received 2 intravenous injections of zoledronate (20 μg/kg). The mesial root of the right mandibular first molar was extracted. Rats were euthanized at 0, 4, and 8 weeks. Bone mineral density (BMD), collagen breakdown (pyridinium [PYD]), vascular regeneration (VEGF), and histology were examined. A trend toward higher PYD values was suggested in control vs experimental groups after wounding. Serum VEGF increased significantly after wounding for both control and experimental groups. After 8 weeks, VEGF continued to rise for the experimental group only. In the extraction socket area, BMD was significantly lower after wounding in control vs. zoledronate-treated rats. Histology sections from experimental groups showed bacteria and bone necrosis. Consistent findings of BRONJ features similar to those in humans were observed after zoledronate treatment.

Key Words: bisphosphonate, osteonecrosis of the jaw, rat model, BRONJ

INTRODUCTION

Bisphosphonates are powerful compounds that have been successfully used for the treatment of complications of bone metastasis, including pathologic fractures, malignant hypercalcemia, osteoporosis, osteopenia, and Paget’s disease. Since 2003, case series reports began to emerge of osteonecrosis of the jaw associated with the use of bisphosphonates, primarily in patients with cancer.1,2

The most important predisposing factors for the development of bisphosphonate-related osteonecrosis of the jaw (BRONJ) appear to be the type and total dose of bisphosphonate, history of trauma, dental surgery, and dental infection.3 Time of exposure to bisphosphonates also appears to be
associated with the development of BRONJ. The risk for osteonecrosis of the jaws is substantially higher for patients taking zoledronate and increases over time.\textsuperscript{2} Although oral lesions may develop after as few as 4 months of bisphosphonate therapy, the median duration of drug use ranges from 22 to 39 months. Trauma to oral tori is also associated with osteonecrosis. The impact of local factors, such as infections and smoking, and underlying medical conditions, such as diabetes or peripheral vascular diseases, remains to be determined. The antiangiogenic property of bisphosphonates and other medications and the presence of other comorbid factors may promote the risk for, or persistence and progression of, this condition.\textsuperscript{3} Patients with BRONJ may present with various complaints. In 2005, Marx and colleagues\textsuperscript{4} reviewed 119 cases of BRONJ and reported that 69\% of patients presented with an area of exposed bone and pain, 31\% presented with asymptomatic exposed bone, 24\% with one or more mobile teeth, and 18\% with a cutaneous fistula or exposed bone through the skin. Patients may have more subtle complaints, such as a heavy feeling or numbness in the jaw. In the 119 patients, 68\% of bone exposures occurred in the mandible alone, 28\% in the maxilla and 4\% in both jaws.\textsuperscript{4}

It has been suggested that BRONJ may result from a marked suppression of bone metabolism that results in accumulation of physiologic microdamage in the jawbones, compromising biomechanical properties.\textsuperscript{5} Trauma and infection increase demand for osseous repair that exceeds the capacity of the hypodynamic bone, resulting in localized bone necrosis.

There are more questions than answers regarding BRONJ; the literature does not provide definitive information about the cause, progression, treatment, or prevention of this condition. Developing a reliable and reproducible animal model is imperative to understand the pathogenic mechanisms and management of BRONJ. So far, there is no established animal model that may be used to test markers and possible treatments for BRONJ. By reproducing the clinical and histologic features of BRONJ in an animal model, a fundamental research tool will be provided. Succeeding studies of the disease would then be able to concentrate on understanding the pathogenic mechanisms of this debilitating clinical condition.

\textbf{MATERIALS AND METHODS}

\textbf{Experimental Model}

We used 34 Sprague-Dawley retired breeder rats (Harlan, Indianapolis, Ind) weighing approximately 300 g. The rats were provided with food and water ad libitum, but no special diet was provided, except for the first week after surgery when the rats were fed a soft diet (Nutra-Gel, BioServ, Frenchtown, NJ). The experimental protocol for this study was reviewed and approved by the Institutional Animal Care and Use Committee. The rats were randomized into one control (n = 15) and one experimental (n = 15) group. The rats were labeled from 1 to 30 to facilitate identification. The experimental group received zoledronate (20 \(\mu\)g/kg intravenous [IV]; diluted to 100 \(\mu\)g/mL in phosphate buffered saline pH 7.4 [PBS]) at baseline and was re-dosed at week 3 after surgery.

\textbf{Surgical Procedure}

Three weeks after the first injection, the rats were anesthetized with an intramuscular injection of a cocktail composed of ketamine (100 mg/mL), xylazine (20 mg/mL), and acepromazine (10 mg/mL) at a dosage of 0.5 mL/kg. The extractions were performed based on a modified Gottardello technique.\textsuperscript{6} Two rubber bands were used to keep the mouth open and to immobilize the tongue. The rubber bands were attached to a rubber dam frame, which allowed for better access to the surgical site. Syndesmotomy was performed by using a small (1-mm) excavator (Darby Perry Excavator 210/211, Instru-med, Warsaw, Ind). The crown of the first mandibular right molar was sectioned using a carbide tapered fissure high-speed bur. Constant irrigation was used to keep the site clean and to avoid overheating. The right mandibular first molar was extracted using a modified mosquito forceps. Any remaining root was removed and the defect reshaped using a tapered carbide bur to a standardized depth of 2 mm from the cemento-enamel junction. At this time, the rats in the experimental group received a second dose of zoledronate. The rats were maintained for observation in individual cages until they recuperated from the anesthesia. The rats were fed a soft diet (Nutra-Gel BioServ, Frenchtown, NJ) for 1 week after surgery to reduce trauma from mastication and hard food.
Five experimental and control rats were euthanized at surgery and at postsurgical weeks 4 and 8. The mandibles were stored in formalin until microcomputerized tomography (CT) reconstruction. A 2-sample $t$ test was again used for statistical analysis.

**Serum Analysis**

Blood samples were collected by amputating a small portion of the tail (about 0.5 cm) at baseline, the day when the extractions were performed, after 4 weeks of healing, and after 8 weeks of healing. The serum bone turnover marker pyridinium (PYD) cross-links of collagen was quantified by using the Metra PYD assay kit (Quidel Corporation, San Diego, Calif), 25 µL of filtered serum was used for each determination.

Vascular endothelial growth factor (VEGF) was also quantified in the serum of the rats. A rat VEGF assay kit (Immuno-Biological Laboratories Company Ltd, Gunma, Japan) was used to quantify the levels of VEGF in the rats’ serum.

**Bone Mineral Density: Micro-CT Analysis**

To evaluate bone mineral density (BMD), the hemimandibles of each group were dissected and then scanned in a micro-CT system (Skyscan 1172, Skyscan, Aartesaaar, Belgium) at an image pixel size of 33 µm. Reconstruction was done using the Skyscan Nrecon program (Figure 1). The data sets obtained after reconstruction were loaded into the Skyscan CT-analyzer software for the measurement of mineral density of these samples. The BMD measurements was obtained from 4 anatomical sites of interest: the socket area (apical to the extraction site), the anterior area (mesial to the first molar), the second molar area (apical to the roots of the second molar), and the ramus (Figure 2). An average of 5 measurements was performed for each of these sites. Each region of interest had the same dimensions and was applied to the same number of cuts in each mandible.

**Observational Histology**

Serial 5- to 10-µm thick sections of decalcified and paraffin-embedded mandibles were cut parallel to the occlusal plane of the molars through the molar roots and the extraction site before mounting on the microscope slides. The sections were then mounted on the microscope slides for hematoxylin and eosin and Brown and Brenn staining (to localize bacteria). A board-certified oral pathologist (R.A.), masked to the treatment protocols, examined the slides at 20×, 40×, 100×, and 200× for changes in bone cellularity, vascularity, inflammatory cell infiltrate, osteoblastic and osteoclastic activity, and necrosis of the bone.

**Results**

**Collagen Breakdown**

The effect of zoledronic acid on collagen breakdown was studied in the control and experimental rats by measuring the PYD cross-links of collagen released into the serum. As shown in Figure 3, there was no measurable effect of zoledronate on...
collagen breakdown in unwounded rats. However, in wounded rats, a trend in the data suggests a greater release of PYD cross-links into the serum of control rats after wounding than in rats treated with zoledronate, though the results were not significant ($P = .2$).

**Angiogenesis**

The VEGF in the serum of zoledronate-treated rats at baseline (123.5 ± 37.6 pg/mL), at wounding (147.8 ± 44.7 pg/mL), at 4 weeks (214.8 ± 47.5 pg/mL), and at 8 weeks (223.4 ± 73.5 pg/mL) after wounding was measured and compared with the same data points for the control group (135.6 ± 51.8 pg/mL, 132.1 ± 44.1 pg/mL, 188.1 ± 47.1 pg/mL, and 152.7 ± 58.8 pg/mL, respectively). The levels of VEGF at 4 weeks after wounding were significantly different than the VEGF levels at wounding for both the control ($P = .03$) and the experimental ($P = .01$) groups. The experimental group showed a trend of increasing values for VEGF at 8 weeks of healing, while the control group, at the same time point, showed a trend of decreasing values for VEGF serum expression (Figure 4). However, the difference between control and experimental values was not significant ($P = .13$).

**Micro-CT Analysis**

The BMD for all areas measured (with the exception of the extraction socket area) showed trends toward higher values in the zoledronic acid-treated rats than in the control rats; however, the differences were not statistically significant. In the extraction socket area, the value for BMD at 4 weeks after wounding was significantly lower ($P = .013$) for the control group compared with the rats treated with bisphosphonates, and it remained different at 8 weeks. The control group also showed a loss in BMD at 8 weeks compared with time 0 (Figure 5).

**Intraoral Examination**

At 8 weeks, 6 of the 8 rats in the bisphosphonate-treated group showed exposed bone in the oral cavity at the site of first molar extraction. In contrast, at 8 weeks all of the rats in the control group showed extraction sites that were healed (Figure 6).

**Histology**

Figure 7 shows one representative rat from the control group and one from the experimental group at 4 weeks after surgery. Figure 8 shows one representative rat from the control group and one from the experimental group at 8 weeks after surgery. The tissue sections obtained from the control groups showed normal healing process with bone trabeculae exhibiting features of remodeling, such as woven bone formation with cellular lacunae and osteoblastic and osteoclastic activity. The tissue sections from the 8-week control groups showed more organized tissue maturation with good evidence of healing. The tissue sections from the experimental groups showed variable degrees of healing, similar to those observed in the control groups. However, all specimens from both the 4-week and the 8-week experimental group showed features of osteonecrosis, such as empty osteocytic lacunae, absence of vascularity in interstices of bone, and irregular resorption bone surfaces. Interestingly, occasional osteoclasts were observed in Howship’s lacunae, which exhibited pyknosis of nuclei and shrinkage of cellular size. These features were interpreted to be consistent with irreversible cellular damage or necrosis. In some specimens, the necrotic bone spicules were surrounded by numer-
FIGURES 6 AND 7. **FIGURE 6.** Intraoral images of the first molar extraction sites in (a) a zoledronic acid–treated rat and (b) a control rat 8 weeks after wounding. Note the appearance of inflammation and exposed bone in the zoledronic acid–treated rat. **FIGURE 7.** Histopathology of mandibular bone from control and zoledronate-treated rats 4 weeks after wounding. (a and b) Hematoxylin and eosin–stained section of a representative control rat showing normal bone remodeling, cellular lacunae, normal osteocytes within lacunae, and vascularity. (c) Representative section from a bisphosphonate-treated rat showing osteonecrosis with sequestration of the buccal cortex (arrow) and mixed inflammatory cellular infiltration associated with bone remodeling (arrow heads) adjacent to osteonecrosis (arrow). (d) Higher-magnification image showing the lack of vascularity and bacteria within interstices (arrow).
uous basophilic bacterial colonies and mixed inflammatory cell infiltrates, including numerous neutrophils.

Figure 9A and B are representative mandibular tissue sections from the 8-week control and bisphosphonate-treated rats, respectively, after staining for bacteria using a modified Brown and Brenn technique. The control section shows characteristic staining of osteocyte nuclei within lacunae characteristic of normal viable bone. The bisphosphonate-treated tissue section shows empty lacunae and filamentous staining characteristic of bacteria by this technique.

**DISCUSSION**

The development of an animal model for BRONJ is important to establish treatment strategies that are evidence-based and associated with valid outcome data. This study attempts to develop a reliable animal model that will reproduce the characteristics of this morbid condition. We replicated clinical conditions that increase the risk of developing BRONJ in adult rats, with high doses of IV zoledronate while undergoing dental extractions of mandibular molars.

In 2008, Hokugo and Nishimura used 35 μg/kg over 2 weeks, and although they observed an increase in 18F-labeled fluorodeoxyglucose, indicating possible chronic inflammation at the healing oral mucosa of experimental animals, their protocol was not successful in altering the bone remodeling metabolic activity. Because BRONJ has been reported as a dose-dependent entity, in our experiment we decided to use a high nonlethal dose of zoledronate (50 μg/kg). To determine the best time for surgery, we executed a pilot study where we observed that the levels of PYD decreased considerably at 3 weeks after the zoledronate injection, whereas at week 4 these levels started to raise to baseline levels. It was decided to perform the surgery 3 weeks after the initial dose of zoledronate. We believed this time point would be the most vulnerable time for healing as the levels of collagen breakdown are diminished. We also estimated that a second dose of zoledronic acid at this time point would increase the possibility of creating healing problems.

Our study found that collagen breakdown is not affected by zoledronate in the unwounded mandible, even after treatment with zoledronic acid. However, after the extractions, the rate of collagen breakdown increased in the control group, as would be expected during healing time, but in the rats treated with zoledronate the rate of collagen breakdown appeared to be slower than for the controls, though the difference was not significant. This may explain the healing delay observed in rats treated with the bisphosphonate.

Because VEGF belongs to a group of proteins involved in angiogenesis, after injury patients may be expected to have elevated levels of these proteins. After 4 weeks of healing, the levels of VEGF in our rat model started to decrease in the control groups, indicating that the tissue has enough blood supply to heal, so the angiogenesis process started to decrease. The levels of VEGF in the experimental group, however, continued to increase, even after 8 weeks of healing. We postulate that the blood supply for the healing zone was insufficient; therefore, the expression of VEGF continued increasing. This prolonged “healing zone” of decreased blood vessel formation and subsequent VEGF elevation may be due to the lack of released cytokines, which would physiologically occur with normal osteoclastic activity.

The values of BMD obtained from the micro-CT analysis confirmed higher levels of BMD for the rats treated with zoledronate in the area of wounding. A trend toward higher BMD was observed in all of the areas of interest measured in our experiment at both 4 and 8 weeks, but these data were not significant.

Rats from our study were also processed for routine histology. Slides from all the rats corresponding to the 4- and 8-week experimental groups showed signs comparable to those reported for cases of BRONJ in humans. Empty lacunae and bacterial infiltration were a common characteristic for the rats in these groups. The slides from the control groups showed signs of normal healing, and osteonecrosis was not observed on these slides.

A few research teams are currently working to develop a BRONJ model. In 2004, Sonis et al in 2008 treated a group of Sprague-Dawley rats with a sequence of zoledronate and dexamethasone weekly for up to 3 weeks, followed by extractions of unilateral maxillary or mandibular molars. They reported the first histologic evidence of necrosis 14 days after extraction in the groups treated with zoledronate. This group found a more robust
Figures 8 and 9. Figure 8. Histopathology of mandibular bone from control and zoledronate-treated rats 8 weeks after wounding. (a) Hematoxylin and eosin–stained section of a representative control rat showing normal bone remodeling, cellular lacunae, normal osteocytes within lacunae, and vascularity. (b and c) Representative sections of bisphosphonate-treated rats after 8 weeks of healing showing osteonecrosis with empty osteocytic lacunae, sequestration of the buccal cortex, and lack of vascularity. Figure 9. Brown and Brenn stain for bacteria in mandibular bone sections from (a) zoledronic acid-treated rats and (b) control rats 8 weeks after wounding.
proliferation of small blood vessels at both time points studied, whereas we did not quantify blood vessels in our specimens. Perhaps the findings of Solis and colleagues may be correlated with our findings of higher expression of serum VEGF at 4 weeks of healing in both experimental and control groups.

Kobayashi et al\textsuperscript{11} used daily subcutaneous injections (250 $\mu$g/kg) of zoledronic acid before and after extracting the first maxillary molar in mice. Although they observed delayed wound healing of the tooth extraction socket, these mice did not manifest representative symptoms of BRONJ, such as necrotic bone exposure and accumulation of oral bacteria colonies, which are typical signs observed in humans developing BRONJ.

In conclusion, the animal model developed in the present study showed several of the most common findings seen in tissues of patients that have been diagnosed with BRONJ. These included bone sequestration and necrosis with empty lacunae, mixed inflammatory cellular infiltration and bacterial colonization. Further development of this model will provide an important tool for combating this devastating disease.

**ABBREVIATIONS**

BMD: bone mineral density  
BRONJ: bisphosphonate-related osteonecrosis of the jaw  
CT: computerized tomography  
PYD: pyridinium  
VEGF: vascular regeneration

**REFERENCES**


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