Original Article
A novel model of bisphosphonate-related osteonecrosis of the jaw in rats

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Abstract: Objective: To establish a rat model of bisphosphonate-related osteonecrosis of the jaw (BRONJ) that realistically mimics major clinical manifestations of the disease. Methods: Female Sprague Dawley rats received intravenous zoledronate 80 μg/kg once a week via the tail vein. Three weeks after intravenous injection, maxillary first molars were extracted under general anesthesia. Then 1, 4 and 12 weeks after tooth extraction, the rats were euthanized, and the intact maxillas were harvested en bloc. Macroscopic analysis, histological analysis and cytokine analysis were performed. Untreated rats with tooth extraction were used as controls. Results: 12 weeks after extraction, rats treated with zoledronate developed BRONJ-like disease, including characteristic features of impaired soft tissue healing, exposed necrotic bone or sequestra, increased inflammatory infiltrates, while the controls showed normal bone healing. 4 weeks after extraction, rats treated with zoledronate exhibited the decreased receptor activator of nuclear factor kappa-B ligand (RANKL) values, the increased osteoprotegerin (OPG) values and the remarkable decreased RANKL/OPG ratio when compared with the controls. Conclusion: The rats treated with zoledronate can be considered a novel, reliable and reproducible animal model to better understand the pathophysiology and pathogenesis of BRONJ and to develop a therapeutic approach.

Keywords: Bisphosphonates, BRONJ, animal model, pathogenesis, RANKL, OPG

Introduction
Bisphosphonates (BPs), which inhibits bone resorption, is widely used to treat osteoporosis and bone metastases of malignant tumors (multiple myeloma, breast cancer, prostatic cancer) and Paget’s disease of bone [1-3]. It effectively increases bone mineral density and bone strength, lowers the risk of bone fracture and greatly improves the quality of life for patients. However, it has been found that long-term large dose of BPs causes osteonecrosis of the jaw. More and more similar cases have been reported since bisphosphonate-related osteonecrosis of the jaw (BRONJ) was first reported in 2003 [4].

In 2009, the American Association of Oral and Maxillofacial Surgeons (AAOMS) clearly defined the BRONJ, which includes the following three characteristics: 1). Exposed bone in the maxillofacial region that has persisted for more than eight weeks; 2). Current or previous treatment with a BP, and; 3). No history of radiation therapy to the jaws [5]. The most typical symptom is unhealed exposed bone, accompanied by pain, mucous swelling, ulcer and another infection. These symptoms have great impact on quality of life for patients. BRONJ is associated with the type of BPs, administration schedule and route. BPs containing nitrogen or administered intravenously is more likely to cause BRONJ compared with BP without nitrogen or administered orally. Surgical alveolar trauma, oral inflammation and combination with immunosuppressive agents or chemotherapeutics also increased risk of osteonecrosis of the jaw [6]. Currently, the pathogenesis of BRONJ remains unknown. The major challenge in the pathogenesis of BRONJ is difficult control of prospective clinical study, especially in the population at high risk of cancer who took multiple drugs to control tumor growth and related skeletal complications. Since no effective treatment is available and the conservative treatment was associated with poor response, it is urgent to stab-
lish animal model of BRONJ to find out the role of BPs in development of BRONJ to facilitate the prevention and treatment options for patients who need relief of pain.

In this current study, we established a rat model of bisphosphonate-related osteonecrosis of the jaw, which realistically recapitulated major clinical manifestations of the human disease, including unhealed mucosa of the alveolar socket, exposed necrotic bone and inflammatory infiltration. This finding showed the rats treated with zoledronate developed BRONJ-like disease after extraction of teeth. We also found that zoledronate was associated with reduced ratio of receptor activator of nuclear factor kappa-B ligand/osteoprotegerin (RANKL/OPG), two key factors in bone formation of bone tissue. Since they are very important in regulation of differentiation of osteoclasts, we determined their expression to study their role in the development of osteonecrosis, which might open a door to further explain the pathogenesis of BRONJ and develop targeting prevention and treatment methods.

Materials and methods

Animals

Male SD rats at the age of 8-10 week weighing approximately 250 g were provided by the Shanghai Slaccas Laboratory Animal Center. The animals were housed in SPF environment at 22±1°C with humidity of 55±10% under an alternating 12-h light and 12-h dark cycle, with food and filtered water ad libitum.

Reagents and equipments

Zoledronate standard was purchased from Sigma, USA. Chloral hydrate, xylene and paraffin wax were purchased from Shanghai Sinopharm Chemical Reagent Co., Ltd. Hematoxylin-eosin staining solution was purchased from Nanjing Jiancheng Biological Engineering Institute, China. Protease inhibitor was purchased from Sigma, USA. Rat RANKL and OPG ELISA kits were purchased from Uscn Life Science Inc, China. Microplate reader was purchased from TECAN, Switzerland. Paraffin embedding machine, paraffin, paraffin slice machine, paraffin stretching palter and paraffin were purchased from Leica, Germany. Optical microscope was purchased from Olympus, Japan.

Establishment of rat model of BRONJ

36 rats were assigned to the test group and the control group. The test group was intravenously injected with 80 μg/kg zoledronate once a week via the tail vein. The control group was intravenously administered with equivalent PBS once a week. Three weeks after administration, the maxillary first molar was non-invasively extracted under anesthesia with 5% chloral hydrate (Figure 1). The bleeding was stopped using gauze. The rats continued to receive treatment after extraction. Then after 1, 4 and 12 weeks after tooth extraction, the rats were euthanized, and the maxillas were separated and harvested.

Hematoxylin-eosin (HE) staining

The bone tissue was completely blocked with 4% paraformaldehyde and then rinsed with water, and stored overnight. Also, the bone tissue was decalcified using 10% EDTA at 4°C for about 2 months, rinsed and stored overnight. After being dehydrated, hyalinized, waxed and embedded, the bone tissue was sectioned at 5 μm and stained with HE staining solution.

Determination of RANKL and OPG in bone tissue

The bone tissues collected were stored in liquid nitrogen at low temperature. After collection of all samples, the samples were removed from the liquid nitrogen and fully ground using mortar. 1 mL of T-PER reagent (25 mM hydroxyethyl glycine, 150 mM sodium chloride, washing solution of pH 7.6, protease inhibitor) was added to each sample. After being mixed, the tissues were lysed and centrifuged to obtain tissue lysate. The concentrations of RANKL and OPG in the bone tissue were determined using
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Control

Zoledronate

Figure 2. Healing of injured mucous membrane 12 weeks after extraction.

Table 1. Summary of bone remodeling in the extraction site 12 weeks after extraction

<table>
<thead>
<tr>
<th></th>
<th>Necrotic bone</th>
<th>Inflammatory infiltration</th>
<th>Soft tissue impairment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zoledronate group</td>
<td>8 (8)</td>
<td>6 (8)</td>
<td>3 (8)</td>
</tr>
<tr>
<td>Control group</td>
<td>0 (6)</td>
<td>0 (6)</td>
<td>0 (6)</td>
</tr>
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</table>

ELISA kits as per the instruction. The number of osteoblasts in the jaw might vary in samples, which might impact the concentrations of RANKL and OPG. Considering this, we also determine the total content of proteins to standardize the concentration.

Statistical analysis

All data was statistically analyzed using SPSS 16.0. The comparison between two groups was performed using independent-samples T test. The level of statistical significance was set at P<0.05, and the significant level of statistical significance was set at P<0.01. The data was expressed as mean ± SD.

Results

Establishment of rat model of BRONJ

12 weeks after extraction, part of rats (3/8) that received zoledronate via tail vein developed impaired soft tissue, unhealed mucus in the extraction site and exposed bone (Figure 2). However, all rats in the control group showed completely healed mucosal epithelium tissue in the extraction site (Figure 2). The statistical analysis of data of soft tissue impairment was shown in Table 1.

The blue circle indicated unhealed mucus in the extraction site and exposed bone in rats treated with zoledronate. The blue arrow indicated normal healing mucus in the extraction site in rats of the control group.

HE staining

HE staining was performed to help determine the condition of osteoblasts, presence of exposed bone, inflammatory infiltration and integrity of epithelial tissue. The finding of sections indicated that the rats in the test group developed typical symptoms of BRONJ including many empty bone lacunae, marginal bone loss, a lot of necrotic bones infiltrated with inflammatory cells, swelling and ulcer 12 weeks after extraction (Figure 3B, 3D). The rats in the control group exhibited normal physical healing of bone, almost completely healed epithelium in the extraction site and approximately complete bone remodeling (Figure 3A, 3C).

HE staining findings of the jaw also suggested the change of osteoblasts induced by zoledronate. 1 week after extraction, the test group showed less osteoclasts than the control group. The control group exhibited active bone remodeling, a great number of osteoclasts and active osteoblasts to promote bone healing (Figure 4).

One of the current clinical diagnostic criteria of BRONJ is exposed bone in the maxillofacial region that has persisted for more than eight weeks. Therefore, statistical analysis was performed for results of all sections. In the control
All jaws showed normal bone remodeling and were free from necrotic bone, inflammatory infiltration and soft tissue impairment. The group treated with zoledronate showed necrotic bone in all samples, inflammatory cell infiltration in most samples (6/8) and soft tissue impairment in some samples (3/8).

**Changes of cytokines-RANKL and OPG for bone remodeling of the jaw**

RANKL and OPG are very important in regulation of maturation and differentiation of osteoclasts. The expressions of the two factors were determined to clarify their role in the development of BRONJ. The results showed that the test group had inhibited expression of RANKL, increased expression of OPG and reduced ratio of RANKL/OPG 4 weeks after extraction compared with the control group (P < 0.01) (Table 2).

**Discussion**

BPs is a class of synthesized inorganic pyrophosphate analogues, which are effective to inhibit activity and function of osteoblasts and induce apoptosis of osteoblasts [7]. BPs is widely used as strong bone resorption inhibitors in clinical practice. However, since the first report of a case with BRONJ, there have been increasing reports in the literature of the occur-

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**Figure 3.** Bone remodeling in extraction site 12 weeks after extraction. A HE staining sections indicated unhealed epithelium in the extraction site, necrotic bone and inflammatory cell infiltration (×100) in rats treated with zoledronate. B HE staining sections of jaw of rats in the control group exhibited normal physical healing of bone, almost completely healed epithelium in the extraction site and approximately complete bone remodeling (×100). C and D were highly magnified figures (×200) of black boxes in A and B. B meant newly formed bone tissue; NB meant necrotic bone; IF meant inflammatory cell infiltration; E meant epithelium; the arrow meant empty bone lacuna that occurred in rats treated with zoledronate.
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**Table 2.** Expressions of RANKL and OPG in bone tissue of the jaw 4 weeks after extraction

<table>
<thead>
<tr>
<th></th>
<th>RANKL</th>
<th>OPG</th>
<th>RANKL/OPG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zoledronate group</td>
<td>134.4±22.6</td>
<td>17.91±1.63</td>
<td>7.54±0.82</td>
</tr>
<tr>
<td>Control group</td>
<td>155.5±17.3</td>
<td>15.12±0.58</td>
<td>13.25±0.65</td>
</tr>
</tbody>
</table>

rence of osteonecrosis of the jaws in patients who were intravenously injected with long-term large dose of BPs. Most researchers believe that BPs exert strong inhibition on osteoclasts after the jaw is injured during extraction. Therefore, bone conversion of the jaw is excessively inhibited, causing osteonecrosis of the jaw. BPs act directly on osteoclasts, inhibit osteolysis of osteoclasts and induce apoptosis of osteoclasts by impacting the activity of lymphocyte precursors [7]. Also, BPs cause blockage in signaling pathway of cells in bone marrow and indirectly inhibit deposition of bone mineral of osteoblasts [8], resulting in bone remodeling inhibition and slower bone remodeling that lead to unsuccessful bone remodeling in the extraction socket. Khosla et al [9] demonstrated that BPs prevent angiogenesis by production of agents that inhibit angiogenesis. The inhibition of vessel remodeling causes inhibition of bone remodeling. Sedghizadeh et al [10] reported that oral infection with bacteria was key factor for occurrence of BRONJ. Whether infection causes necrosis or necrosis induces infection during BRONJ remains controversial.

The pathogenesis of BRONJ is unclear and there is no effective clinical treatment, so it is important to establish a simple, effective and reliable animal model of BRONJ to determine the role of BPs in occurrence of BRONJ, which will provide a route for targeted prevention and treatment.

To study the pathogenesis of BRONJ, many researchers have tried to establish animal models including rats [11-16], beagles [17, 18] and mini-pigs [19], revealing that application with BPs during extraction and other adjuvant therapy induces inhibition of bone remodeling in extraction socket that causes osteonecrosis of the jaw. However, the success rate of the BRONJ model establishment was low with BPs alone in most studies. Although adjuvant application with immunosuppressive agents and chemotherapeutic agents increase the success rate of model establishment, the influence of other drugs on osteonecrosis of the jaw can’t be excluded. Models with beagles and mini-pigs are time-consuming and expensive. Biasotto et al [20] successfully established a rat model with BRONJ through intravenous injection with zoledronate, but they also created expanded bone defect of 4 mm in diameter in addition to trauma caused by extraction. The success rate of the model was 100%, but the risk factors were not in conformity with those of patients undergoing just extraction.

In this current study, we established a rat model of BRONJ, which reproduced major clinical manifestations of the human disease, including unhealed mucosa of the alveolar socket, exposed necrotic bone and inflammatory infiltration. To avoid the impact of tumor growth, we...
selected healthy rats for the experiment because osteonecrosis is associated with dose and type of drugs rather than property and malignancy of tumors. Zoledronate, the strongest third generation of BPs, was intravenously administered to increase success rate of the model of BRONJ. The extraction was also performed for rats, since bone injury is the most common risk factor of BRONJ. The results of all sections were statistically analyzed. Necrotic bone was found in all samples. Inflammatory cell infiltration was observed in most samples (6/8). Soft tissue impairment was observed in some samples (3/8). The success rate of the model was relatively high compared with other animal studies of BRONJ.

The RANKL/RANK/OPG system is one of the most important signaling pathways that regulate bone resorption and osteogenesis [21]. RANKL is mainly present in surface of osteoblast precursors, and binds to receptor RANK on the surface of osteoclast precursors, which activates signal transduction pathway to promote differentiation and maturation of osteoclast precursors, and enhance bone resorption. OPG is a key cytokine for osteoblast precursors to inhibit bone resorption. It can compete with RANKL for binding to RANK, and inhibit formation and activation of osteoclasts, thereby inhibiting bone resorption by osteoclasts. In the current study, the zoledronate group had significantly lower osteoclasts than the control group 1 week after extraction. The control group exhibited active bone remodeling and a large number of osteoclasts and active osteoblasts to facilitate bone healing. Also, we found that the test group had inhibited expression of RANKL, increased expression of OPG and reduced ratio of RANKL/OPG 4 weeks after extraction compared with the control group, demonstrating that zoledronate not only directly impacts proliferation and differentiation of osteoclast precursors and inhibits osteolysis by osteoclasts on the bone surface after injury of the jaw, but also indirectly inhibits function of osteoclasts by regulation of expressions of genes such as RANKL/OPG on surface of osteoblast precursors, which breaks the balance between osteolysis and osteogenesis. The inhibition and reduction of bone remodeling after extraction results in abnormal bone repair of the jaw. If the trauma remains unhealed for a long time and become infected, the possibility of osteonecrosis of the jaw increases.

In conclusion, the rat model of BRONJ successfully reproduced major clinical manifestations of the human disease and provided a route for explaining pathogenesis of BRONJ. The model might also be used as a tool to determine the effectiveness of treatments for BRONJ.

Disclosure of conflict of interest

None.

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