The Effect of Bisphosphonate on Bone Formation After Tooth Extraction in Ovariectomized Rats

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Abstract: Because dentists often see patients who are taking bisphosphonate (BIS) drugs, it is important for dentists to be aware of bisphosphonate-related osteonecrosis of the jaw (BRONJ). However, many aspects of the pathogenesis of BRONJ have not yet been clarified. The present study examined the healing process of tooth extraction sockets in ovariectomized rats to elucidate the pathogenesis of BRONJ, particularly the impact of BIS administration in new bone formation. Nine-week old female Wistar rats (6 weeks old during surgical removal of the ovaries) were divided into control (saline treated) and BIS-treated groups (6 in each condition). Alendronate was used for BIS treatment. The maxillary second molar was extracted. Specimens were decalcified with EDTA and embedded in paraffin for serial section. New bone formation and osteoclast counts were determined in H-E and tartrate-resistant acid phosphatase stain, respectively. Bone mineral density was also measured using micro-CT in non-decalcified samples. Contact microradiography (CMR) was taken in polished specimens. Granulation tissue was observed in extraction sockets of both groups at 1 week, and new bone formation was observed at 4 weeks. In the BIS-treated group, the number of multinucleated osteoclasts away from the bone surface increased. New bone formation after tooth extraction increased over time, but it was clearly less in the BIS-treated group compared to the control group. Results showed that BIS inhibited the formation of new bone at extraction socket during the early stage, causing low-density bone trabeculae and the spread of abnormal osteoclasts leading to BRONJ.

Key words: Bisphosphonate, Alendronate, Tooth extraction, Osteoclasts

Introduction

Bisphosphonate (BIS) is a drug used to treat osteoporosis and Paget’s disease and has an effect on bone resorption\(^1\)\(^-\)\(^3\). Moreover, it is also used in the treatment of malignancy associated with hypercalcemia including the prevention of bone metastasis in malignant tumors\(^4\)\(^-\)\(^5\).

Due to the increase in the number of patients with osteoporosis recently, the chance of encountering patients taking BIS for dentists also increased. Thus, dentists must be aware of the so-called Bisphosphonate-Related Osteonecrosis of the Jaw (BRONJ) in treating patients under BIS treatment\(^6\)\(^-\)\(^7\). Numerous studies in Europe and America have reported the occurrence of BRONJ, however, the pathogenesis is still unknown\(^8\)\(^-\)\(^9\).

Failure of extraction socket to heal was shown to be an early symptom of BRONJ and invasive dental procedures such as tooth extraction is an important factor in BRONJ\(^10\)\(^-\)\(^11\). Therefore, to find the effect of BIS on the healing of tooth extraction socket would be a stepping-stone to reveal the mechanism of BRONJ.

Patients with osteoporosis are more than 80% women in which the decrease in female hormones after menopause may be the major cause. The rapid decrease in estrogen after menopause has a major effect in bone throughout the body but it is not clear whether it has the same effect in alveolar bone.

After administration, BIS is deposited in bone and its pharmacological effect is on osteoclasts, which destroy bone by phagocytosis. The basic mechanism of action is the inhibition of farnesyl pyrophosphate synthase, which can cause apoptosis of osteoclasts\(^12\)\(^-\)\(^13\). Alendronate is a typical second generation BIS used in this experiment. Studies showed that Alendronate is the most extensive clinical drug for the prevention of fractures\(^14\)\(^-\)\(^15\) and has strong inhibition of bone resorption by inducing apoptosis of osteoclasts, thus becoming the first line in the treatment of osteoporosis\(^14\)\(^-\)\(^16\). Moreover, it is also used in the treatment of malignant tumor with hypercalcemia in Japan\(^17\).

A study of postmenopausal osteoporosis in ovariectomized

rat is the most commonly used animal model and many basic data have been gathered so far\textsuperscript{8,19}. Thus, the animal model can be used to examine the healing of tooth extraction socket healing in postmenopausal osteoporosis administered with BIS. The aim of the study was to determine the effect of BIS administration in the healing process after tooth extraction in ovariectomized rats with reduced estrogen and to clarify the pathogenesis of BRONJ in postmenopausal osteoporosis

Materials and Methods

Alendronate Sodium Hydrate (Tiroc\textsuperscript{®} Injection 10 mg, Teijin Pharma, Tokyo) was used for BIS treatment. The drug was administered through intraperitoneal injection with a dose of 20 microgram per 100 g body weight (0.2 mg/g).

Thirty, 9-week old (about 180 g) Wistar ovariectomized rats (OVX rats, Sankyo Lab Service, Tokyo; ovariectomy was done at 6 weeks old) were used in the study. The OVX rats were divided into 2 groups, control and BIS-treated groups. The drugs were first administered at 9 weeks old. For the control group, saline water was administered in the same manner and amount. The maxillary second molar on both sides of the jaw (M2) were extracted at age 10 weeks in both groups. Dental hand instruments (YDM, Tokyo) were used for tooth extraction. Pentobarbital (30-40 mg/kg) and diethyl ether were used for general anesthesia. Postoperative medications were administered to each group once after tooth extraction. Then after, the animals were sacrificed at 1, 2 and 4 weeks by pentobarbital overdose. The maxillary bone with the remaining molars were collected as samples and fixed in 10% formaldehyde. The groups were further divided into non-decalcified and decalcified groups. The experiment was approved by Tokyo Dental College Animal Experiment Committee (approval number 250202).

Samples were decalcified in 10% EDTA (pH 7.3) at room temperature for 28 days. Then after, the samples were placed in series of alcohol following routine fixation, embedded in polyester resin (Nissin EM, Tokyo Rigolac). Tomography was taken using micro-CT (HMX225=ACTIS +4 TESCO, Tokyo) with the following conditions; thickness of 50 micrometer, voltage of 140 kV, current of 140A, and SID/SOD of 600/60. Focusing range was from the mesial surface of M1 to the distal surface of the maxillary third molar (M3). CT images were taken using TRI3D-BON (Ratoc, Engineering, Tokyo) configuring the 3D image to measure the bone density of the mesial root plate of M2 extraction socket. Measurement range was set to 1 mm\textsuperscript{3} on the floor and one side the wall of socket (Fig. 1).

For a detailed observation of calcification, a polished specimen of 100-micrometer thickness from the sample block was prepared using Soft X-ray generator (CMR-3, SOFTEX, Tokyo) for contact microradiography (CMR). Radiograph was taken with a voltage of 15kV, a current of 3 mA, a focus distance of 44.4 mm and an exposure time of 13 min. Glass plates (HRP-SN-2 2X2 30Z, KONICA MINORTA OPTO, TOKYO) were used for the imaging. Developing was done for 5 min at 20\degree C using a developing solution (D-19, Kodak, USA), fixed, washed and dried. Plates were then covered with glass.

The number TRAP-positive cells at the extraction socket was counted and subjected to multiple comparative test using non-repeated measures of ANOVA and SNK test to compare between groups. A p-value of <0.05 was considered significant.

Results

H-E stain

One week after tooth extraction, the socket is filled with granulation tissue in both control and BIS-treated groups. In the control group, fine immature bone extending from the extraction socket wall was observed. Newly formed bone extended from the socket wall toward the middle of the extraction socket. Osteoblasts were observed near the edge of the apical third of the extraction socket. Granulation tissue was seen at the center of the extraction socket as well as an image of reminiscent osteoid (Fig. 2a, b).
Resorption of the interalveolar septum around the edges of the extraction socket was also noted. In BIS-treated group, only a small amount of new, immature bone was observed on the wall and floor of extraction socket (Fig. 2c, d). Resorption of the interalveolar septum reaching the apical end was detected (Fig. 2d).
bone in the socket has an irregular mesh with a wide gap in between the floor of the socket and granulation tissue (Fig. 3b). Moreover, the newly formed bone lacks the bone-like fiber bundle with thickening of the cavity wall. Furthermore, a relatively clear boundary was observed between the newly formed bone and the socket wall (Fig. 3b).

Four weeks after extraction, the new bone in the socket has matured; bone quality was better and became denser in both groups. In the control group, the trabecular bone became thick compact bone; the boundary surrounding the alveolar bone disappeared and became an integral part of the bone structure (Fig. 4a). In BIS-treated group, the bone became denser in quality although still filled with gaps of granulation tissue and the boundary between alveolar bone and new bone was relatively clear (Fig. 4b). Lack of ruffled border and several multinucleated bone cells with various abnormalities situated away from the bone surface were observed away (Fig. 5a, b).

**TRAP staining**

One week after extraction, in the control group, although TRAP-positive cells in the socket wall were not expected, multinucleated bone cells were seen in the bone marrow of the alveolar bone and in the resorbed interalveolar septum. TRAP-positive cells were also observed in BIS-treated group in a similar trend with the control group except near the interalveolar septum. Two weeks after extraction, in the control group, TRAP-positive cells corresponding to bone resorption were noted. In BIS-treated group, TRAP-positive cells with abnormal nuclear condensation were found away from the bone surface. The ruffled border was also obscured (Fig. 5c). Even four weeks after extraction, in BIS-treated group, the ruffled border was still unclear and the multinucleated cells were still noted away from the bone surface. Furthermore, the multinucleated osteoclasts are smaller than the usual, TRAP-positive cells were also present in the surrounding connective tissue away from the bone surface (Fig. 5d).

Comparing the number of TRAP-positive cells per unit area, the number increased significantly at 2 weeks compared to 1 week (P<0.05). However, no significant difference was observed between 4 and 2 weeks (Fig. 6).

Comparing the percentage of TRAP-positive cells, which were either free or attached to the bone surface, no significant difference was obtained in each week for the control group. However, in BIS-treated group, free cells were more compared to attached
cells in each week. Significant difference was observed in 2 and 4 weeks (P<0.05, Fig. 7).

**Micro-CT image**

Measuring the bone density of the mesial palatal root of M2 in each week, an increase in bone density of volume was observed in the control group over time (P<0.05). In BIS-treated group, an increase in bone density was also observed in the same way as the control group but no significant difference was observed between 2 and 4 weeks (Fig. 8).

Comparing the bone density in same period, bone density is clearly less in BIS-treated group than in the control group but significant suppression of bone formation was observed at 1 and 4 weeks (P<0.05). Nevertheless, no significant difference was obtained between the two groups at 2 weeks (Fig. 8).

**CMR image**

One week after extraction, in the control group, the socket is filled with immature bone with irregular mesh and weak calcification (Fig. 9a). The newly formed bone has needle-like structure with low degree of mineralization. In BIS-treated group, newly formed bone in the extraction socket was hardly seen although a small amount of immature bone was detected near the floor of the socket (Fig. 9b).

Two weeks after extraction, in the control group, calcification of bone tissue increased compared to 1 week and the trabecular structure became clear (Fig. 10a). In BIS-treated group, trabecular structure in the socket became clear but it was less than the control group. A thin immature bone was somehow more commonly observed (Fig. 10b).

Four weeks after extraction, in the control group, the extraction socket became unclear, calcification of the bone increased, trabecular structure became thick and dense bone was generally observed (Fig. 11a). In BIS-treated group, compared to 2 weeks, calcification and degree of bone density increased. However, in comparison with the control group at 4 weeks, the bone density was lower and trabecular structure was slightly irregular (Fig. 11b).

**Discussion**

**New bone formation**

Regarding the formation of new bone, the amount of newly formed bone one week after extraction is clearly less in BIS-treated group than in the control group and low degree of bone mineralization was seen in CMR. The results suggest that BIS administration inhibited new bone formation delaying the healing of extraction socket in the early stage. Moreover, the inhibitory effect influenced the calcification of the newly formed bone. Normally, angiogenesis is enhanced in the early stage of healing of tooth extraction socket with granulation tissue and remarkable invasion of new blood vessels in the socket. Bone formation in tooth extraction socket occurs as osteoblasts derived from undifferentiated mesenchymal cells are present in the granulation tissue although reports showed that BIS administration prevents blood vessel formation having similar results with Alendronate\(^{20,21}\). In other words, the delay in the proliferation of granulation tissue and formation of new blood vessels would also delay the induction of osteoblasts and early formation of new bone.

Postmenopausal osteoporosis is said to be highly rotational and bone resorption is enhanced by the decrease in estrogen concentration\(^{22}\). Cytokines such as TGF-\(\beta\) and IGF-1 present in the bone matrix are released during bone formation and by their action, bone formation also increased transiently after a delay in
bone resorption. However, in this experiment, the increase in bone density over time was seen in the control group suggesting that bone formation proceeded predominantly even the estrogen level was lowered in active areas such as the extraction socket. On the other hand, only a weak increase in the degree of bone density in BIS-treated group was detected two weeks after extraction. This is because, bone metabolism dropped in BIS-treated group causing a delay in the formation of new bone in the extraction socket suggesting that BIS affected the increase in bone density.

Furthermore, considering the impact in the decline of bone metabolism for the quality of new bone, there is a possibility of the effect of micro-damage as a factor in osteoporosis. Micro-damage is a fine crack, which occurs in bone tissue. Bone metabolism is reduced by strong inhibition of bone resorption in BIS-treated group thus accumulation of micro-damage

20. BIS has an extended half-life in long bones and the reduction in bone metabolism is caused by excessive and long-term intake.

In the oral cavity, the interalveolar septum is subjected to a lot of load during tooth extraction and accumulation of micro-damage occurs to the jaw since the alveolar bone itself is brittle due to poor bone metabolism than the normal. The newly formed trabecular bone in BIS-treated group was irregular; inflammation may have caused the extensive expansion of granulation tissue in a short period of time, suggesting that it can induce BRONJ.

Abnormality of the osteoclasts

Many theories have been proposed that the main cause of bone loss is estrogen deficiency leading to increase osteoclasts activation enhancing bone resorption. In the inflammatory cytokine theory, estrogen deficiency caused the production of IL-1, IL-6, TNF-β, GM-CSF and other inflammatory cytokines. An increase in inflammatory cytokines enhances bone resorption by inhibiting apoptosis, promoting osteoclasts differentiation by M-CSF and RANKL. Also, several studies indicated the possible direct effect of estrogen in osteoclasts and the increase in bone resorption caused by an increase in ovarian hormone stimulation. The path leading to the increase in the activation of osteoclasts by estrogen deficiency is extensive.

In the present study, in both control and BIS-treated groups, a significant difference in the number of TRAP-positive cells between 1 and 2 weeks after extraction was obtained. It was thought that the increase in bone mass was due to healing of the extraction socket together with the rapid remodeling of the bone due to estrogen deficiency and increase in TRAP-positive cells. In BIS-treated group, an increase in the number of TRAP-positive cells was observed in comparison with the control group but those TRAP-positive cells were away from the bone surface. A significant difference was observed between the free and attached cells. BIS inhibited the function of osteoclasts in the same manner as cell adhesion ability to the bone surface was decreased.

Osteoclasts adhere to bone surface creating ruffled border during bone resorption. Therefore, it is considered that osteoclasts away from the bone surface are non-functional. Results showed an increase in the number of non-functional osteoclasts in BIS-treated group. This is consistent with the findings in one study. Moreover, the TRAP-positive cells observed in BIS-treated group showed unusual multinucleation with partial or complete lacking of ruffled border compared to the control group. The study showed that estrogen deficiency promoted the differentiation and increase in the number of osteoclasts and BIS promoted apoptosis of osteoclasts coupled with abnormal cells.

Acknowledgement

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References

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