Effects of Bisphosphonate Administration on Peri-Implant Bone in Vitamin D-Deficient Rats

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Abstract: The aim of this study was to examine histomorphologically how bisphosphonate (BP) injected before and after implant placement surgery affects the peri-implant bone in vitamin D-deficient animal model. This study used 60 six-week-old male Sprague-Dawley (SD) rats which were given a vitamin D-deficient diet. These experimental animals were divided into Group 1 (BP administration starting before implant placement), Group 2 (control group) and Group 3 (BP administration starting after implant placement). Threaded titanium implant was placed 0.50 mm mesial to the first molar. The samples were tissues from Groups 1 and 2 obtained at 1, 2, 4 and 8 weeks after implant placement and tissues from Group 3 obtained after the start of BP administration. Evaluation by light microscope and micro-CT imaging were performed. In Group 1, bone density around implants significantly increased as time progressed from after implant placement. In Group 3, bone density significantly decreased as the duration of bisphosphonate use increased. Group 1 had a significantly higher proportion of lacunae without osteocytes compared with Group 2 after implant placement. In Group 3, the proportion of these lacunae increased significantly from one to two weeks after the start of bisphosphonate administration. There were significantly higher proportions at 4 weeks and 8 weeks after the start of administration compared with 1 week after the start. This histological result suggests the need to exercise caution in using implant treatment on patients taking bisphosphonates and in administering bisphosphonates in patients after implant placement.

Key words: Implant, Bisphosphonate, Osteonecrosis, Osteocyte, Osteoclast

Introduction

Implant treatment achieves a highly functional restoration, and good prognosis can be expected. Therefore, it has rapidly become a widely used, effective treatment for missing teeth. Implant treatment is often performed in elderly patients who have a history of various systemic diseases. Thus, they are usually undergoing treatment for these diseases.

Bisphosphonates (BP) are used to treat bone metastasis and systemic bone diseases such as Paget disease and osteoporosis⁴⁻⁶. Bisphosphonate-related osteonecrosis of the jaw (BRONJ) was first reported by Marx and Wang et al. in 2003⁷⁻⁶. It is known that tooth extraction can lead to BRONJ⁷⁻⁶, and BRONJ related to implant treatment has also been reported⁷⁻¹⁰. There is a tendency for multiple osteonecrosis to occur in myeloma patients and breast cancer patients with vitamin D-deficiency. Vitamin D deficiency can increase the risk for osteonecrosis of the jaw⁷⁻¹².

Therefore, it is necessary to elucidate the effects of vitamin D deficiency and bisphosphonate administration on peri-implant bone to safely perform implant treatment. The aim of this study was to examine histomorphologically how bisphosphonate injected before and after implant placement surgery affects the peri-implant bone in vitamin D-deficient animal model.

Materials and Methods

This study used 60 six-week-old male Sprague-Dawley (SD) rats (weight: 180-210 g). These experimental animals were divided into 3 groups as will be described later.

Animal model and surgical procedure

Each treatment on experimental animals was performed according to the time points indicated in Figure 1.
Group 1 (bisphosphonate (BP) administration starting before implant placement)

The rats were given a vitamin D-deficient diet (Diet11: 0.47% Ca, 0.30% P; Japan-CLEA, Tokyo, Japan) beginning at 6 weeks old to obtain vitamin D-deficient rats. The rats were raised on 12-h light-12-h dark cycles. When the vitamin D-deficient diet was begun, the rats also began to receive administration of zoledronate (Zometa, Novartis Pharma, Tokyo, Japan). Zoledronate (4.0 mg) was diluted with 100 ml physiological saline, and the resulting solution was injected into the tail vein of each rat (dose: 35 μg/kg). The injection was performed once every 2 weeks. The vitamin D-deficient diet and bisphosphonate administration were continued until the rats were sacrificed. At 1, 2, 4 and 8 weeks after the start of bisphosphonate administration (5, 6, 8 and 12 weeks after implant placement), samples were collected using the same method as in Group 1.

Evaluation of biomarkers of bone metabolism

When the rats were 6 and 9 weeks old, blood tests and urinalysis were performed in Group 2. An evaluation was performed on the changes in biomarkers of bone metabolism due to vitamin D deficiency (examined items: 25(OH)D, Ca, P, ALP, PTH, OC, TRACP-5b, DPD, Cre, and DPD/CRE).

Micro-CT imaging and evaluation

Bone density was measured in samples obtained at 1, 2 and 4 weeks after implant placement in Groups 1 and 2 and samples obtained at 1, 2 and 4 weeks after the start of BP administration for Group 3. CT images (SkyScan 1176, Bruker micro CT, Belgium) were taken of these samples and a phantom for QCT to calculate bone density. The imaging data were used for 3-dimensional image reconstruction (NRecon, Bruker micro CT, Belgium). Bone density was measured in the peri-implant area within 500 μm from the implant surface (CT-An, Bruker micro CT, Belgium).

Preparation of light microscopy sections

Decalcified tissue specimens

The samples were tissues from Groups 1 and 2 obtained at 1, 2, 4 and 8 weeks after implant placement and tissues from Group 3 obtained at 1, 2, 4 and 8 weeks after the start of BP administration. These samples were immersed in half-strength Karnovsky’s fixative (2.5% glutaraldehyde and 2% paraformaldehyde) for 48 h and decalcified in 0.50M EDTA for 14 days. After decalcification, each implant body was carefully removed so that the interface was not disrupted, and the samples were embedded in paraffin. The resulting paraffin blocks were sliced into 3.0 μm-thick sections using a sliding microtome (Leica, Solms, Germany). The sections were stained with hematoxylin and eosin (H&E staining).

Tissue specimens stained with tartrate-resistant acid phosphatase (TRAP)

The same method as for the decalcified tissue specimens was used to prepare 3.0 μm-thick sections for TRAP-staining. The sections were deparaffinized and washed with water. They were placed in the TRAP solution for 40 min. washed, and stained with hematoxylin for 5-6 seconds.

Evaluation by light microscope and statistical analysis
Table 1. Measured serum levels of biomarkers of bone metabolism (*p<0.05)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Serum chemistry measurement</th>
<th>ALP (U/l)</th>
<th>Ca (mg/dl)</th>
<th>IP (mg/dl)</th>
<th>25OHD (ng/ml)</th>
<th>PTHIN (pg/ml)</th>
<th>Osteonectin (ng/ml)</th>
<th>TRACP (mU/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without vitamin D</td>
<td>1717.7±150.2</td>
<td>9.3±1.4</td>
<td>18.4±6.5</td>
<td>9.7±0.8</td>
<td>5.3±3.8 or 0 10</td>
<td>&lt;10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>deficiency feeding</td>
<td>766.3±94.2</td>
<td>10.8±0.5</td>
<td>22.1±1.4</td>
<td>3.1±0.1</td>
<td>10±0.0</td>
<td>1±0</td>
<td>&lt;10</td>
<td>-</td>
</tr>
<tr>
<td>P value</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.049</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Statistical significance was set at p<0.05.

Table 2. Measured urinary levels of biomarkers of bone metabolism (*p<0.05)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Urine chemistry measurement</th>
<th>DPD (nM/l)</th>
<th>DPD/Cre (nM/mMCRE)</th>
<th>Cre (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without vitamin D</td>
<td>651.7±290.6</td>
<td>378.6±81.3</td>
<td>0.2±0.04</td>
<td></td>
</tr>
<tr>
<td>deficiency feeding</td>
<td>1113.0±1193.4</td>
<td>504.5±25.3</td>
<td>0.25±0.3</td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td>0.30</td>
<td>0.031</td>
<td>0.35</td>
<td></td>
</tr>
</tbody>
</table>

*Statistical significance was set at p<0.05.

The prepared sections were examined under a light microscope (BX51, Olympus Co., Tokyo, Japan). Peri-implant bone was histomorphologically evaluated for each group using decalcified specimens (H&E staining). Evaluation of lacunae without osteocytes was performed in the region within 500 µm of the bone-implant interface (calculation of proportion of lacunae without osteocytes).

Statistical analysis was performed by parametric t-test. Statistical significance was set at p<0.05. In TRAP-stained sections, the number, morphology, and distribution of TRAP-positive cells were examined.

Figure 1. Time table of animal experiment

Figure 2. Changes in peri-implant bone density over time

Figure 2a. Groups 1 and 2: changes in peri-implant bone density over time. Groups 1 and 2 showed significantly increasing bone density over time. (*p<0.05)

Figure 2b. Group 3: changes in peri-implant bone density over time. Group 3 showed significantly decreasing bone density over time. (*p<0.05)

Results

Changes in biomarkers of bone metabolism
Figure 3. Groups 1 and 2: light microscopic images at 1 week after implant placement

Figure 3a. Whole image (Upper image, Group 1, H&E staining): One week after implant placement, no histological finding of inflammation was observed in the peri-implant area. There were lacunae without osteocytes in the cortical bone and marrow regions near the bone-implant interface. (M: mesial side, D: distal side, O: oral cavity side, R: rhinal side, red arrow: mature bone type, black arrow: callus-like tissue type, green arrow: fibrous tissue type)

Magnified image of black frame (Lower image, Group 1, H&E staining): In the cortical bone and marrow regions, immature, fibrous callus-like tissue was observed in the area between implant threads (black arrow). (blue frame: lacuna without osteocyte)

Figure 3b. Whole image (Group 2, H&E staining): One week after implant placement, histological findings showed lacunae without osteocytes in the cortical bone and marrow regions near the bone-implant interface. Group 2 had peri-implant bone formation as in Group 1 (arrows), and continuity of the plate-like trabeculae was observed in the peri-implant region. (M: mesial side, D: distal side, O: oral cavity side, R: rhinal side, red arrow: mature bone type)

Figure 4. Groups 1 and 2: light microscope images at 4 weeks after implant placement

Figure 4a. Group 1 had more peri-implant trabeculae compared with Group 2 (control group), and there was formation of dense trabeculae. The number of lacunae without osteocytes was lower at 4 weeks after implant compared with the number at 1 or 2 weeks after placement, indicating the maturation of the peri-implant bone tissue. (M: mesial side, D: distal side, O: oral cavity side, R: rhinal side, red arrow: mature bone type, black arrow: callus-like tissue type, green arrow: fibrous tissue type)

Figure 4b. There was dense peri-implant bone on the oral side, particularly in the cortical bone region. A large amount of peri-implant connective tissue was observed on the nasal side, and there were inflammatory cell infiltration, mainly of neutrophils (black frame). Lower image shows the region of black frame. (M: mesial side, D: distal side, O: oral cavity side, R: rhinal side, red arrow: mature bone type, green arrow: fibrous tissue type)
Figure 5. Groups 1 and 2: light microscopic images at 8 weeks after implant placement

Figure 5a. In Group 1, the area surrounding the implant body was mostly covered with fibrous connective tissue. There was inflammatory cell infiltration in the fibrous connective tissue, involving the area of two implant threads on the oral side. There were no osteoclast-like cells (multinucleated giant cells) near the implant body. There were scarcely any lacunae without osteocytes in the area distant from the implant body, and normal bone tissue was seen. (M: mesial side, D: distal side, O: oral cavity side, R: rhinal side, green arrow: fibrous tissue type)

Figure 5b. In Group 2, the peri-implant region on the oral side was filled with connective tissue, particularly on the mesial side, and slight inflammatory cell infiltration was observed. Dense, matured bone tissue was seen distal to the implant body. There were osteoclast-like cells on the trabecular surface near the implant body (black frame). Lower image shows the region of black frame. (M: mesial side, D: distal side, O: oral cavity side, R: rhinal side, blue arrow: osteoclast-like cell)

Figure 6. Group 1: light microscope images at 8 weeks after implant placement

Whole image (Upper figure, Group 1, H&E staining): the peri-implant area in the marrow region was mostly covered with fibrous connective tissue. There was no area where the implant body contacted the bone. Severe inflammatory cell infiltration was observed. In addition, an isolated sequestrum was observed in the connective tissue (black frame). In the region distant from the implant body, the bone tissue was normal and lacunae without osteocytes were very scarce. Osteoclast-like cells were observed on the surface of the bone distant from the implant (black frame). (M: mesial side, D: distal side, O: oral cavity side, R: rhinal side)

Magnified image of the area in the black frame (Lower image, Group 1, H&E staining): An isolated sequestrum was observed in the connective tissue. In the surroundings, severe inflammatory cell infiltration was seen with neutrophils and plasma cells.

Figure 7. Group 3: light microscopic image at 8 weeks after the start of bisphosphonate administration

At 5 weeks after implant placement, peri-implant bone was plate-like and mature. Lacunae without osteocytes were scarce in the bone tissue near the implant. There were osteoblast-like cells and many osteoclast-like cells near the bone-implant interface. (M: mesial side, D: distal side, O: oral cavity side, R: rhinal side, red arrow: mature bone type)
Figure 8. Changes over time in the proportion of lacunae without osteocytes in the peri-implant area

Compared with Group 2, Group 1 showed significantly higher proportions of these lacunae at all time points. (*p<0.05)

Figure 8a. Groups 1 and 2: changes over time in the proportion of lacunae without osteocytes in the peri-implant area

Figure 8b. Group 3: changes over time in the proportion of lacunae without osteocytes in the peri-implant area

Group 3 showed significantly increased proportion of these lacunae at one to two weeks after the start of bisphosphonate administration. Group 3 had significantly higher proportion at 4 weeks and 8 weeks after the start of administration compared with that 1 week after the start. (*p<0.05)

Figure 9. Group 1: light microscopic images of a TRAP-stained section at 2 weeks after implant placement

Whole image (Group 1, TRAP staining): TRAP-positive cells (osteoclast-like cells; multinucleated giant cells) were observed in the trabecular surface near the bone-implant interface. The overall number of TRAP-positive cells was small. (M: mesial side, D: distal side, O: oral cavity side, R: rhinal side)

Figure 10. Group 1: light microscopic image of a TRAP-stained section at 4 weeks after implant placement

The number of TRAP-positive cells (osteoclast-like cells; multinucleated giant cells) was very small compared to that at 2 weeks after implant placement. TRAP-positive cells were flat near the bone-implant interface. (M: mesial side, D: distal side, O: oral cavity side, R: rhinal side)

Figure 11. Group 3: light microscopic images of a TRAP-stained section at 8 weeks after the start of bisphosphonate administration

Overall image (Left image, Group 3, TRAP-staining): The number of TRAP-positive cells was very small, and these cells were scarce in the bone tissue near the implant body. (M: mesial side, D: distal side, O: oral cavity side, R: rhinal side)

Magnified image (right image, Group 3, TRAP-staining): Some TRAP-positive cells (blue arrow) were flat in the area distant from the implant body.

Serum ALP and 25(OH)D levels were significantly lower 3 weeks after a vitamin D-deficient diet was begun compared with before (p<0.05) (Table 1). Serum PTH and urinary DPD/Cre were significantly increased (p<0.05) (Table 2).
Evaluation of bone density

In Group 1, bone density significantly increased as time progressed from 1, 2 and 4 weeks after implant placement (Figure 2a). However, no statistical difference in density was observed between Group 1 and Group 2 (control group) at any time point. In Group 3, bone density significantly decreased as the duration of bisphosphonate use increased (Figure 2b).

Evaluation of light microscopy images

Group 2 had a larger amount of bone in the peri-implant area compared with Group 1 at 1, 2 and 8 weeks after implant placement (Figures 3a, b). A similar tendency was observed at 2 weeks after implant placement but there was no significant difference between the groups. Group 1 had a larger amount of bone in the peri-implant area compared with Group 2 at 4 weeks after implant placement (Figures 4a and b). As mentioned previously, Group 2 had a larger amount of peri-implant bone compared with Group 1 at 8 weeks after implant placement (Figures 5a and b). Sequestrum formation was observed in tissue sections of Group 1 at 8 weeks after implant placement (Figure 6). In tissue sections of Group 3, the areas between threads were filled with bone at all time points (Figure 7).

Evaluation of lacunae without osteocytes

Group 1 had a significantly higher proportion of lacunae without osteocytes compared with Group 2 at all time points of 1, 2, 4, and 8 weeks after implant placement (Figure 8a). In Group 3, the proportion of these lacunae increased significantly from one to two weeks after the start of bisphosphonate administration. There were significantly higher proportions at 4 weeks and 8 weeks after the start of administration compared with 1 week after the start (Figure 8b).

Evaluation of TRAP-stained tissue specimens

Group 2 had the smallest number of TRAP-positive cells (osteoclast-like cells) in tissue specimens obtained at 2 weeks after implant placement (Groups 1 and 2). TRAP-positive cells were very scarce. TRAP-positive cells were more concentrated in the area closer to the bone-implant interface in Group 1 than in Group 2 (Figure 9). A similar tendency was seen in sections obtained at 4 weeks after implant placement. In Group 1, flattening of TRAP-positive cells was observed (Figure 10). Group 2 had the largest number of TRAP-positive cells in tissue sections obtained at 8 weeks after implant placement. These cells were very scarce in Group 1. In Group 3, there were many TRAP-positive cells at 2 and 4 weeks after the start of bisphosphonate administration. However, there was only a small number of these cells at 8 weeks after the start, and these cells were flat (Figure 11).

Discussion

Implant treatment has already become an important prosthodontic treatment option for missing teeth. It has become widely used as a general treatment method. Implant treatment is often performed on elderly patients who are not necessarily in good health. Osteoporosis is a disease that is often seen in elderly individuals, particularly women. It is known that the risk for bone fracture becomes high as the disease progresses. Osteoporosis itself is not considered a risk factor in implant treatment. Bisphosphonates are drugs widely used to treat systemic bone diseases in clinical practice. However, they have potential side effects such as inflammation and osteonecrosis of the jaw after surgical treatment. Thus, there are discussions on the pros and cons of implant treatment in bisphosphonate users. Bisphosphonates have a high affinity for hydroxyapatite of bone tissue and are bone resorption inhibitors that suppress osteoclast activity. They have been used widely as a first-line drug for osteoporosis in the fields of general surgery and orthopedic surgery. Bisphosphonates are also used in diseases characterized by bone fragility such as bone metastasis and multiple myeloma. Presently, oral bisphosphonates are mainly used for osteoporosis and injectable bisphosphonates for bone metastasis of malignant tumor. BRONJ was first reported by Marx and Wang et al. in 2003, and this condition has subsequently gained much attention. In recent years, reports have increased on osteonecrosis of the jaw after dental surgery in bisphosphonate users. BRONJ occurs at a particularly high frequency in patients using injectable bisphosphonates such as zoledronate. Oral bisphosphonates such as alendronate had been considered to have a low risk. However, osteonecrosis of the jaw has also been reported in patients using oral bisphosphonates for osteoporosis. The pathogenesis is unknown, and there have been only a small number of reports on histological changes. Yarom et al. conducted a survey on BRONJ patients and found that 8.9% of these patients were oral bisphosphonate users. Thus, one cannot ignore the occurrence of BRONJ due to oral bisphosphonate use.

Our study used an animal experimental model. In one group of rats, implants were placed following bisphosphonate administration after implant placement. In another group of rats, bisphosphonate was administered after osseointegration was achieved. The response of peri-implant tissue was evaluated histologically in both groups.

In blood tests and urinalysis, serum concentrations of ALP, 25(OH)D, PTH and DPD/Cre were significantly changed after 3 weeks of a vitamin D-deficient diet compared to before the diet. Thus, it was speculated that bone metabolism would change from a continued vitamin D-deficient diet.

In the evaluation of bone density using CT, peri-implant bone density of Group 1 increased significantly as time progressed after implant placement. There was no significant difference in bone density between Group 1 and Group 2 (control group). This result...
is consistent with that of Morinaga et al.29, who studied normal rats. In our study, detailed examination was performed on lacunae in bone tissue using a light microscope. In peri-implant bone, the proportion of lacunae without osteocytes was significantly higher in Group 1 than in Group 2. This finding suggests that bisphosphonates might have only a small effect on peri-implant bone density during osseointegration after implant placement. However, bisphosphonates can increase the areas of necrosis because decalcified bone tissue is not normal. According to the definition of BRONJ established by the American Association of Oral and Maxillofacial Surgeons, patients with this condition have the following characteristics: a history of treatment with bisphosphonate, no history of radiation treatment to the jaws, and persistent exposed necrotic bone. In our animal model, no exposed necrotic bone was observed. However, Group 1 had a significantly higher proportion of lacunae without osteocytes in the bone tissue compared to Group 2. Thus, osteonecrosis related to bisphosphonate use was observed.

In the evaluation of bone density, Group 1 showed increasing peri-implant bone density over time but Group 3 showed decreasing density over time. Both groups showed an increasing proportion of lacunae without osteocytes over time.

Group 1 showed a large number of TRAP-positive cells in the early stages after the start of bisphosphonate administration. However, the number decreased as the duration of administration increased. In addition, cells became flattened. Similar findings were obtained from Group 3, and it was suspected that the activity of TRAP-positive cells decreased and their functions were inhibited. Therefore, our results suggest that bisphosphonate affects the activity of osteoclasts that accumulate in the jaw, regardless of whether bisphosphonate is administered before or after implant placement. Thus, there are concerns of negative effects on implant prognosis even after osseointegration is achieved. These effects include osteonecrosis in the peri-implant area, decreased bone density, and changes in TRAP-positive cells.

In conclusion, our study examined the effects of bisphosphonate administration in rats before and after implant placement. The evaluation was performed on H&E-stained sections and TRAP-stained sections which were examined under a light microscope. Lacunae without osteocytes in peri-implant bone and osteonecrosis were observed in Group 1 with bisphosphonate administration starting before implant placement. This histological result suggests the need to exercise caution in using implant treatment on patients taking bisphosphonates. In Group 3 with bisphosphonate administration after osseointegration, peri-implant bone density decreased over time and the number of lacunae without osteocytes increased. This result suggests the need for caution in administering bisphosphonates in patients after implant placement.

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