Experimental study of influence of direct bisphosphonate administration on bone substitute
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Articular cartilage has a low self-repair ability and natural healing cannot be expected. Treatment using various bone substitutes has been performed, but these have various disadvantages. Compared to autologous bone substitutes, the osteochondral repair ability of allogeneic bone substitutes is low, but the effective and safe utilization of these as a bone substitute may be possible by improving their osteochondral repair ability. We prepared a full-thickness osteochondral defect in the patellar fossa of rabbits, added a bisphosphonate preparation to bovine tooth-derived Demineralized Dentin Matrix (DDM), which has its own osteochondral repair ability, and investigated osteochondral repair ability in the defects. As a result, we suggest that the addition of high-dose BPs inhibits the osteochondral repair ability of DDM.

Keywords: Demineralized dentin matrix, Bisphosphonate, Osteochondral repair

INTRODUCTION
Articular cartilage has a low self-repair ability because it contains no blood, lymphatic vessels or nerves, and its dividing and proliferative abilities are poor. Accordingly, once articular cartilage is damaged by trauma or degeneration, deformation readily occurs. Particularly, when full-thickness damage of articular cartilage is not appropriately treated, secondary osteoarthritis develops, and the patient’s quality of life is markedly impaired by pain and joint dysfunction. Therefore preserving treatment to severe degenerative disorder of mandibular condyle is difficult.

Establishment of surgical joint cartilage repairing procedure is expected.

Articular cartilage of the four extremities has been treated with autologous osteochondral transplantation of an osteochondral fragment en bloc to defective regions. However, there are many disadvantages of fresh autologous bone transplantation, including surgical stress, limitation of bone graft collection, and the possibility of prolonged dysfunction of the donor region. These disadvantages may be overcome using natural bone tissue and artificial biomaterials as a bone substitute, but the biocompatibility, bioabsorbability, and osteoconductivity of these are inferior to autologous bone.

Bisphosphonate preparations (BPs) are used to treat abnormal bone metabolism, such as osteoporosis and Paget’s disease, as a potent bone resorption inhibitor. In addition, it has been reported that BP administration near the extraction socket and extraction site and below the maxillary periosteum on the opposite side improved the osteochondral repair ability of the extraction socket. However, it is unclear whether or not direct BP administration to the defective bone region exhibits a similar effect. Since serious adverse effects of BPs on localized bone defects, such as jawbone necrosis, have been reported, the systemic or extensive administration of BPs may induce clinical problems. Thus, we chose the alendronate that has high bone affinity. And it was second generation BPs acted only on osteoclasts. We chose alendronate used clinically frequently in that. In addition we investigated the direct addition of BPs to a bone substitute, implanted it into defective bone regions, and investigated the influence of the localized BPs addition on the osteochondral repair ability of the bone substitute.

We thought that we improved osteochondral repair ability of DDM by BPs inhibiting bone resorption. To investigate the influence of the direct addition of BP to a bone substitute on the osteochondral repair ability, a full-thickness cartilage defect was prepared in the osteochondral patellar fossa of the knee joint of rabbits formed by osteochondral factor similar to jaw joint. Bovine tooth-derived DDM, reported to have a high osteochondral repair ability, was transplanted with BP into the defect as a bone substitute, and the influence of direct BP administration on the osteochondral repair ability of the bone substitute was investigated.

MATERIALS AND METHODS
Forty eight 13-week-old male rabbits weighing 3.0 kg (Chubu Kagaku Shizai, Aichi, Japan) were used (48 rabbits for the control and experimental groups, respectively). The rabbits were maintained under the same conditions, and given free access to food and drinking water. This experiment was performed following the animal experiment guidelines of the School of Dentistry, Aichi-Gakuin University.

DDM was prepared from bovine lower frontal teeth
by modifying the method reported by Urist et al.\textsuperscript{13}. Bovine lower frontal teeth were extracted, adhering surrounding soft tissue and the dental pulp were removed, and the teeth were stored at −80°C for 24 h. After complete demineralization (0.6 N HCl, 1 week), the sample was washed with distilled water and defatted (chloroform/methanol, 24 h). After freeze-drying, the sample was ground using FREEZE/MILL (Spec Industries, MA, USA). The ground sample was sieved to standardize the particle size at 250–500 μm, sterilized with EO gas, and used in the experiment (Figs. 1 A and B).

Surgery was performed under general anesthesia with pentobarbital sodium injection (Somnopentyl, Kyoritsu seiyaku, Japan) at 0.5 mL/kg into the auricular vein. The knee joint region was shaved, and infiltration anesthesia with lidocaine hydrochloride (Xylocaine, Fujisawa Pharmaceutical, Japan) was applied to the surgical region. After disinfection of the surgical field, longitudinal median incisions were made in the bilateral knees, and the joint was approached through a medial parapatellar incision. The patella was dislocated, and a full-thickness defect of 5.0-mm diameter and 6.0-mm depth reaching the cancellous bone was made in the articular cartilage by penetrating the subchondral region of the femoral patellar fossa with a trephine bar (Fig. 2). The same treatment was applied to the bilateral femoral patellar fossa in each rabbit. The animals were divided into 6 groups for subsequent treatments. Group 1 \((n=8)\) was assigned for bone defects alone, Group 2 \((n=8)\) was assigned for implantation of 100 mg of DDM alone (The bone defect is 5.0 mm in diameter, 6.0 mm in depth (volume 117.75 cube mm). Bone defects was filled with DDM), and these were regarded as control groups. For the experimental groups, 100 mg of DDM combined with 1, 10, 100, and 200 μg of a bisphosphonate preparation (BP) (alendronate sodium hydrate, Teiloc, Teijin Pharma, Japan) were implanted in Groups 3, 4, 5, and 6 \((n=8\) each), respectively.

In the wound, the synovial membrane was reduced, and the epithelium was sutured with nylon thread and adhered with a surgical adhesive. After being maintained for 9 weeks after surgery, the animals were sacrificed, and the knee joints were excised for measurement.

Images of the excised rabbit knee joints were acquired at 70 kV and 55 μA using μCT scanner (SMX 225CT-SV3, Shimadzu, Kyoto, Japan). The architecture and morphology were measured in the stereoscopic images\textsuperscript{10} using analytical software (TRI/3D-BON, RATOC System Engineering, Japan), and soft and hard tissues were selected based on the CT values. Only hard tissue was extracted, the morphology was measured, and the new Bone Volume/Total Volume of the defective region \((BV/TV)\) was determined\textsuperscript{10}. In addition, the maximum vertical distance of the deepest region of the patellar fossa from its parietal region was measured as the maximum depth in 2-dimensional cross-sectional images of the knee joint.

For histological analysis, serial sections of 5-μm thickness were prepared following the standard method after the preparation of non-demineralized, ground sections (embedded in GMA resin), stained with Safranin-O, and observed under a light microscope.

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**Fig. 1** A: DDM powder after freeze-drying and passing through 250–500 μm sieve.
B: SEM image of DDM powder (100 kV×100).

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**Fig. 2** Knee joint patellar fossa in which a full-thickness bone defect was prepared (sagittal plane). Volume of defects is 117.75 cubic millimeter (diameter 5.0 mm, depth 6.0 mm). DDM is filled in all amount bone defects.
A modified version of the system described by Wakitani et al.\textsuperscript{16}) (Eclipse E600, Nikon, Tokyo, Japan). In addition, using the modified standardization scale reported by Wakitani et al.\textsuperscript{16}), the repair of defective articular cartilage tissue at 9 weeks was quantified in each group, in which a maximum score of 13 points was divided into 5 categories, and the following parameters were evaluated: Cell morphology was graded from 0 (when cartilaginous tissue was absent) to 4 points (for tissue that was normal, compared with the adjacent, uninjured cartilage). Surface regularity, proportion of the surface of the defects that smooth compared with the entire surface, was graded from 0 (when less than one-quarter of the surface was smooth) to 3 points (when more than three-quarters was smooth). Integration of donor to host adjacent cartilage was graded from 0 (a complete lack of integration, which we refer to as dissociation) to 2 points (no gap between the donor and the host cartilage). Matrix staining was graded from 0 (no metachromatic staining) to 3 points (for tissue that was normal, compared with the adjacent, uninjured cartilage). Thickness of cartilage, or the average thickness of the cartilage in the defect compared with that of the surrounding cartilage, was graded from 0 (when the average thickness of the cartilage in the defect was less than 50% that of the surrounding cartilage) to 2 points (when the average thickness was more than 81%, less than 120% that of the surrounding cartilage). But, when the average thickness was more than 120% that of the surrounding cartilage, thickness of cartilage was graded 1 point (Table 1). The obtained experimental data are presented as the mean ± standard error, and the significance of differences was analyzed using one-way layout analysis of variance (Tukey’s multiple comparison test). All statistical analyses were performed using Graph Pad Prism v.4 (Graph Pad Software, San Diego, CA, USA), and \( p<0.05 \) was regarded as significant.

### RESULTS

#### Macroscopic findings

Macroscopic findings at nine weeks after implantation are shown in Fig. 3. In Group 1, the defective bone region was concave, and the boundary with the marginal region of the bone defect was clear, seemed incompletely repair. In Group 2, almost no concavity was noted in the repaired region, and the repaired cartilage surface was smooth, seemed favorably repair. In Group 3, the cartilage surface was smooth, similarly to that in Group 2, and the boundary with the margin was unclear, seemed favorably repair. In Group 4, although the condition was inferior to Groups 2 and 3, the surface property and boundary with the defect seems to be favorably repaired. In contrast, in Group 5, the defective bone region was concave and the boundary was clear, showing incomplete repair. In Group 6, no cartilage-like tissue was noted on the superficial layer of the defective region, and repair of the defect was the poorest among all groups.

#### μCT analysis

μCT images of the region with the maximum defective width at 9 weeks after surgery are shown in Fig. 4.

<table>
<thead>
<tr>
<th>Cell morphology</th>
<th>Matrix staining (metachromasia)</th>
<th>Surface regularity</th>
<th>Integration of donor to host adjacent cartilage</th>
</tr>
</thead>
</table>
| hyaline cartilage      | normal (compared to host)       | smooth (>
\(3/4\))                         | both edges integrated                        |
| mostly hyaline cartilage| slightly reduced                | moderate (1\(2~3/4\))              | one edge integrated                           |
| mostly fibrocartilage  | significantly reduced           | irregular (1\(4~1\)2)              | both edges not integrated                      |
| mostly noncartilage    | no metachromatic stain          | severely irregular (<1\(4\))       |                                                  |
| noncartilage           |                                  |                                     |                                                  |
| Thickness of cartilage (\% ) |                                |                                     |                                                  |
|                        | 121–150                         |                                     |                                                  |
|                        | 81–120                          |                                     |                                                  |
|                        | 51–80                           |                                     |                                                  |
|                        | 0–50                            |                                     |                                                  |
| Total Maximum          | 14                               |                                     |                                                  |

A modified version of the system described by Wakitani et al.\textsuperscript{16})

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In Group 1, new bone-like tissue formation from the margin of the bone defect was noted, but no new bone had formed in the center of the defect. In Group 2, new bone-like tissue formation was noted in the center of the bone defect, but it was only slight, and a deep concavity remained. In Group 3, active bone repair was noted throughout the bone defect, and the center was sufficiently repaired. In Group 4, new bone had formed slightly in the center of the bone defect, but a deep concavity remained, showing poor bone repair. In Group 5, new bone had formed only slightly in the center of the defect, and a deep concavity remained. In Group 6, new bone had formed slightly at both ends of the defect, but no bone repair was noted in the defect.

The amounts of repaired bone in the defective region at 9 weeks after implantation are compared in Fig. 5. In Group 3, the BV/TV value was higher than that in Group 1, and showed a tendency to be higher than

Fig. 3 Macroscopic findings of femoral patellar fossa at 9 weeks after implantation.

Fig. 4 Two-dimensional μCT images acquired at 70 kV and 55 μA using an SMX 225CT-SV3 μCT scanner (Shimadzu).

Fig. 5 Comparison of the BV/TV of the defective bone region among the experimental groups at 9 weeks after implantation.
in Group 2, although the difference was not significant. In Groups 5 and 6, the BV/TV value was lower than in Groups 2 and 3.

**Histological analysis**

The amounts of repaired bone in the defective region at 9 weeks after implantation are compared in Fig. 5. In Group 3, the BV/TV value was higher than that in Group 1, and showed a tendency to be higher than in Group 2, although the difference was not significant. In Groups 5 and 6, the BV/TV value was lower than in Groups 2 and 3.

Safranin-O-stained tissues at a low magnification at 9 weeks after implantation are shown in Fig. 6. Bone repair was evaluated based on these preparations. In Group 1, trabeculae extending from both ends of the bone defect were connected, but new bone was thin in the center of the defect. In Group 2, repaired bone with the same thickness as that of the surrounding bone was noted throughout the defective bone region. In Group 3, sufficient subchondral bone was noted. In Group 4, bone repair was incomplete, and a deep concavity was present in the center of the defect. In Group 5, the thickness of new bone was thin, bone repair was slight in the center, and the trabeculae of new bone in the defect were not continuous. In Group 6, no bone repair was noted, and implanted DDM remained.

Using the modified standardization scale (Table 1), the level of cartilage repair was measured. Its quantitative evaluation is shown in Table 2. In Group 6, no cartilage regeneration was noted, being scored 0. The cartilage repair scores in Groups 1, 4, 5 and 6 were significantly lower than that in Group 2.

**Table 2  Histological scores (at 9 weeks after implantation)**

<table>
<thead>
<tr>
<th></th>
<th>Cell morphology</th>
<th>Surface regularity</th>
<th>Integration of donor to host adjacent cartilage</th>
<th>Matrix staining</th>
<th>Thickness of cartilage</th>
<th>Total score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group1</td>
<td>3.7±0.3</td>
<td>2.7±0.3</td>
<td>2.0±0</td>
<td>2.7±0.3</td>
<td>1.3±0.3*</td>
<td>12.4±1.2*</td>
</tr>
<tr>
<td>Group2</td>
<td>4.0±0</td>
<td>3.0±0</td>
<td>2.0±0</td>
<td>3.0±0</td>
<td>2.0±0</td>
<td>14.0±0</td>
</tr>
<tr>
<td>Group3</td>
<td>4.0±0</td>
<td>3.0±0</td>
<td>2.0±0</td>
<td>3.0±0</td>
<td>2.0±0</td>
<td>14.0±0</td>
</tr>
<tr>
<td>Group4</td>
<td>4.0±0</td>
<td>2.4±0.3*</td>
<td>2.0±0</td>
<td>2.7±0.3</td>
<td>1.7±0.3</td>
<td>12.8±0.9*</td>
</tr>
<tr>
<td>Group5</td>
<td>3.7±0.3</td>
<td>1.7±0.3**</td>
<td>1.0±0</td>
<td>2.4±0.3*</td>
<td>1.3±0.3*</td>
<td>10.1±1.2**</td>
</tr>
<tr>
<td>Group6</td>
<td>0***</td>
<td>0***</td>
<td>0</td>
<td>0***</td>
<td>0***</td>
<td>0***</td>
</tr>
</tbody>
</table>

Values represent the average score ±S.E.M. for each category.

*$$p<0.01$$, **$$p<0.001$$, ***$$p<0.0001$$ (vs group 2). (Tukey’s multiple comparison test)
DISCUSSION

The shape of the bone defect was set at a diameter of 5.0 mm and depth of 6.0 mm, for which natural healing of cartilage is not expected, based on studies reported by Otsuka et al.17 and Reddi et al.18,19). Regarding DDM, Urist et al. demonstrated that organic substances account for about 20% of tooth dentin in weight; the principal component is type I collagen, similarly to bone, with a trace amount of bone morphogenetic protein (BMP) having osteoinduction ability20). In addition, the presence of various bone growth factors in tooth dentin has been clarified21-24). If DDM can be prepared from an autologous extracted tooth and used as an autologous bone substitute, its biocompatibility as a transplantation material will be high, and risks of new infection and immunogenic problems, which occur after allogeneic and xenogeneic transplantsations, will decrease. Since extracted wisdom tooth and teeth extracted for orthodontic treatment could be used, this may be a superior graft material without secondary invasiveness due to harvesting25). A granular shape was adopted to increase the contact area with tissue, and the particle diameter was set at 250–500 μm, for which osteoinduction was reported25,26).

Regarding the dose of BPs, Binderman et al. (150 μg/kg)9) and Hikita et al. (1.0 mg/kg)10) investigated bone repair of defective bone regions after BP administration, and observed an increase in bone regeneration. However, since the BP administration was nearly all systemic in their studies, the level of BP reaching the defective bone region may have been low. Our experiment was performed on the assumption of local BP administration to bone defects. Thus, the BP dose was set at 1, 10, 100, or 200 μg (0.3, 3.3, 33.3, and 66.6 μg/kg, respectively). BPs cause serious adverse effects, such as renal disorder and jawbone necrosis after extraction in dental patients treated with BPs for a prolonged period, but they have a high affinity for bone tissue and exhibit a potent bone resorption-inhibitory effect on osteoclasts. We conceived this experiment based on the consideration that BPs inhibit bone resorption by acting on osteoclasts, improving the relative bone formation by osteoblasts.

Although no significant difference was noted in the group treated with 1 μg of BP combined with DDM, compared to the group treated with DDM alone, new bone slightly increased, and new bone formation decreased as the addition of BP increased in a concentration-dependent manner. In the groups treated with 100 and 200 μg of BP combined with DDM, the amount of new bone was significantly lower than that in the group treated with DDM alone. Similarly, the repaired cartilage surface was smoother in the 1 μg BP+DDM than in the DDM alone group, and no cartilage repair was noted in the high-dose group (200 μg BP+DDM group).

The reason for these results was considered to be as follows: DDM has been shown to induce the differentiation of undifferentiated mesenchymal cells into osteogenic cells and to induce them to form cartilage and bone27-31). Osteogenesis occurs in spaces between DDM particles after implantation, and DDM becomes the core of new bone and promotes its growth, with which DDM is slowly absorbed and replaced with bone through tissue collagenase28). The bone repair mechanism of DDM may have been inhibited by BP in the 100 and 200 μg BP+DDM groups. BPs have been reported to inhibit the entire function of osteoclasts, and induce osteoclast apoptosis and the inhibition of RANK expression in osteoclast precursors32). Moreover, they were reported to influence the functions of not only osteoclasts but also osteoblasts and the amount of new bone formation in other studies33,34). In the bone repair process, bone formation by osteoblasts and bone resorption by osteoclasts repeats as bone metabolic turnover35). Therefore, it is also possible that the addition of BP at high doses (100 and 200 μg) influenced osteoclasts, osteoclast precursors, and osteoblasts, and excessively inhibited bone metabolic turnover. In contrast, new bone increased in the 1 μg BP+DDM group, suggesting that this additive BP dose (1 μg) was close to the dose that reached the defective bone region in the above-mentioned studies performed by Binderman et al. (150 μg/kg)9) and Hikita et al. (1.0 mg/kg)10). The influence may have been limited to slight inhibition of osteoclasts and may have been too small to influence the entire bone metabolism at this dose.

Cartilage repair was also favorable in the low additive BP dose group, but it was markedly inhibited, similarly to bone repair, in the high additive dose group. Repair of subchondral bone, which serves as a scaffold for cartilage regeneration, may have been inhibited by the addition of high-dose BP.

CONCLUSIONS

To investigate the influence of BPs directly added to a bone substitute on osteochondral repair ability, full-thickness defects were prepared in the patellar fossa of rabbits, bovine tooth-derived DDM combined with BP was implanted in the defects, and the osteochondral regeneration ability was investigated.

1. When 100 mg of DDM combined with 1 μg of BP was implanted into full-thickness defects in the knee joint patellar fossa, new bone formation increased compared to that in the group of bone defects alone, and showed an increased tendency compared to that in the group implanted with DDM alone.

2. When 100 mg of DDM combined with 100 and 200 μg of BP was implanted, osteochondral regeneration was inhibited in the 100 μg BP-added group, and repair of the bone defect was markedly inhibited in the 200 μg BP-added group.

We suggest that the addition of high-dose BPs inhibits the osteochondral repair ability of DDM, and attention should be paid when BPs are used to treat regions requiring bone repair.
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REFERENCES