TRAUMA: RESEARCH

Effects of the systemic administration of alendronate on bone formation in a porous hydroxyapatite/collagen composite and resorption by osteoclasts in a bone defect model in rabbits

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Several bisphosphonates are now available for the treatment of osteoporosis. Porous hydroxyapatite/collagen (HA/Col) composite is an osteoconductive bone substitute which is resorbed by osteoclasts. The effects of the bisphosphonate alendronate on the formation of bone in porous HA/Col and its resorption by osteoclasts were evaluated using a rabbit model. Porous HA/Col cylinders measuring 6 mm in diameter and 8 mm in length, with a pore size of 100 μm to 500 μm and 95% porosity, were inserted into a defect produced in the lateral femoral condyles of 72 rabbits. The rabbits were divided into four groups based on the protocol of alendronate administration: the control group did not receive any alendronate, the pre group had alendronate treatment for three weeks prior to the implantation of the HA/Col, the post group had alendronate treatment following implantation until euthanasia, and the pre+post group had continuous alendronate treatment from three weeks prior to surgery until euthanasia. All rabbits were injected intravenously with either saline or alendronate (7.5 μg/kg) once a week. Each group had 18 rabbits, six in each group being killed at three, six and 12 weeks post-operatively. Alendronate administration suppressed the resorption of the implants. Additionally, the mineral densities of newly formed bone in the alendronate-treated groups were lower than those in the control group at 12 weeks post-operatively. Interestingly, the number of osteoclasts attached to the implant correlated with the extent of bone formation at three weeks.

In conclusion, the systemic administration of alendronate in our rabbit model at a dose-for-weight equivalent to the clinical dose used in the treatment of osteoporosis in Japan affected the mineral density and remodelling of bone tissue in implanted porous HA/Col composites.

A wide variety of bone substitutes have been developed that include sintered calcium phosphates and their composites, with their clinical use having increased over the past decade.1 Recently, other types of bone substitutes have also been developed, including a hydroxyapatite/collagen (HA/Col) composite.2,3 In contrast to sintered hydroxyapatite (HA), which is barely resorbed after implantation, HA/Col is resorbed by osteoclast-like cells and is involved in natural remodelling of bone. The coupling mechanisms between osteoblasts and osteoclast-like cells are thought to contribute to this material's osteoconductibility. The porous nature of HA/Col has a sponge-like elasticity and affords excellent biocompatibility and osteoconductibility.4,5 It has a large surface area of 75 m²/g owing to its composition of 80% nanoscale HA crystals enabling it to adsorb a large quantity of proteins, such as growth factors, and other molecules, as well as drugs and ions.3,6

Bisphosphonates are synthetic analogues of pyrophosphate, and were first synthesised in 1865 and used in industry as a corrosion inhibitor.7,8 Etidronate was the first bisphosphonate to be used clinically in the treatment of myositis ossificans.7 In 1976, the effectiveness of etidronate for the treatment of osteoporosis was reported, although the biological mechanisms had not been clarified.9 Subsequently, the mechanism of action of bisphosphonate was established, permitting the development of other bisphosphonates,
including alendronate, which is more effective than first-generation bisphosphonates in the treatment of osteoporosis. Bisphosphonates have a high affinity for HA and are adsorbed onto the HA contained in bone after they are administered. When osteoclasts dissolve HA and resorb bone, bisphosphonates are released and taken up by the osteoclasts to initiate apoptosis, thereby leading to decreased bone resorption.\textsuperscript{10-14} Administered bisphosphonates are also adsorbed onto the HA surfaces of implanted porous HA/Col. Whereas the HA nanocrystals contained in natural bone are bound to each other so compactly that bisphosphonates adsorb only onto HA at the surface of the bone,\textsuperscript{15,16} the HA nanocrystals in HA/Col are not bound to each other as tightly. This permits bisphosphonates to penetrate into the material through the numerous pores in the HA/Col fibres and water. The pore size ranged from 100 $\mu$m to 500 $\mu$m and was measured using the intercept method. Owing to the preparation method for the ice crystals, there was some unevenness in the distribution of pore sizes and shapes among the implants, which could not be evaluated quantitatively.

**Materials and Methods**

HA/Col nanocomposite fibres were synthesised from atelocollagen derived from porcine skin, Ca(OH)$_2$, and $\text{H}_3\text{PO}_4$ using a co-precipitation method described previously.\textsuperscript{2} In brief, atelocollagen (Nitta Gelatin Co., Osaka, Japan) was dissolved in an $\text{H}_3\text{PO}_4$ solution. Both the dissolved collagen and a Ca(OH)$_2$ suspension were added to distilled water and placed in a water bath that was thermostatically stabilised at 40°C. The speed of each addition was controlled to maintain the pH at 9.0. The initial materials were measured so that the final HA/Col nanocomposites would have an 80:20 weight-to-ratio composition. The HA/Col fibres were lyophilised to prepare porous HA/Col implants. Porous HA/Col implants were produced by homogenising the HA/Col fibres (1 g) with 6.5 ml of phosphate-buffered saline and alkaliising with 50 $\mu$l of a 1 M sodium hydroxyapatite solution. The resultant solution was mixed with 1.5 ml of a 0.6% collagen solution dissolved in phosphoric acid (pH 2.0). The mixture, now at pH 7.0, was infused into a mould. In order to initiate the gelation of the collagen as a binder, the mould containing the mixture was incubated at 37°C for two hours. The HA/Col gel produced was frozen at -60°C for two hours. The HA/Col gel produced was frozen at -60°C, and the liquid within the mixture formed ice crystals in the gel. The spaces occupied by the ice crystals were converted into pores by subsequent lyophilisation. The lyophilised porous HA/Col constructs were cross-linked by thermal dehydration at 140°C under vacuum, cut into cylindrical shapes 6 mm in diameter and 8 mm long, and sterilised by irradiation (Fig. 2). The porosity of the implants was 95%, as calculated from the compositional ratio of the HA/Col fibres and water. The pore size ranged from 100 $\mu$m to 500 $\mu$m and was measured using the intercept method. Owing to the preparation method for the ice crystals, there was some unevenness in the distribution of pore sizes and shapes among the implants, which could not be evaluated quantitatively.

**Surgical Procedure.** The experiment was conducted using 72 three-month-old male Japanese white rabbits. Their mean weight was 3.15 kg (2.8 to 3.3) (CLEA Japan Inc., Tokyo, Japan), and the study was approved by the Animal Committee of Tokyo Medical and Dental University. The rabbits were anaesthetised by an intramuscular injection of medetomidine sodium (Domitor, 0.5 ml/kg; Nippon Zenyaku Kogyo Co. Ltd, Fukushima, Japan) and ketamine hydrochloride (Ketalar, 0.5 ml/kg; Daiichi Sankyo Co. Ltd, Tokyo, Japan). Under sterile conditions, an incision was made in the lateral aspect of the right distal femur. The periosteum was detached and a full-thickness unicortical and trabecular bone resection was performed on the lateral condyle using a 5 mm diameter trephine drill with continuous saline irrigation to prevent thermal necrosis of the margins. After the drilling, the debris and remnants of the bone marrow were removed by flushing the area with saline, and the implants were inserted into the bone bed that had been created.
Injection protocol. The total experimental period was 15 weeks and consisted of a three-week ALN pre-treatment period and a 12-week post-operative observation period. The rabbits were divided randomly into four groups (n = 18), a control group and three systemic alendronate sodium hydrate (ALN; Teijin Pharma Ltd, Tokyo, Japan) administration groups. The three active groups were the 'pre' group, which received ALN pre-treatment for three weeks before surgery, the 'post' group, which received ALN treatment beginning post-operatively until euthanasia, and the pre+post group, which received continuous ALN treatment from three weeks before surgery until euthanasia. All rabbits received either 0.9% saline or ALN (7.5 μg/kg) intravenously once a week throughout the experimental period (Fig. 3). The dose of alendronate was adjusted according to the usual dose for the treatment of osteoporosis in Japan (35 mg/week) and the extent of bioavailability.27 A total of six rabbits from each group were killed at three, six and 12 weeks post-operatively, and the right distal femora were collected and cleansed of soft tissues. These bone specimens were fixed in 4% paraformaldehyde and 0.025% glutaraldehyde for one week at 4°C.

Three-dimensional micro-CT analysis of bone. Imaging of the femora was performed using a micro-CT apparatus (Scan Xmate-E090; Comscan Techno Co., Kanagawa, Japan), and three-dimensional (3D) micro-CT images were analysed using image analysis software (TRI/3D-BON; Rato System Engineering Co., Tokyo, Japan). The mineral density of the extracted bone tissue and the region of implantation were calculated. Briefly, the evaluation zone was defined as the implanted cylindrical site without a cortical aperture, of which both the diameter and the length were 6 mm. The mineral density of each voxel in the area (Fig. 4) was calculated from the CT Hounsfield number using a standard curve. Before analysis, the mineral densities of porous HA/Col pieces, excluding the pores, were found to be uniformly 220 mg/cm³. Accordingly, in order to identify which voxels represented bone tissue, a mineral density threshold was set at 400 mg/cm³, and the area above the threshold was defined as bone tissue (Fig. 4).

Histological examination. The dissected distal femora were decalcified in 20% ethylenediaminetetra-acetic acid, dehydrated with a gradient ethanol series, and embedded in
paraffin. Based on macroscopic observation, coronal sections 5 μm thick, including the centre of the implant area, were prepared. Haematoxylin and eosin (H&E) staining was used to quantify the residual HA/Col area and the bone-tissue area; tartrate-resistant acid phosphatase (TRAP) staining was used to quantify the number of osteoclasts present. The border between the residual HA/Col and the surrounding bone tissue was clearly recognised because residual HA/Col was easily identified by its distinctive layer structure and staining intensity which is lower than bone in H&E-stained sections. The residual HA/Col area, bone-tissue area and TRAP-positive cell number were measured using ImageJ software (NIH, Bethesda, Maryland).

Statistical analysis. In each experiment, the overall differences between groups were determined by two-factor analysis of variance (ANOVA), and Pearson’s product-moment correlation coefficient (r) was used to study the relationship between the number of osteoclasts and the area of bone formation. Statistical significance was set at p < 0.05.

Results
Micro-CT analysis. Tissue from the cylindrical implant area with mineral density > 400 mg/cm³ was identified as bone and was used for the analysis. The mineral densities of the extracted material, which are thought to correlate with the maturity and quality of the bone formed, are shown in Figure 5. Although the overall differences between the groups were not significant (ANOVA, p = 0.077), the mean density of the control group tended to be higher than that of the other groups at six weeks; however, there was considerable variability in the individual samples. In addition, the mean densities of the control and pre groups also tended to be higher than those of the post and pre+post groups at 12 weeks. The total mineral content, defined as the product of the mineral density and bone volume, was also calculated. There were no significant differences between the groups, and the content values were highly variable (p = 0.268).

Histological evaluation. Figure 6 shows representative H&E-stained coronal sections. At three weeks after surgery, the implant structures in every group were generally well-defined shapes, containing inconsistent pore shapes. Active bone formation and formed bone were observed adjacent to the implants and inside the pores, although there were individual differences, which might be due to the irregular surfaces of the implants rather than to differences between the groups. At six weeks the areas of the implants in each group were reduced, as also was the area of the formed bone, especially in the ALN-treated groups, compared with the results at three weeks. In particular, in the post and pre+post groups, the exposed remnants of the implant without surrounding bone tissue were more numerous. At 12 weeks, the residual HA/Col became smaller and more scattered in the implant areas. In the control group most of the remnants were surrounded by formed bone and active osteoblasts were still attached to some of the surrounding bone. In the post and pre+post groups, not only were the residual implants larger than in the control group, but the amounts of remnants exposed directly to bone marrow without surrounding bone tissue were larger than that in the control group (Fig. 6).

Quantitative evaluation of histological findings. In a coronal section at the centre of the implant, the areas of residual HA/Col (Fig. 7) and the areas of bone tissue in the bone marrow cavity were measured. The areas of HA/Col decreased over time, especially after six weeks. Although the overall differences between the groups were not significant (ANOVA, p = 0.115), the residual HA/Col area of the
control group tended to be lower throughout the experimental period than in the other groups, in which the HA/Col areas were almost equivalent to every time point. The measurement of the bone-tissue area varied widely in each sample, as shown in the micro-CT analysis of the total mineral content.

In TRAP-stained sections, there were large numbers of TRAP-positive osteoclasts (Fig. 8a). Most of the TRAP-positive cells were not attached to bone but instead to the implant throughout the experimental period, but the number of cells varied widely in each section. Figure 8b shows the ratio of the mean number of osteoclasts attached directly to the HA/Col material to the total length of the edge of the implant. At six weeks, despite the increased ratio in the control group, the continuously ALN-treated groups did not show increases in this ratio. However, the overall difference between the groups was not statistically significant (ANOVA, p = 0.184) and the ratios varied extensively, especially in the control group and the ALN pre-treatment group. Correlations between the bone area and number of osteoclasts at three weeks, regardless of the group, are shown in Figure 8c. A relatively high statistically positive correlation was observed (r = 0.691).

Discussion

We evaluated the effect of ALN on bone formation in the presence of a porous HA/Col implant. The amount of ALN administered to the rats in this study was chosen to represent the dose for weight equivalent for patients being treated for osteoporosis in Japan (35 mg/week). This dose was selected to allow extrapolation of the effects of the clinical use of bisphosphonate after the implantation of bone substitutes. It should be noted that in most other countries doses twice as large (70 mg/week) are used.

The mineral densities in the post and pre+post groups tended to be lower than those of the control and pre group at 12 weeks. Bisphosphonates are synthetic analogues of pyrophosphate, which has an affinity for HA and inhibits mineralisation. Therefore, first-generation bisphosphonates strongly inhibit mineralisation and have been used as anti-calcification agents. ALN is a second-generation bisphosphonate which has little anti-calcification effect at the dose used to treat osteoporosis, but at much higher doses does exert an anti-calcification action. However, in this study, the dose of ALN reduced the mineral density of the bone tissue formed at the implant site. The porous HA/Col of the ALN-treated groups were thought to contain ALN at high densities, and the drilling of a hole and the implantation of HA/Col induced a higher bone turnover as a reaction to the implantation itself. In general, bisphosphonates tend to deposit bone tissue with vigorous bone turnover. These facts suggest the possibility that local redistribution of the ALN occurs as a result of the HA/Col being resorbed by osteoclasts to the newly formed bone, producing a higher concentration of ALN than appropriate for the treatment of osteoporosis. As a result, not only would the osteoclast activity and related osteoblast activity be inhibited by the ALN in the implant, but the ALN
accumulating in the new bone would prevent mineralisation, and thereby the mineral density might be reduced.

The quantitative analysis of the HA/Col remnant revealed an inhibitory effect of ALN on osteoclast-induced resorption of HA/Col. Analysis of the ratio of osteoclast numbers to the length of the HA/Col surface showed no increase at six weeks after surgery in the continuously ALN-treated groups, although the control and pre groups showed relatively high ratios. These results suggest that systematically administered ALN inhibits osteoclast activity and resorption of the transplanted HA/Col.

At three weeks, most of the osteoclasts at the transplanted site were attached to the HA/Col and actively resorbing it. In contrast to the HA/Col resorption, the number of osteoclasts attached to the bone tissue of the transplanted site was very small, and the bone tissue resorption by the osteoclasts was negligible at three weeks postoperatively. Therefore, the bone volume at the transplanted site at three weeks is thought to be an indicator of the bone formation activity of the osteoblasts, and the strong correlation between the number of osteoclasts attached to HA/Col and the area of bone-tissue suggests coupling.
mechanisms between osteoblasts and the osteoclasts that resorb HA/Col. HA in the bone tissue has an affinity for a wide variety of molecules, including growth factors, which can affect bone remodelling. Therefore, the following mechanism is thought to be involved in bone remodelling. HA is a reservoir of growth factors, which are released when the HA is resorbed by osteoclasts, thereby affecting bone remodelling. The importance of this mechanism in natural bone has recently been questioned. The HA in porous HA/Col also has the same affinity for such molecules, and the adsorptive area of HA/Col is larger than that of natural bone, as stated above. Therefore, this osteoclast-osteoblast coupling mechanism might not be negligible at HA/Col transplanted sites.

In conclusion, our data suggest that the systemic administration of ALN affects the mineral density and remodelling of bone tissue formed at the implant site of HA/Col, although a statistically significant difference was not demonstrated in our model when using a dose representing the dose used in the treatment of osteoporosis in Japan. This study suggests that it might be beneficial to suspend the administration of bisphosphonates after the implantation of HA/Col or other bioabsorbable bone substitutes, at least until the implants are resorbed, although further studies are needed to understand this process better.

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References