The Influence of Alendronate on Bone Formation and Resorption in a Rat Ectopic Bone Development Model

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Background: Most bone grafting techniques that include bone marrow, alloplastic materials, and extracellular bone matrix produce new bone mass, filling bone defects unpredictably. In most cases, the new bone undergoes resorption due to low local strains, resulting in significant bone loss. Recently, it was shown that alendronate and other bisphosphonates reduce bone loss when administered systemically or locally. The aim of this study was to investigate whether alendronate is effective on bone formation or bone resorption.

Methods: A total of 64 rats were divided into 2 main groups. In all the rats, fresh bone marrow removed from DA young rats was placed into demineralized rat femur cylinders (DBMC) and implanted into subcutaneous sites of host DA rats, to form new bone. Group A served as an alendronate treatment group, and group B served as a non-treated control. Group A received 100 µl of 1.5 mg/ml alendronate solution at 1, 2, and 3 weeks (group A1) and at 3, 4, and 5 weeks (group A2). At designated times, the rats were sacrificed, and the implanted DBMC was dissected out of the thorax and processed for histological and microradiography image analysis.

Results: Alendronate given at 1, 2, and 3 weeks (during the bone formation phase) did not increase the amount of bone or the visual bone density in comparison to the time-matched control, after 4 and 8 weeks. When alendronate was injected at 3, 4, and 5 weeks, the bone mass increased by 70% and by 166% after 6 and 10 weeks, respectively, in comparison to the untreated control. The visual bone density in group A2 was maintained at the level of 140 ± 15 at 6 weeks and 152 ± 15 at 10 weeks. The matched, non-treated control group B2 was significantly lower, 106 ± 20 and 108 ± 15, respectively. The histological sections showed that alendronate treatment at 3, 4, and 5 weeks maintained the normal appearance of the ossicle at 6 and 10 weeks in comparison to the osteopenic bone appearance in the matched controls.

Conclusions: This study suggests that alendronate is effective in inhibiting bone loss, but ineffective during the bone formation phase. We suggest, therefore, that alendronate should be administered in procedures where bone resorption is expected. *J Periodontol 2003;74:44-50.*

KEY WORDS

Alendronate/therapeutic use; bisphosphonates; bone loss/prevention and control; bone marrow; bone reconstruction; bone regeneration; bone resorption/prevention and control.

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Bone regeneration, replacement, and reconstruction are needed for therapy of numerous clinical conditions of bone loss. The regeneration of injured or excised bone tissue is comprised of a complex sequence of events that begin with the recruitment, attachment, and proliferation of progenitor cells, followed by cell differentiation into appropriate phenotypes that are capable of restoring the damaged tissue.[1,2] Bone marrow and autologous bone are considered to be the main source for osteoprogenitor cells and may contribute to repair of bone defects in periodontal and other regeneration procedures.[3-6] Normally, bone marrow cells can differentiate into the hematopoietic lineage and to the stroma cell lineage. Furthermore, the stroma lineage has the ability to differentiate into fibroblasts, adipocytes, myoblasts, chondroblasts, and osteoblasts.[7-9]

Most of the bone grafting techniques that include bone marrow, alloplastic materials, and extracellular bone matrix produce new bone mass, filling bone defects unpredictably. Nevertheless, the reconstruction of bone is not fully accomplished by merely establishing bone mass. For long-term success, the newly formed bone has to be implemented into the normal function of the surrounding tissues. In most cases, the new bone undergoes re-

sorption due to low local strains, resulting in significant bone loss.

Recently, it was shown that alendronate and other bisphosphonates reduce bone resorption when administered systemically or locally.[10-12] Their biological effects are attributed mainly to their incorporation in bone, enabling direct interaction with osteoclasts and osteoblasts through a variety of biochemical pathways.[13-15] While their effect on reducing bone resorption is well documented,[16,17] their effect on bone formation is still uncertain.

We have recently employed a modified procedure for the development of ectopic bone in growing rats. This procedure was previously described by Nimni et al.[18] In brief, demineralized rat bone cylinders (DBMC) are filled with fresh full marrow, which is removed from femurs and tibias of donor rats and implanted at thoracic subcutaneous sites of DA young host rats. In this model system, the transplanted marrow develops into bone tissue, filling the space of the DBMC during 3 to 4 weeks. The "ossicle"-like structure includes trabecular bone surrounded by cortical bone. New hematopoietic marrow occupies the intertrabecular spaces. Our previous observations[19] have revealed that the newly formed bone undergoes gradual resorption due to low local strains, losing up to 80% of its peak bone mass 6 to 8 weeks later. We could therefore distinguish between the bone formation phase, reaching its peak at 4 weeks, and the subsequent resorption phase.

The aim of this study was to investigate whether alendronate is effective in the bone development phase or in the bone resorption phase. The bone mass was estimated from contact microradiographic films of the harvested DBMC ossicles. Histology sections of the ossicles were examined for cell and matrix composition.

MATERIALS AND METHODS

The research was carried out in accordance with the Helsinki Accord at the animal care unit of the Faculty of Medicine, Tel Aviv University. The experimental model is based on the ability of fresh bone marrow to form bone in DA rat thorax subcutaneous sites. The fresh bone marrow is removed from DA young rats, placed into demineralized rat femur cylinders (DBMC), then implanted into subcutaneous sites of host DA rats. The DA rats consistently formed bone tissue in this model.

Preparation of DBMC

Following sacrifice of 3-month-old (240 to 280 g) DA rats by CO_2, the femurs were removed and cleaned of tendon and muscle. The metaphyses were cut off, and the medullary tissue was removed using dental endodontic reamers. Most of the cortical bone was left, revealing a cylinder shape. The bone cylinders were then rinsed for 15 minutes in distilled water, followed by their immersion in 0.6 N HCl solution for 48 hours. Twenty to 30 cortical bone cylinders were placed in 40 ml of HCl solution and slowly stirred. After demineralization of the cylinders was completed, the HCl solution was decanted and the bones were rinsed 2 to 3 times in 200 ml of sterile water and immersed in 60 ml of sterile saline for 60 minutes. Then, the saline was replaced by 70% ethyl alcohol. Prior to their implantation, the alcohol was washed out with sterile phosphate buffered saline solution.

Experimental Procedure

Prior to implantation of the fresh marrow, the DBMC were washed with sterile PBS and put on sterile gauze to soak most of the PBS solution. The DBMC were cut to get sections 7 to 8 mm long. The inner diameter of the DBMC was 3 to 4 mm.

The bone marrow donor DA rats were sacrificed using CO_2, and the femurs and tibias were bluntly dissected out. The epiphysis ends were excised using sharp scissors. The marrow tissue was pushed out using a blunt trocar. The bone marrow thus harvested was packed into the DBMC to be readily implanted in thoracic subcutaneous sites of 2-month-old host DA rats. The DBMC ends were not sealed and were open to interaction with the host.

The male DA rats in whom the cylinders were implanted were anesthetized prior to surgery using a mixture of 25 mg/kg body weight of ketalar§ and 42 mg/kg body weight of xylazine‖ injected intraperitoneally (i.p.). The thorax area was shaved, and the skin was gently cleaned using an antiseptic solution.

A small cut was made in the skin just above the lower border of the ribs, using surgical scissors. A blunt dissection of soft tissues created a space between subcutaneous tissue and thorax muscles, allowing the placement of 2 DBMC apart from each other, on both sides of the thorax far from the incision.

A total of 64 rats received 2 implanted cylinders each. The rats were divided into 2 groups, which were again subdivided in a randomized fashion. Group A served as an alendronate treatment group, and group B served as a non-treated (control) group (32 rats in each experimental group). Rats received the i.v. injection via the dorsal vein of the penis.

Group A was treated by i.v. injection of 100 µl of 1.5 mg/ml alendronate solution (0.5 mg/kg body weight) in the following time intervals: Group A1 received alendronate by i.v. injection at 1, 2, and 3 weeks following implantation; group A2 received alendronate injections at 3, 4, and 5 weeks following implantation (Fig. 1).

Group B, the non-treated group, received saline injection by i.v. at the same time intervals as group A and were sacrificed at the same times as groups A1

§ Malgene 1000, Rhone Merieux, Lyon, France.
‖ Rampun, Bayer, Leverkusen, Germany.

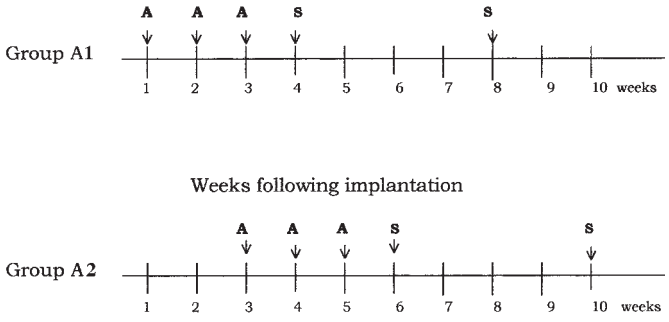

Figure 1.
Flow chart of study design and experimental groups. A = alendronate i.v. administration; S = sacrifice of animals.

and A2 (Fig. 1). Group B was subdivided into groups B1 and B2.

At designated times (Fig. 1), the rats were sacrificed by CO_2, and the implanted DBMC were dissected out of the thorax and fixed in 4% buffered formalin. Half of the rats in group A1 and B1 were sacrificed 4 weeks following implantation, and half were sacrificed after 8 weeks. In groups A2 and B2, half of the rats were sacrificed after 6 weeks, and half of the rats after 10 weeks (Fig. 1).

High-resolution x-ray microradiography analysis of the cylinders was performed no later than 24 hours after fixation. The x-ray analysis was performed using safety film in a cabinet x-ray system for 5 seconds at 20 kV(p). An aluminum gradient scale was attached to each of the x-ray films for standard. The DBMC specimens were put on the film to contact its widest surface. The high-resolution microradiographs were then scanned, image processed, and analyzed for amount of bone mass and its mineralized content.[10,11]

The microradiographs were scanned at a resolution of 1,200 × 1,200 DPI, and 256 gray level per pixel. The gray level range between 120 and 240 was determined by scanning trabecular bone of normal male DA rat femurs, and was found to be similar to ectopic bone ("ossicle") formed in DBMC in this model (Fig. 2). We measured in pixels the area of the mineralized bone relative to the total area of the DBMC cylinder, expressed as percentage of mineralized bone mass. The mean gray level of the DBMC was considered as the visual mean bone mineral density of the ossicle.[20]

Histological sections were prepared and stained by hematoxylin and eosin. Three to 4 sections were cut and stained through the long axis of the DBMC. Analysis of tissue on the outer surface of the DBMC and the tissue content inside the DBMC was performed. Light microscopy semiquantitative observations were described by at least 2 independent observers. Cortical and trabecular bone, osteoblasts, resorptive surfaces, and the cellular content of the marrow tissue were evaluated in the treated and non-treated controls.

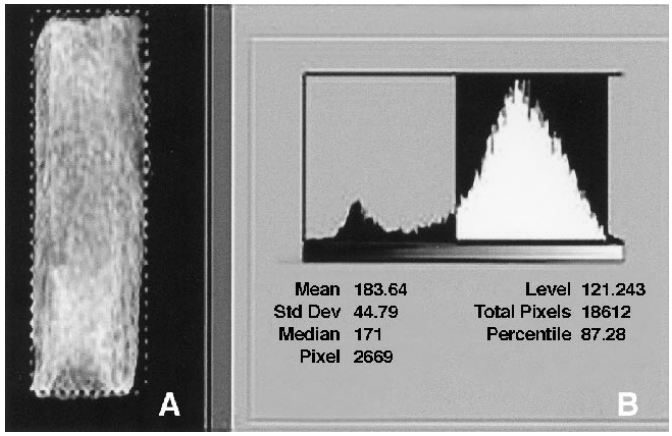

Figure 2.
*A typical bone mass histogram **(B)** of a DBMC microradiography 4 weeks after implantation **(A)**, demonstrating the bone mass surface area (in white) and its mineralized content surface area (in pixels and percent of total area).*

Statistical Analysis

Data are presented as the mean ± standard deviation from 2 experiments. Differences in means between the groups were analyzed by 2-way analysis of variance (ANOVA), followed by Scheffe's comparison test. Significance was determined at a $P<0.05$ level or greater.

RESULTS

In the present experiments, we investigated the effect of alendronate on the amount of bone, its mineral content, and its structure in an ectopic bone development model. Normally, an ossicle was formed in a thoracic subcutaneous site 3 to 4 weeks after implantation (group A1 and group B1, Fig. 2A). The mineralized surface area as depicted from the microradiograph relates to the amount of bone, expressed in pixels, and the mean visual mineral density, expressed in level of gray scale between 120 and 240 (Fig. 2B). This range of gray level was measured in normal trabecular bone of rat femurs. The ossicle consisted of cortical bone at the periphery and many trabeculae interconnected, with extensive areas of active bone formation surfaces as seen in most of the histology sections, 3 to 4 weeks after marrow implantation (Fig. 3). Figure 4 shows cortical bone rich with osteocytes at the periphery of the ossicle and trabecular bone surrounded by hematopoietic marrow, with only a few areas of fat cells. Numerous blood vessels were seen in the marrow spaces and in the bone matrix. The trabeculae were covered with cuboidal osteoblasts secreting new matrix on many of its surfaces (Fig. 4).

Four weeks after implantation, the mean amount of bone of the non-treated DBMC was 2,783 ± 290 count pixels, which is 93 ± 7% of the total surface area, as measured from the microradiographs. The mean visual

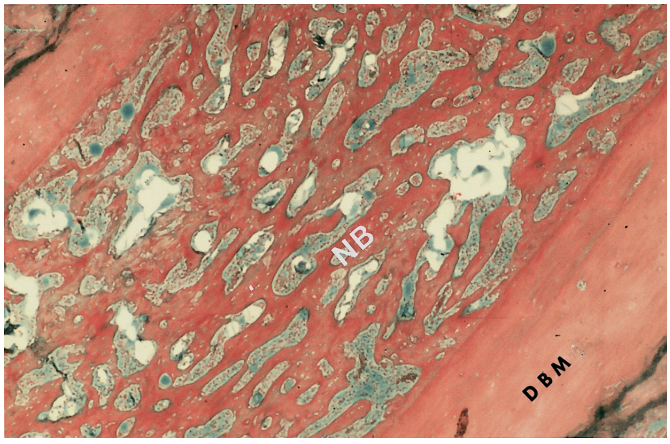

Figure 3.
Histological section of DBMC ossicle 4 weeks after implantation. DBM is the residual demineralized bone matrix (non-viable). NB is the newly formed bone (hematoxylin and eosin; original magnification ×4).

mineral density was 150 ± 17 gray levels (Table 1). Alendronate i.v. injections after 1, 2, and 3 weeks did not significantly change the relative bone content or the visual mineral density (BMD) at 4 weeks (group A1) in comparison to non-treated controls (group B1) (Table 1).

After 4 weeks, the bone that reached maximum content and density underwent a resorption phase most probably due to lack of mechanical stimulation of the ossicle.[22] At 6 weeks, the mineralized area decreased dramatically by 50% (P <0.001) in comparison to 4 weeks, and was gradually reduced after 8 weeks and 10 weeks (Table 1) in the non-treated controls. In the histological sections of the ossicle, osteopenic bone (Fig. 5) concurred with the microradiographic estimations (Table 1). The trabeculae were sparse, small, and thin, and they lost their connectivity (Fig. 5). The marrow became fibrous and fat tissue was prominent. Few active sites of bone formation were observed; however, resorption surfaces with osteoclasts were dominant (Fig. 6). It seems that the unstrained newly formed ossicle underwent a rapid and prominent resorption phase (groups B1 and B2).

In the present study, alendronate treatment given during the bone formation phase (Group A1) did not significantly affect bone loss in comparison to untreated controls tested at 4 and 8 weeks after implantation, indicated by relative bone content and by visual bone mineral density (Table 1). Histological observations support the x-ray analyses. In contrast, in another set of experiments where alendronate was injected i.v. at 3, 4, and 5 weeks after marrow implantation (group A2), bone loss was significantly reduced (Table 1, experimental group at 6 and 10 weeks). The amount of mineralized bone was higher by 70% and 166% after 6 and 10 weeks, respectively, in comparison to the

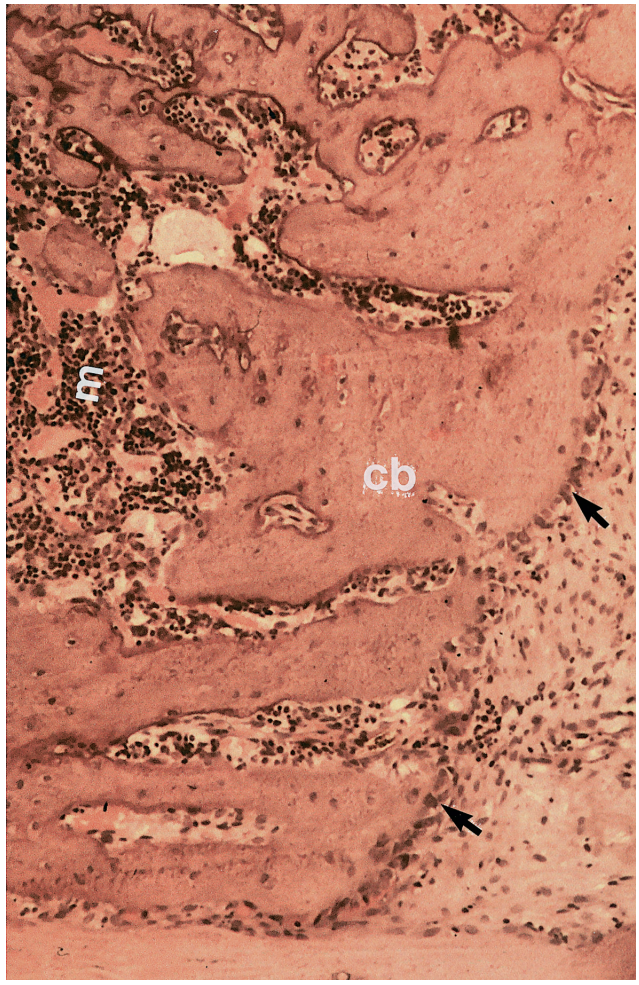

Figure 4.
Histological section at the periphery of DBMC ossicle, showing part of new cortical bone (cb), and trabecular bone extending from the cortical bone and surrounded by new hematopoietic marrow (m). Osteoblasts (arrows) line the bone surface (H & E; original magnification ×10).

non-treated controls. Also, the visual bone mineral density (BMD) was significantly higher than in the non-treated controls (Table 1). The histology sections of alendronate-treated rats (group A2) showed a normal pattern of bone similar to that observed after 4 weeks of implantation. Interestingly, the marrow spaces in this group were filled with active hematopoietic marrow, reminiscent of normal bone marrow.

DISCUSSION

The present paper describes a system for bone development and its remodeling at ectopic subcutaneous thoracic sites. We used fresh marrow removed from DA rat femurs, placed into a cylinder DBMC, and immediately implanted in another rat as described by Nimni et al.[18] We have shown that an ossicle of mineralized bone develops in the growing rat, with all the characteristics of long bone without the epiphyseal

Table 1.

Effect of Alendronate on Amount and Density of Ectopic Bone

Period of Implantation	Relative Bone Content (% of surface area)			BMD (gray level)		
	Control	Experimental	Significance	Control	Experimental	Significance
4 weeks	93 ± 6[B1]	87 ± 7[A1]	N.S.[X]	150 ± 17	144 ± 19	NS[X]
6 weeks	44 ± 6[B2]	74 ± 8[A2]	P <.001[Y]	106 ± 20	140 ± 15	*P* <0.01[Y]
8 weeks	31 ± 3[B1]	33 ± 2[A1]	N.S.[X]	114 ± 12	122 ± 15	NS[X]
10 weeks	24 ± 6[B2]	64 ± 9[A2]	P <.001[Y]	108 ± 15	152 ± 15	*P* <0.01[Y]

X = group B1 saline only, and group A1 alendronate treatment at 1, 2, and 3 weeks.
Y = group B2 saline only, and group A2 alendronate treatment at 3, 4, and 5 weeks.
BMD = visual bone mineral density.
NS = not significant.

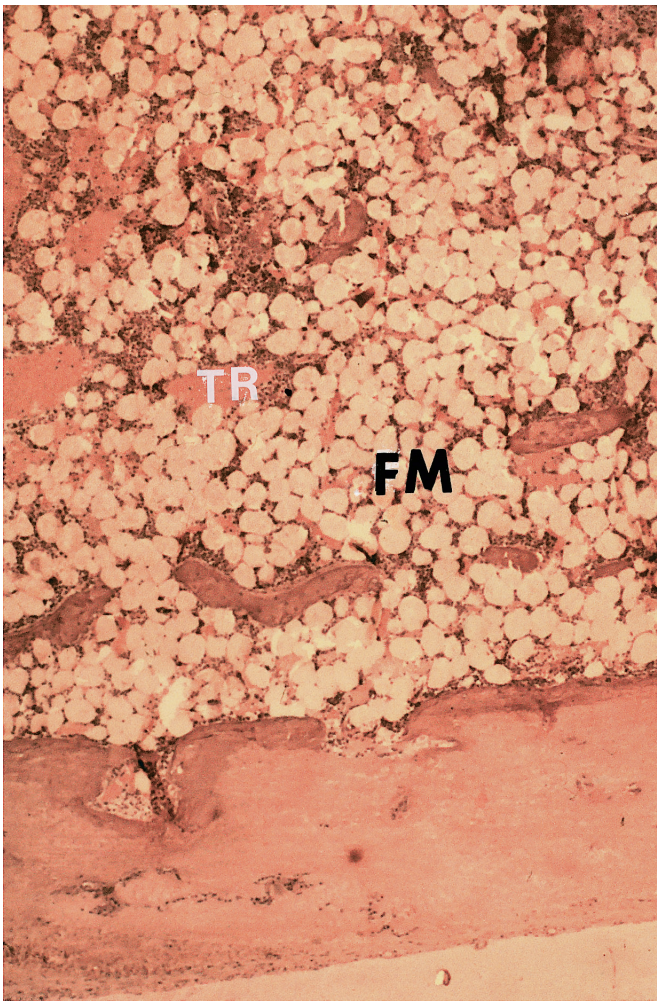

Figure 5.
Histological section of DBMC ossicle 8 weeks after implantation. Most of the area is occupied by fat marrow (FM). Small trabeculae that lost their connectivity can be seen (TR) (H & E; original magnification ×10).

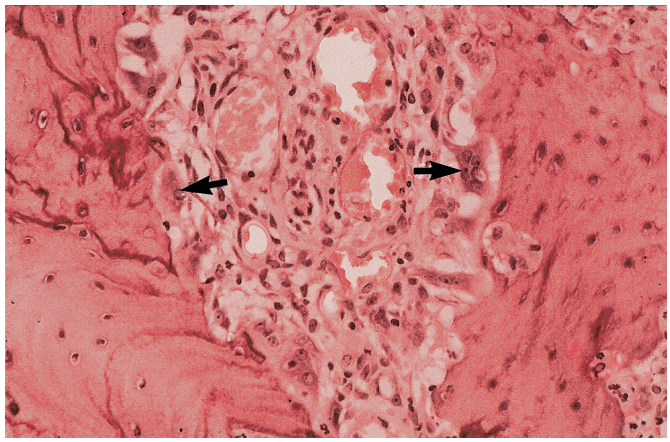

Figure 6.
Active osteoclasts (arrows) are visible on bone surface areas undergoing resorption (H & E; original magnification ×10).

cartilage plate. The ossicle reaches its maximal bone mass during 4 weeks. After 4 weeks, probably because of unstrained conditions, the ossicle undergoes remodeling with dominant bone resorption.[17,20-22] We have therefore employed this model to study the effect of alendronate on bone mass and morphology when administered during bone development or during the bone resorption phase. The results revealed that alendronate effectively reduced bone resorption when given subsequent to bone development. Alendronate did not increase the amount of bone mass in comparison to the corresponding non-treated rats, when the drug was delivered during the bone development period of the first 3 weeks. It is possible that alendronate given at doses presented in this study (0.5 mg/kg injected i.v. at weeks 1, 2, and 3) was not effective on bone formation or preventing bone resorption.

It was proposed that bisphosphonates, under certain conditions, increase bone formation in vivo.[21] In addition, studies performed on bone cell cultures, using very low concentrations of bisphosphonates,[23,24] found increased parameters for bone formation. It was previously suggested that alendronate treatment of osteoporotic patients increased bone mass,[16] and pamidronate has similar effects.[25] Although there are several hypotheses to explain the increase in bone mass resulting from inhibitors of bone resorption, most of these hypotheses propose an indirect effect.[26] Rodan suggested that mechanical strain is a dominant factor, which increases the amount of bone as a feedback mechanism for inhibition of resorption, which explains the efficacy of alendronate.[26] In Rodan's study, the ossicle, which is formed under low mechanical strains, underwent extensive bone resorption after 4 weeks. Therefore, this model can separate to some extent the direct effect of alendronate during the bone development period (up to 4 weeks) and its effect on bone remodeling (after 4 weeks). Interestingly, bone mass increased in the present study only when alendronate was given during the resorptive phase. Bisphosphonates are known to bind strongly to the surface by chemisorption onto calcium and their high affinity for calcium phosphate solid phase.[27,28] It may explain their effectiveness after bone mineralization has occurred. Thus, since most of the mineralization occurred 2 weeks after implantation, we postulate that alendronate given early (during 1, 2, and 3 weeks) was not effective in preventing bone resorption.

Many studies have demonstrated that alendronate is a potent bisphosphonate that inhibits osteoclast bone resorption.[14,23,27] The direct effects are made possible by the uptake of bisphosphonates by osteoclasts during the resorption process, a process favored by the fact that alendronate also is deposited preferentially under the osteoclasts, where they attain high concentrations.[15,29] It is therefore very effective when active bone resorption is stimulated. Since our ectopic bone ossicle formation system separated the bone formation phase during 4 weeks from the remodeling and extensive bone loss later on, our results suggest that alendronate is not effective during the bone formation phase, but is very effective during the bone resorption phase. It is of interest to note that alendronate passes through the placenta and is accumulated in the fetal skeleton, thus increasing bone mass due to a decrease of bone resorption.[27] It was also noted in our study that BMD decreased after 4 weeks in the ossicles of non-treated rats, and that alendronate given both early or later increased the BMD. This increase was significant in comparison to time-matched controls, when alendronate was given at 3, 4, and 5 weeks following implantation.

In the present ectopic bone development model in the rat, we have confirmed previous reports that alendronate inhibits bone loss.[10-13,16,17,22,24,25,28] It is of interest that alendronate maintained the normal characteristics of hematopoietic bone marrow during the entire experiment in contrast to the non-treated animals.

In conclusion, this study suggests that alendronate is effective in inhibiting bone loss, while at the same time, not increasing the amount or bone density during the bone formation phase. We suggest, therefore, that alendronate should be administered in procedures where bone resorption is expected.

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