Osteoinductivity of Demineralized Bone Matrix Is Independent of Donor Bisphosphonate Use

Zvi Schwartz, DMD, PhD, Sharon L. Hyzy, BS, Mark A. Moore, PhD, MBA, Shawn A. Hunter, PhD, Chad J. Ronholdt, MBA, MoonHae Sunwoo, PhD, and Barbara D. Boyan, PhD

Investigation performed at the Georgia Institute of Technology, Atlanta, Georgia

Background: Demineralized bone matrix is commonly used as a bone graft substitute, either alone or to supplement an osteoconductive material, because of its osteoinductive properties. The aging of the population has led to an increase in the number of prospective donors of demineralized bone matrix who have taken bisphosphonates to prevent osteoclast-mediated bone resorption. The aim of this study was to determine whether oral bisphosphonate usage affects the osteoinductivity of demineralized bone matrix from donors.

Methods: Sex-matched and age-matched pairs of samples were provided by four tissue banks (three or four pairs per bank). Demineralized bone matrix donors without bisphosphonate treatment had a mean age (and standard deviation) of 69.1 ± 2.5 years, and donors with bisphosphonate treatment had a mean age of 68.9 ± 2.0 years. Each pair included one donor known to have taken bisphosphonates and one who had not taken bisphosphonates. Demineralized bone matrix previously confirmed as osteoinductive was the positive control, and heat-inactivated demineralized bone matrix was the negative control. Demineralized bone matrix incubated with 1 mL of phosphate-buffered saline solution containing 0, 0.002, 2.0, or 2000 ng/mL of alendronate was also tested. Gelatin capsules containing 15 mg of demineralized bone matrix were implanted bilaterally in the gastrocnemius muscle of male nude mice (eight implants per group). The mice were killed thirty-five days after implantation, and hind limbs were recovered and processed for histological analysis. Osteoinductivity was measured with use of a qualitative score and by histomorphometry.

Results: Nine of fifteen samples from donors who had had bisphosphonate treatment and ten of fifteen samples from patients who had not had bisphosphonate treatment were osteoinductive. Qualitative mean scores were comparable (1.7 ± 0.4 for those without bisphosphonates and 1.9 ± 0.7 for those with bisphosphonates). Osteoinductive demineralized bone matrix samples produced ossicles of comparable size, regardless of bisphosphonate usage. Histomorphometric measurements of the area of new bone formation and residual demineralized bone matrix were also comparable. The addition of alendronate to control demineralized bone matrix did not affect its osteoinductivity.

Conclusions: Demineralized bone matrix samples from donors treated with bisphosphonates and donors not treated with bisphosphonates have the same ability to induce bone formation. However, it is not known if the quality of the new bone is affected, with subsequent consequences affecting bone remodeling.

Clinical Relevance: These results show that demineralized bone matrix can be safe and effective as an osteoinductive material, even when the original bone graft is obtained from donors who have used bisphosphonates.

Bisphosphonates are a class of compounds characterized by two phosphate groups covalently bound to a central carbon and, as such, are resistant to enzymatic cleavage. They act as surfactants and bind to the surface of calcium phosphate

crystals in a manner similar to polyphosphates like pyrophosphate, thereby acting as inhibitors of crystal growth. However, they are chemically stable, and therefore they are longer lasting in their effects than pyrophosphate. Bisphosphonates have a high

Disclosure: One or more of the authors received payments or services, either directly or indirectly (i.e., via his or her institution), from a third party in support of an aspect of this work. In addition, one or more of the authors, or his or her institution, has had a financial relationship, in the thirty-six months prior to submission of this work, with an entity in the biomedical arena that could be perceived to influence or have the potential to influence what is written in this work. No author has had any other relationships, or has engaged in any other activities, that could be perceived to influence or have the potential to influence what is written in this work. The complete **Disclosures of Potential Conflicts of Interest** submitted by authors are always provided with the online version of the article.

affinity for bone, where they are adsorbed to the mineral surface¹, making bone tissue an important target for their pharmacologic action. They are widely used clinically to treat diseases in which stability of bone mineral and bone quality are at risk². Bisphosphonates have been shown to be effective in increasing bone mass in postmenopausal women³⁻⁵ and osteoporotic men⁶, thereby preventing vertebral body and femoral neck fractures⁷. They are also successful therapeutics to treat osteolytic lesions induced by bone resorbing tumors⁸.

The mechanisms by which bisphosphonates exert their effects in bone are not well understood. Not only do they have direct inhibitory effects on osteoclasts, but they also have effects on osteoblasts. Various forms of bisphosphonate have been shown to enhance alkaline phosphatase activity and expression of bone morphogenetic protein (BMP)-2, type-I collagen, and osteocalcin⁹. In addition to influencing proliferation and maturation of normal human osteoblasts¹⁰, bisphosphonates also regulate apoptosis of osteoblasts and osteocytes¹¹. Many of the effects of bisphosphonates are dose-dependent and vary with the chemistry of the compounds being tested¹². These compounds have been reported to enhance proliferation and osteoblastic differentiation of mesenchymal stem cells¹³, but they have also been shown to dose-dependently inhibit calcium phosphate deposition in cultures of human mesenchymal stem cells induced to undergo osteoblast differentiation¹¹.

In addition to their effects on bone cells, bisphosphonates have effects on other tissues as well, including inhibiting angiogenesis¹⁴, potentially by reducing endothelial cell proliferation and survival¹⁵. Bisphosphonates modulate myeloid cell differentiation and function¹⁶, affect neural tissues^{17,18}, regulate cementoblast function¹⁹, and inhibit breast cancer metastases²⁰. Bisphosphonates have also been associated with osteonecrosis of the jaw²⁰, but the cells involved in this condition are not known.

The population of patients being treated with bisphosphonates has continued to grow as therapeutic applications increase. As a result, there is increased potential for tissue donors to have taken bisphosphonates, particularly postmenopausal women with osteoporosis. The chemical properties of these compounds cause them to be retained in the mineral phase of the bone, but whether this affects the organic composition is not known. Bisphosphonates do affect the biosynthetic characteristics of osteoblasts^{21,22}, suggesting that there may be alterations in the extracellular matrix as a consequence of use. Recently, it has been found that the addition of bisphosphonates to implanted allograft bone affects the rate of allograft resorption and can affect the success of implants used with allograft^{23,24}.

Demineralized bone matrix is commonly used as a bone graft substitute, either alone or to supplement an osteoconductive material, because of its osteoinductive properties. In the present study, we examined the effects of exposure to bisphosphonates on the properties of bone allograft by specifically focusing on the osteoinductive property of demineralized bone matrix. Since the discovery of the osteoinduction²⁵, there has been considerable research into the osteogenic

properties of demineralized bone matrix, both in vitro and in vivo²⁶⁻²⁸. Clinical use of demineralized bone matrix has led to variable reports of success²⁹⁻³². Demineralization of bone exposes the bone-inductive proteins located in the matrix, but the amounts of these proteins may vary among donors. Donor age may play a role^{33,34}. Human demineralized bone matrix is classified as a transplant tissue in the United States, and it is processed and distributed by tissue banks, whose methods are not uniform. Thus, the osteoinductivity of demineralized bone matrix may also vary between sources^{33,35}. In addition, bisphosphonate usage may be a contributing factor. Although the demineralization process should remove any bisphosphonate together with the bone mineral³⁶, it is not known whether exposure to these compounds may have had an effect on the osteoinductive properties of the demineralized bone matrix.

This study was based on the hypothesis that demineralized bone matrix from donors treated with oral bisphosphonates has different osteoinductivity in comparison with demineralized bone matrix from donors who had not received oral bisphosphonates. To test this hypothesis, the osteoinductive ability of demineralized bone matrix from donors known to have taken oral bisphosphonates was compared with demineralized bone matrix from donors who had not been on bisphosphonate therapy. Osteoinductivity was assessed with use of a standardized animal model in which demineralized bone matrix is implanted in the gastrocnemius muscle of nude mice and the presence of new bone is determined by quantitative histomorphometry. This assay has been shown previously to be sensitive enough to assess dose-dependent effects of a variety of additives to demineralized bone matrix, as well as differences in demineralized bone matrix donors and variations between bone banks, and the effects of ovariectomy and estrogen replacement therapy^{33-35,37-44}. Demineralized bone matrix from three or four pairs of donors from each of four different bone banks was used for the study, enabling us to compare the effect of processing methods used, either terminally irradiated or nonirradiated. Moreover, the study examined whether the addition of bisphosphonates to mineralized bone allograft with or without demineralization had an effect on bone induction ability.

Methods and Materials

Sample Preparation

This study was conducted in two parts. For the first part, demineralized bone matrix processed for clinical use was provided by four different tissue banks: LifeNet Health, Community Tissue Services (CTS), Allosource, and Musculoskeletal Transplant Foundation. All samples provided to the surgical and evaluation team were coded to blind the reviewers to the tissue bank source as well as donor age, sex, and history of bisphosphonate therapy. The code was held in a locked file and was broken only once the results were tabulated. Code numbers were applied randomly so that the bank of origin would not be discernible in the overall reporting of the results. Each tissue bank provided three or four matched batches of demineralized bone matrix: one batch from a donor who had a

reported history of using bisphosphonates and one batch from an age and sex-matched donor who had not taken bisphosphonates (see Appendix). It was not possible to control samples for the type of bisphosphonate used or the duration of use because the bone banks do not currently collect this information from donors. Demineralized bone matrix that had been previously shown to have high osteoinductivity was used as a positive control. Demineralized bone matrix from the same lot as the positive control was heated for twenty-four hours at 105°C to inactivate any biologically active protein factors present and served as a negative control. For the second part, mineralized allograft and demineralized bone matrix from the same donor as the positive control above were soaked in 1 mL of phosphate-buffered saline solution with 0, 0.002, 2.0, or 2000 ng/mL of alendronate (Merck, Whitehouse Station, New Jersey)³⁶ and implanted.

Although each tissue bank used a slightly different method for processing demineralized bone matrix, all tissue banks follow a general system that involves cleaning, defatting, and disinfecting cortical bone; grinding the defatted bone to particle sizes between 400 and 1000 µm; demineralizing the bone particles in dilute hydrochloric acid solutions, resulting in a residual calcium content of <8% per the American Association of Tissue Banks standards; and freeze-drying the final product. Some banks also chose to add a terminal sterilization step. Particle size was well controlled by all four tissue banks and was maintained between 400 and 1000 µm.

Demineralized bone matrix (15 mg per sample) was placed in a size-9 gelatin capsule (Torpac, Fairfield, New Jersey) in order to facilitate implantation and to prevent displacement while in situ. We previously demonstrated that these capsules dissolve quickly and play no role in the outcome of the assay^{33,35}. Samples were prepared under aseptic conditions and were kept at room temperature until implantation.

Study Design

The study was conducted under a protocol approved by the Institutional Animal Care and Use Committee at the Georgia Institute of Technology. In the first phase of the study, sixty male athymic nu/nu (nude) mice (Harlan, Indianapolis, Indiana) were divided into fifteen groups of four mice each. This strain of mouse, which has reduced helper T cells, was selected because it reduces the chance that a response is due to immunogenicity rather than to the implants themselves. Demineralized bone matrix samples (with and without bisphosphonates) from the four tissue banks, as well as the positive and negative control demineralized bone matrix samples, were implanted in the gastrocnemius muscle, one implant per limb. Thus, each mouse received two implants, both of the same type to reduce any systemic influences, resulting in eight implants per sample.

In the second part of the study, thirty-two mice of the same strain were divided into eight groups of four each, and mineralized bone allograft or positive control demineralized bone matrix that had been treated with alendronate was implanted bilaterally.

Implantation Protocol

Athymic male nu/nu mice were anesthetized by inhalation of 5% isoflurane in O₂. Both hind legs of each mouse were disinfected with use of isopropanol and chlorhexidine. Skin incisions of 0.5 cm were made over each gastrocnemius, and a muscle pouch was prepared by blunt dissection. The implant was inserted into the muscle pouch, and the skin incision closed with wound clips.

The mice were housed in conditions appropriate to their immunocompromised status and given food and water ad libitum for thirty-five days. We have previously shown that this period is sufficient for demonstration of even low levels of osteoinductive activity in this model, despite potential variability of preparations^{33,35}.

Histological Evaluation

The animals were killed at thirty-five days after implantation by asphyxiation with carbon dioxide. The hind limbs were dissected and fixed in 10% neutral buffered formalin and examined radiographically (Faxitron, Lincolnshire, Illinois) to localize the implant site. Following fixation, the tissue was decalcified in 5% formic acid, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Three consecutive cross sections (3 to 4 µm each) of the implant were obtained at three different levels on the longitudinal axis of the sample. We found that this helped to ensure that a section of the implanted region containing a cross section of the tibia and the fibula was obtained to confirm correct orientation, since some of the implants shifted position when the mice returned to weight-bearing. When more than one section with these features was found, the section with the largest cross-sectional area was selected for analysis.

The ability of the samples to induce new bone was qualitatively evaluated by two independent observers, who were blinded to treatment, and was rated according to the following previously published semiquantitative scheme. A score of 1 indicated the presence of particles without any bone; 2, the production of new bone in one site within the section and covering <40% of the surface area examined; 3, the production of new bone in more than one site, covering >40% but <70% of the surface area examined; and 4, the production of new bone in more than one site, covering >70% of the surface area examined^{33,35}.

New bone formation can be found even when heat-inactivated demineralized bone matrix is used, possibly because of particle packing, condensed hematoma, or isolated areas of active factors. If only one or two implants per donor exhibit focal areas of new bone, the mean qualitative score would be <1.5. Therefore, to reduce the likelihood of false positives and to ensure that osteoinduction is a general property of the demineralized bone matrix being tested, demineralized bone matrix implants receiving scores of ≥1.5 were considered osteoinductive.

With use of this method, there was similar induction ability across bone banks, eliminating concerns about demineralized bone matrix quality. Observer scoring was calibrated to an experienced reviewer (Z.S.) prior to beginning the

TABLE I Histological Score and the Number of Bone-Inducing Samples from the Total Number of Samples

Treatment	All Banks		Bank A		Bank B		Bank C		Bank D	
	Score*	No.	Score*	No.	Score*	No.	Score*	No.	Score*	No.
Demineralized bone matrix	1.71 ± 0.40	10/15	1.81 ± 0.27	3/4	1.67 ± 0.16	3/4	1.64 ± 0.28	2/4	1.71 ± 0.07	3/3
Demineralized bone matrix and bisphosphonate	1.85 ± 0.66	9/15	1.72 ± 0.41	2/4	1.99 ± 0.26	3/4	1.78 ± 0.38	2/4	1.90 ± 0.43	2/3

*The values are given as the mean and the standard error of the mean.

experiment. When the observers' ratings disagreed, a third observer (B.D.B.) evaluated the disputed sections. The overall grade for each implant was obtained by averaging the scores from all specimens in the group.

On the same histological sections, a histomorphometric analysis was performed with use of a computerized histomorphometric system (version 4.5.1, Image-Pro Plus; Media Cybernetics, Bethesda, Maryland). The section that exhibited the most demineralized bone matrix particles and new bone was selected to represent that sample for area analysis, and the section was scanned with use of a microscope equipped with 1×, 5×, and 10× objectives. The image was captured by video camera, and the data were stored and analyzed with use of the imaging software. Calibration was

performed according to the instructions accompanying the software. The areas of new cortical bone and residual demineralized bone matrix particles in each section were measured. Ossicle formation, the sum of new bone and new bone marrow, was also quantified. Representative histological images are presented in Figure 1.

Statistical Analysis

The results of the morphometric analysis are shown as the mean and the standard error of the mean of the specimens examined, with N being the number of bone graft implant sites. For this study, type I error was set as 0.05. For a two-sample t test, assuming alpha = 0.05 and power of 0.8, and with use of a standard deviation of 0.2 from previous experiments, a sample

Fig. 1

Representative histological images of samples analyzed in this study for heat-inactivated (**Figs. 1-A and 1-B**) and active (**Figs. 1-C through 1-F**) demineralized bone matrix samples (×5). Thin arrows indicate new bone, stars indicate bone marrow, and wide arrows mark demineralized bone matrix.

TABLE II Effect of Demineralized Bone Matrix Processing on Osteoinduction

Treatment	Irradiated Samples* (n = 18)		Nonirradiated Samples* (n = 14)	
	Score	Ossicle Formation (mm^2)	Score	Ossicle Formation (mm^2)
Demineralized bone matrix and bisphosphonate	1.83 ± 0.17	0.09 ± 0.03	1.69 ± 0.09	0.09 ± 0.03
Demineralized bone matrix without bisphosphonate	1.80 ± 0.29	0.13 ± 0.06	1.95 ± 0.23	0.21 ± 0.08

*The values are given as the mean and the standard error of the mean.

size of eight is adequate to determine effect sizes of 30%. Thus, the sample size we used, eight implants, was appropriate. We previously also calculated our data with use of each animal as an N, averaging the results from the two implants per animal and then assessing the data with an N = 4 and found that the statistical comparisons remained the same^{33,35}. For this reason, we used each implant as an independent N. Significant differences between groups were determined by analysis of variance and the use of the Bonferroni modification of the Student t test. P values of <0.05 were considered significant. The sample size was sufficient to compare samples with respect to processing method: irradiated versus nonirradiated. In addition, it was possible to assess osteoinductivity as a function of donor age, either younger than or older than seventy years. This age was selected on the basis of previous studies showing that demineralized bone matrix from donors older than seventy years is less osteoinductive than bone from younger donors in this model^{33,34}.

Source of Funding

This research was supported by a grant from the Scientific and Technical Affairs Committee of the American Association of Tissue Banks.

Results

Effect of Donor Bisphosphonate Use on Demineralized Bone Matrix Osteoinduction

The average age of donors who had been treated with bisphosphonates was 68.9 ± 2.0 years (see Appendix). Two of

these donors were male, and thirteen were female. The average age of donors who had not been treated with bisphosphonates was 69.1 ± 2.5 years, which was not significantly different from the bisphosphonate group. Three of these donors were male and twelve were female.

There was no difference in the semiquantitative assessment of osteoinduction between the two groups. Demineralized bone matrix in the experimental groups had scores similar to the positive control demineralized bone matrix and higher than the negative control demineralized bone matrix (Fig. 2-A). This was the case for demineralized bone matrix from all tissue banks. Ten of fifteen samples from donors not treated with bisphosphonates and nine of fifteen samples from donors treated with bisphosphonates were osteoinductive, on the basis of the mean score (Table I). There were no tissue bank-specific differences in the number of demineralized bone matrix samples that were osteoinductive. New bone formation was significantly higher in all samples compared with the negative control (Fig. 2-B). There was no difference in new bone formation between the experimental groups and the positive control. Only Bank B had significantly increased new bone in the bisphosphonate groups; there was no difference in any of the other groups. The area of newly formed ossicles was increased in demineralized bone matrix samples soaked in bisphosphonate compared with demineralized bone matrix alone (Fig. 2-C). All samples had a larger ossicle area compared with the negative control. Demineralized bone matrix-only groups had a significantly lower ossicle area than the positive control samples.

TABLE III Effect of Donor Age on Demineralized Bone Matrix Osteoinduction

Treatment	Age*			
	≥70 Yr		<70 Yr	
	Score	Ossicle Formation (mm^2)	Score	Ossicle Formation (mm^2)
Demineralized bone matrix and bisphosphonate	1.84 ± 0.13	0.12 ± 0.03	1.71 ± 0.13	0.09 ± 0.02
Demineralized bone matrix without bisphosphonate	2.16 ± 0.27	0.21 ± 0.06	1.95 ± 0.23	0.13 ± 0.07

*The values are given as the mean and the standard error of the mean.

Fig. 2

Effect of donor bisphosphonate (Bis) usage on demineralized bone matrix (DBM) osteoinductivity as determined by qualitative score (**Fig. 2-A**), new bone formation (**Fig. 2-B**), and ossicle size (**Fig. 2-C**). The values for the implants were calculated by determining the average for each of the eight implants per donor (three to four donors per treatment) and then by determining the mean and standard error of the mean of the values for each of the treatments. # $p < 0.05$ for sample vs. negative control (NC); • $p < 0.05$ for sample vs. positive control (PC); and * $p < 0.05$ for DBM vs. DBM + bisphosphonate.

The areas of residual demineralized bone matrix remaining after thirty-five days were measured (**Fig. 3**). All samples had significantly more demineralized bone matrix than the negative control, with the exception of Bank C. Only Bank B samples of demineralized bone matrix with bisphosphonates had significantly higher residual demineralized bone matrix than the positive control sample. Bank C had significantly less residual demineralized bone matrix than the positive control samples. There was no difference between demineralized bone matrix only and demineralized bone matrix and bisphosphonate for any of the bone banks.

Because the samples came from four tissue banks with separate preparation methods, we compared the effect of the sterilization methods used, either irradiated or nonirradiated (**Table II**). We found no difference in qualitative scores or ossicle formation between demineralized bone matrix with or without bisphosphonate in samples with either sterilization method. The age of the demineralized bone matrix donor was also considered (**Table III**). Ossicle formation and qualitative score were not significantly different between samples of demineralized bone matrix with or without bisphosphonate from donors who were less than seventy years old and from donors who were seventy years and older. Moreover, no significant

Fig. 3

Effect of donor bisphosphonate usage on demineralized bone matrix (DBM) osteoinductivity as determined by residual demineralized bone matrix. The values for the implants were calculated by determining the average residual demineralized bone matrix in each of the eight implants per donor (three to four donors per treatment) and then by determining the mean and standard error of the mean of the values for each of the tissue banks. # $p < 0.05$ for sample vs. negative control (NC); • $p < 0.05$ for sample vs. positive control (PC); and * $p < 0.05$ for DBM vs. DBM + bisphosphonate.

Fig. 4

The effect of the addition of bisphosphonate on demineralized bone matrix osteoinductivity as determined by qualitative score (**Fig. 4-A**), new bone formation (**Fig. 4-B**), and ossicle size (**Fig. 4-C**). Data shown are the mean and the standard error of the mean for each of the eight implants per treatment. # $p < 0.05$ for sample vs. 0 ng/mL of bisphosphonate.

association was found between donor age and ossicle formation or qualitative score.

Effect of Bisphosphonate Addition on the Osteoinductivity of Demineralized Bone Matrix

To examine the effects of bisphosphonate on demineralized bone matrix osteoinductivity, positive control samples of demineralized bone matrix with the addition of alendronate were examined in our model. Osteoinduction, as measured by qualitative score, was unchanged with increasing doses of alendronate (**Fig. 4-A**). All samples scored higher than the heat-inactivated demineralized bone matrix samples. The samples also induced a greater area of new bone formation (**Fig. 4-B**) and ossicle formation (**Fig. 4-C**) than heat-inactivated samples did, but there was no difference between the treatment doses.

Finally, we examined the effect of bisphosphonate addition on the osteoinductivity of mineralized bone (see Appendix). The qualitative score of osteoinductivity was unchanged for all samples, regardless of bisphosphonate dose. The score of the samples (i.e., 1) indicates that the samples did not induce new bone formation.

Discussion

This study showed that there was no difference in overall osteoinductivity of demineralized bone matrix prepared from allograft from donors who were known to have been medicated with bisphosphonates and that from donors who were not. This observation was independent of whether the demineralized bone matrix was processed by any specific tissue bank, although different methods of production were used. We used samples that were prepared with use of routine processing methods in each of the tissue banks, and not samples prepared specifically for this study. Thus, which of the several different kinds of bisphosphonates the donors were prescribed, if more than one form of bisphosphonate was used, or the length of time the donors were on bisphosphonate therapy were not known. A comprehensive medical and social history of each donor was used to identify donors who had or had not taken bisphosphonates, but it cannot be entirely ruled out that the donors identified as not having taken bisphosphonates at the time of death had had no prior exposure. It is possible that the failure to detect an effect of bisphosphonate usage on osteoinductivity was because of a failure to definitively screen for bisphosphonate-free donors. Given that each bone bank contributed multiple age-matched pairs of donors, however, the sample population in each treatment group was drawn from a broad spectrum of the population. Therefore, despite the relatively limited sample size, the results strongly support the conclusion that demineralized bone matrix osteoinductivity is not affected by bisphosphonate use.

The fact that we used routinely processed preparations of demineralized bone matrix also allowed us to make important observations. All of the tissue banks produced demineralized bone matrix that was osteoinductive as well as some batches of demineralized bone matrix that were not osteoinductive in the

mouse muscle implant model. This suggests that the failures were due to donor-dependent differences and not to a difference in preparation. Although some of the demineralized bone matrix was terminally sterilized by gamma irradiation, there was no difference in its osteoinductivity compared with nonirradiated demineralized bone matrix preparations. The sample size from each bank, and therefore each processing method, was relatively small (six to eight samples, of which one-half were from donors who had used bisphosphonates). Thus, we cannot state in an absolute way that processing is not a concern³⁵.

Bisphosphonates have been shown to modify the response to bone allograft used in bone sites, either enhancing or inhibiting allograft resorption and new bone formation^{24,45-49}. A recent report showed this depended on the concentration of bisphosphonate in an allograft²³. When allograft is implanted ectopically, it is resorbed without new osteogenesis^{34,50}, indicating that it is not osteoinductive. We hypothesized that inhibition of osteoclastic activity by alendronate could reduce the resorption of the allograft, potentially allowing it to stimulate bone formation, but this did not occur. Whether or not alendronate was added to the allograft, no bone formation was noted. In contrast, demineralized bone matrix was osteoinductive with or without the addition of alendronate, consistent with another study demonstrating that alendronate did not affect osteogenesis⁵¹.

Demineralized bone matrix batches used in the present study were generally from older donors (more than fifty-six years old), as this is the age group most likely to be medicated. We previously showed that demineralized bone matrix from donors more than fifty-six years old had reduced osteoinductivity compared with that from young donors³³; however, in the present analysis, all samples exhibited similar new bone formation when implanted in the mouse muscle model with no correlation to age. No young donors were used in the present study; therefore, it is not known if there would have been a higher percent of the samples with osteoinductive properties in demineralized bone matrix processed from a young donor population. Our results support previous observations from our group³⁴ and others⁵²⁻⁵⁴, showing that donors who are sixty to seventy years of age continue to possess osteoinductive bone, and suggest that any loss of osteoinductivity occurs later in life.

The results of this study show that demineralized bone matrix from donors treated with or without bisphosphonates has the same ability to induce bone formation. The addition of alendronate to samples of demineralized bone matrix known to

be osteoinductive had no effect on osteoinduction. The results suggest that bone formation by means of demineralized bone matrix was not affected by oral bisphosphonates or the addition of bisphosphonates to demineralized bone matrix. However, remodeling of the new bone was not studied, so it is possible that bisphosphonates do not affect bone formation but have longer-term effects on the bone created, possibly during bone remodeling.

Appendix

eA A table showing the age and sex of the demineralized bone matrix donors, and a figure showing the effect of the addition of bisphosphonate on mineralized bone osteoinductivity, as determined by qualitative score, are available with the online version of this article as a data supplement at jbjs.org. ■

NOTE: The authors thank Erin Plute and Leang Chhun for their technical assistance.

Zvi Schwartz, DMD, PhD
Sharon L. Hyzy, BS
Barbara D. Boyan, PhD
Department of Biomedical Engineering,
Georgia Institute of Technology,
315 Ferst Drive N.W., Atlanta, GA 30332-0363.
E-mail address for B.D. Boyan: barbara.boyan@bme.gatech.edu

Mark A. Moore, PhD, MBA
LifeNet Health, Bio-Implants Division,
1864 Concert Drive, Virginia Beach, VA 23453.
E-mail address: mark_moore@lifenethealth.org

Shawn A. Hunter, PhD
Community Tissue Services,
Center for Tissue Innovation and Research,
2900 College Drive, Kettering, OH 45420.
E-mail address: shunter@communitytissue.org

Chad J. Ronholdt, MBA
LABS Inc., 6933-B South Revere Parkway,
Centennial, CO 80112.
E-mail address: cronholdt@labsinc.org

MoonHae Sunwoo, PhD
Musculoskeletal Transplant Foundation,
125 May Street, Edison, NJ 08837.
E-mail address: moonhae_sunwoo@mtf.org

References

1. Carano A, Teitelbaum SL, Konsek JD, Schlesinger PH, Blair HC. Bisphosphonates directly inhibit the bone resorption activity of isolated avian osteoclasts in vitro. *J Clin Invest.* 1990;85:456-61.
2. Russell RG. Bisphosphonates: from bench to bedside. *Ann N Y Acad Sci.* 2006; 1068:367-401.
3. Ensrud KE, Black DM, Palermo L, Bauer DC, Barrett-Connor E, Quandt SA, Thompson DE, Karpf DB. Treatment with alendronate prevents fractures in women at highest risk: results from the Fracture Intervention Trial. *Arch Intern Med.* 1997; 157:2617-24.
4. Bell NH, Johnson RH. Bisphosphonates in the treatment of osteoporosis. *Endocrine.* 1997;6:203-6.
5. Fleisch HA. Bisphosphonates: preclinical aspects and use in osteoporosis. *Ann Med.* 1997;29:55-62.
6. Finkelstein JS, Leder BZ, Burnett SM, Wyland JJ, Lee H, de la Paz AV, Gibson K, Neer RM. Effects of teriparatide, alendronate, or both on bone turnover in osteoporotic men. *J Clin Endocrinol Metab.* 2006;91:2882-7.
7. Landis BN, Richter M, Dojcinovic I, Hugentobler M. Osteonecrosis of the jaw after treatment with bisphosphonates: is irreversible, so the focus must be on prevention. *BMJ.* 2006;333:982-3.
8. Krempien R, Niethammer A, Harms W, Debus J. Bisphosphonates and bone metastases: current status and future directions. *Expert Rev Anticancer Ther.* 2005; 5:295-305.

9. Im GI, Qureshi SA, Kenney J, Rubash HE, Shanbhag AS. Osteoblast proliferation and maturation by bisphosphonates. *Biomaterials*. 2004;25:4105-15.
10. Fromig O, Body JJ. Bisphosphonates influence the proliferation and the maturation of normal human osteoblasts. *J Endocrinol Invest*. 2002;25:539-46.
11. Plotkin LI, Manolagas SC, Bellido T. Dissociation of the pro-apoptotic effects of bisphosphonates on osteoclasts from their anti-apoptotic effects on osteoblasts/osteocytes with novel analogs. *Bone*. 2006;39:443-52.
12. Schindeler A, Little DG. Osteoclasts but not osteoblasts are affected by a calcified surface treated with zoledronic acid in vitro. *Biochem Biophys Res Commun*. 2005;338:710-6.
13. von Knoch F, Jaquiere C, Kowalsky M, Schaeren S, Alabre C, Martin I, Rubash HE, Shanbhag AS. Effects of bisphosphonates on proliferation and osteoblast differentiation of human bone marrow stromal cells. *Biomaterials*. 2005;26:6941-9.
14. Ribatti D, Nico B, Mangieri D, Maruotti N, Longo V, Vacca A, Cantatore FP. Neridronate inhibits angiogenesis in vitro and in vivo. *Clin Rheumatol*. 2007;26:1094-8.
15. Hasmmim M, Bieler G, Regg C. Zoledronate inhibits endothelial cell adhesion, migration and survival through the suppression of multiple, prenylation-dependent signaling pathways. *J Thromb Haemost*. 2007;5:166-73.
16. Wolf AM, Rumpold H, Tilg H, Gastl G, Gunsilius E, Wolf D. The effect of zoledronic acid on the function and differentiation of myeloid cells. *Haematologica*. 2006;91:1165-71.
17. Dickson PV, Hamner JB, Cauthen LA, Ng CY, McCarville MB, Davidoff AM. Efficacy of zoledronate against neuroblastoma. *Surgery*. 2006;140:227-35.
18. Berthelot JM. Current management of reflex sympathetic dystrophy syndrome (complex regional pain syndrome type I). *Joint Bone Spine*. 2006;73:495-9.
19. Chun YH, Foster BL, Lukasavage PA, Berry JE, Zhao M, Tenenbaum HC, Somerman MJ. Bisphosphonate modulates cementoblast behavior in vitro. *J Periodontol*. 2005;76:1890-900.
20. Brufsky A. Management of cancer-treatment-induced bone loss in postmenopausal women undergoing adjuvant breast cancer therapy: a Z-FAST update. *Semin Oncol*. 2006;33(2 Suppl 7):S13-7.
21. Xiong Y, Yang HJ, Feng J, Shi ZL, Wu LD. Effects of alendronate on the proliferation and osteogenic differentiation of MG-63 cells. *J Int Med Res*. 2009;37:407-16.
22. Orriss IR, Key ML, Colston KW, Arnett TR. Inhibition of osteoblast function in vitro by aminobisphosphonates. *J Cell Biochem*. 2009;106:109-18.
23. Jakobsen T, Baas J, Bechtold JE, Elmengaard B, Sballe K. The effect of soaking allograft in bisphosphonate: a pilot dose-response study. *Clin Orthop Relat Res*. 2010;468:867-74.
24. Jakobsen T, Baas J, Bechtold JE, Elmengaard B, Sballe K. Soaking morselized allograft in bisphosphonate can impair implant fixation. *Clin Orthop Relat Res*. 2007;463:195-201.
25. Urist MR. Bone: formation by autoinduction. *Science*. 1965;150:893-9.
26. Alper G, Bernick S, Yazdi M, Nimni ME. Osteogenesis in bone defects in rats: the effects of hydroxyapatite and demineralized bone matrix. *Am J Med Sci*. 1989;298:371-6.
27. Cheung S, Westerheide K, Ziran B. Efficacy of contained metaphyseal and periarticular defects treated with two different demineralized bone matrix allografts. *Int Orthop*. 2003;27:56-9.
28. Narang R, Wells H, Laskin DM. Experimental osteogenesis with demineralized allogeneic bone matrix in extraskelatal sites. *J Oral Maxillofac Surg*. 1982;40:133-41.
29. Barnett JD, Mellonig JT, Gray JL, Towle HJ. Comparison of freeze-dried bone allograft and porous hydroxylapatite in human periodontal defects. *J Periodontol*. 1989;60:231-7.
30. Quintero G, Mellonig JT, Gambill VM, Pelleu GB Jr. A six-month clinical evaluation of decalcified freeze-dried bone allografts in periodontal osseous defects. *J Periodontol*. 1982;53:726-30.
31. Becker W, Becker BE, Caffesse R. A comparison of demineralized freeze-dried bone and autologous bone to induce bone formation in human extraction sockets. *J Periodontol*. 1994;65:1128-33.
32. Michelson JD, Curl LA. Use of demineralized bone matrix in hindfoot arthrodesis. *Clin Orthop Relat Res*. 1996;325:203-8.
33. Schwartz Z, Somers A, Mellonig JT, Carnes DL Jr, Dean DD, Cochran DL, Boyan BD. Ability of commercial demineralized freeze-dried bone allograft to induce new bone formation is dependent on donor age but not gender. *J Periodontol*. 1998;69:470-8.
34. Boyan BD, Lohmann CH, Somers A, Niederauer GG, Wozney JM, Dean DD, Carnes DL Jr, Schwartz Z. Potential of porous poly-D, L-lactide-co-glycolide particles as a carrier for recombinant human bone morphogenetic protein-2 during osteoinduction in vivo. *J Biomed Mater Res*. 1999;46:51-9.
35. Hunter SA, Orheim R, Sazon M, Newman H, Woll JE, Bergevin M. Demineralization removes residual alendronate in allograft bone procured from donors with a history of bisphosphonate use. *J Periodontol*. 2011;82:281-6.
36. Hunter SA, Orheim R, Sazon M, Newman H, Woll JE, Bergevin M. Demineralization removes residual alendronate in allograft bone procured from donors with a history of bisphosphonate use. *J Periodontol*. 2011;82:281-6.
37. Hunter SA, Orheim R, Sazon M, Newman H, Woll JE, Bergevin M. Demineralization removes residual alendronate in allograft bone procured from donors with a history of bisphosphonate use. *J Bone Joint Surg Am*. 2007;89:139-47.
38. McMillan J, Kinney RC, Ranly DM, Fatehi-Sedeh S, Schwartz Z, Boyan BD. Osteoinductivity of demineralized bone matrix in immunocompromised mice and rats is decreased by ovariectomy and restored by estrogen replacement. *Bone*. 2007;40:111-21.
39. Ranly DM, McMillan J, Keller T, Lohmann CH, Meunch T, Cochran DL, Schwartz Z, Boyan BD. Platelet-derived growth factor inhibits demineralized bone matrix-induced intramuscular cartilage and bone formation. A study of immunocompromised mice. *J Bone Joint Surg Am*. 2005;87:2052-64.
40. Boyan BD, Ranly DM, McMillan J, Sunwoo M, Roche K, Schwartz Z. Osteoinductive ability of human allograft formulations. *J Periodontol*. 2006;77:1555-63.
41. Boyan BD, Weesner TC, Lohmann CH, Andreacchio D, Carnes DL, Dean DD, Cochran DL, Schwartz Z. Porcine fetal enamel matrix derivative enhances bone formation induced by demineralized freeze dried bone allograft in vivo. *J Periodontol*. 2000;71:1278-86.
42. Boyan BD, Lohmann CH, Somers A, Niederauer GG, Wozney JM, Dean DD, Carnes DL Jr, Schwartz Z. Potential of porous poly-D, L-lactide-co-glycolide particles as a carrier for recombinant human bone morphogenetic protein-2 during osteoinduction in vivo. *J Biomed Mater Res*. 1999;46:51-9.
43. Schwartz Z, Weesner T, van Dijk S, Cochran DL, Mellonig JT, Lohmann CH, Carnes DL, Goldstein M, Dean DD, Boyan BD. Ability of deproteinized cancellous bovine bone to induce new bone formation. *J Periodontol*. 2000;71:1258-69.
44. Schwartz Z, Somers A, Mellonig JT, Carnes DL Jr, Wozney JM, Dean DD, Cochran DL, Boyan BD. Addition of human recombinant bone morphogenetic protein-2 to inactive commercial human demineralized freeze-dried bone allograft makes an effective composite bone inductive implant material. *J Periodontol*. 1998;69:1337-45.
45. Baas J. Adjuvant therapies of bone graft around non-cemented experimental orthopedic implants stereological methods and experiments in dogs. *Acta Orthop Suppl*. 2008;79:1-43.
46. Aspenberg P, Astrand J. Bone allografts pretreated with a bisphosphonate are not resorbed. *Acta Orthop Scand*. 2002;73:20-3.
47. Astrand J, Aspenberg P. Systemic alendronate prevents resorption of necrotic bone during revascularization. A bone chamber study in rats. *BMC Musculoskelet Disord*. 2002;3:19.
48. Zou X, Xue Q, Li H, Bnger M, Lind M, Bnge C. Effect of alendronate on bone ingrowth into porous tantalum and carbon fiber interbody devices: an experimental study on spinal fusion in pigs. *Acta Orthop Scand*. 2003;74:596-603.
49. Jeppsson C, Astrand J, Tgil M, Aspenberg P. A combination of bisphosphonate and BMP additives in impacted bone allografts. *Acta Orthop Scand*. 2003;74:483-9.
50. Bauer TW, Muschler GF. Bone graft materials. An overview of the basic science. *Clin Orthop Relat Res*. 2000;371:10-27.
51. Yaffe A, Kollerman R, Bahar H, Binderman I. The influence of alendronate on bone formation and resorption in a rat ectopic bone development model. *J Periodontol*. 2003;74:44-50.
52. Traianedes K, Russell JL, Edwards JT, Stubbs HA, Shanahan IR, Knaack D. Donor age and gender effects on osteoinductivity of demineralized bone matrix. *J Biomed Mater Res B Appl Biomater*. 2004;70:21-9.
53. Nyssen-Behets C, Delaere O, Duchesne PY, Dhem A. Aging effect on inductive capacity of human demineralized bone matrix. *Arch Orthop Trauma Surg*. 1996;115:303-6.
54. Zhang M, Powers RM Jr, Wolfenbarger L Jr. A quantitative assessment of osteoinductivity of human demineralized bone matrix. *J Periodontol*. 1997;68:1076-84.