Extremely Small-magnitude Accelerations Enhance Bone Regeneration

A Preliminary Study

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Received: 27 February 2008 / Accepted: 16 September 2008 / Published online: 15 October 2008
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Abstract High-frequency, low-magnitude accelerations can be anabolic and anticatabolic to bone. We tested the hypothesis that application of these mechanical signals can accelerate bone regeneration in scaffolded and nonscaffolded calvarial defects. The cranium of experimental rats ($n = 8$) in which the 5-mm bilateral defects either contained a collagen scaffold or were left empty received oscillatory accelerations (45 Hz, 0.4 g) for 20 minutes per day for 3 weeks. Compared with scaffolded defects in the untreated control group ($n = 6$), defects with a scaffold and subject to oscillatory accelerations had a 265% greater

fractional bone defect area 4 weeks after the surgery. After 8 weeks of healing (1-week recovery, 3 weeks of stimulation, 4 weeks without stimulation), the area (181%), volume (137%), and thickness (53%) of the regenerating tissue in the scaffolded defect were greater in experimental than in control animals. In unscaffolded defects, mechanical stimulation induced an 84% greater bone volume and a 33% greater thickness in the defect. These data provide preliminary evidence that extremely low-level, high-frequency accelerations can enhance osseous regenerative processes, particularly in the presence of a supporting scaffold.

One or more of the authors (SJ) received funding from the Wallace H. Coulter Foundation, the Whitaker Foundation, and the National Science Foundation.

Each author certifies that the Institutional Animal Care and Use Committee at the State University of New York at Stony Brook approved the animal protocol for this investigation and that all investigations were conducted in conformity with ethical principles of research.

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Introduction

Orthopaedic or maxillofacial osseous defects typically are repaired by bone transplantation with either autologous graft or nonautologous bone substitutes [4]. Although autologous grafts are considered the gold standard for bone grafts, donor site morbidity is a possible outcome and nonautologous or synthetic bone materials often are preferred by patients and clinicians [5, 16, 41]. Unfortunately, most bone substitutes possess only osteoconductive properties and often are associated with limited and delayed bone regeneration [16, 44].

Tissue-engineering approaches, including stem cells and growth factors [18, 24] or externally applied signals such as stimulation with electric currents [7, 11], ultrasound [10, 43], or laser [28], are promising in overcoming these problems, but they also have several shortcomings. Current cell culture-based techniques require considerable resources to reach the number of required cells, bear risks for transfer of infectious diseases, and involve invasive surgery [40]. Many externally applied physical stimuli are

clinically irrelevant, are inconvenient because of the complicated manner by which they are applied [30], or have shown inconsistent results [10, 43].

Among the many factors influencing bone modeling and remodeling, functional mechanical loading from muscle contractility may be sensed by bone cells as a potent extrinsic signal [12, 14]. Muscle loading not only includes large forces generated by vigorous physical activities such as running or weightlifting, but also has much more prevalent, very small-magnitude forces associated with subtle events such as posture [12]. When these low-level mechanical signals are delivered at sufficiently high frequencies, such as by standing on a vertically oscillating plate (whole body vibration), they can stimulate bone cells to enhance bone formation, decrease resorption, and remodel into a stiffer and stronger structure with improved architectural characteristics [20, 36, 48]. Application of these extremely low-magnitude, high-frequency mechanical stimuli in recent human studies suggest beneficial effects in a healthy skeleton and in skeletons disturbed by local or systemic stimuli, including inactivity, hormonal changes, or low bone mass [17, 35, 46].

The physical mechanisms by which cells can perceive a signal this small have not been identified. Although the efficacy of the much larger exercise-induced mechanical signals has been related to the magnitude of the generated tissue deformation [8], matrix deformation per se does not appear necessary for high-frequency, low-level mechanical stimuli to influence cell activity [22], and high-frequency oscillatory accelerations applied as unconstrained motions may serve as a regulatory physical signal to increase bone formation in healthy bone [14] and to attenuate the decline in bone formation [15] and mechanical properties [31] during disuse. Oscillatory accelerations, rather than deformations, can be readily applied to any skeletal segment, weightbearing or not, and the potential clinical advantages of a stimulus that is barely perceptible and involves motions on the order of only 100 μm are apparent. Whether such a stimulus can enhance osteogenesis in a bone defect is unknown.

Using a well-established rat calvarial defect model [32], we addressed the following research questions: (1) Can application of extremely low-amplitude, high-frequency accelerations increase the size and morphologic features of regenerating bone in scaffolded and unscaffolded defects in 4 weeks? (2) Can a treatment effect induced by the mechanical signal during the first 4 weeks be retained during a subsequent 4-week rest period? (3) Does the presence of a scaffold potentiate the efficacy of the mechanical signal? (4) Does the stimulus produce a systemic response in the calvaria and is the quality of the regenerating bone affected by the stimulus?

Materials and Methods

We divided 16 male Sprague-Dawley rats (Charles River Laboratory, Wilmington, MA) randomly into control and experimental animals ($n = 8$ each). At 4 months of age, both groups of animals received a bilateral calvarial defect measuring 5 mm in diameter (Fig. 1). The rat calvarial defect model has been used extensively for testing the efficacy of biomaterials to accelerate bone regeneration with or without the presence of supplied cells and growth factors [23, 27]. It was selected because of the relative ease by which bone defects can be created, scaffolds can be added, and mechanical signals can be applied, resulting in a lesser likelihood of complications than in many other models. All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee at the State University of New York at Stony Brook.

All operations were performed with the animals under general anesthesia (isoflurane; Halocarbonyl Products Corp, North Augusta, SC). After disinfection of the calvarial skin, we made an incision along the sagittal suture. The periosteum was elevated carefully without any rupture, and two calvarial bone defects (5 mm in diameter) on both parietal sides were created with a trephine burr. The size of the defect was chosen to be less than the critical defect size and to allow for generation of two bilateral calvarial defects in parietal bone that did not involve the sagittal suture or the temporal bone. As determined by micro-computed tomography (μCT), the average ($\pm$ standard deviation) thickness of the calvarial bone around the defect was 0.57 (0.06) mm across all rats and the volume of a defect comprised approximately 10% of the total calvarial bone volume. After a random distribution pattern, we left one defect empty during surgery, whereas the contralateral defect was filled with a collagen sponge scaffold as described previously [33, 39]. After reunion of the periosteum with resorbable suture materials (Vicryl; Ethicon Inc, Somerville, NJ), we closed the wound with silk suture (Ethicon Inc). Preoperatively, all rats received one dose of antibiotics (20 mg/kg cefazolin; Cura Pharmaceutical Co Inc, Eatontown, NJ). Before and after surgery (3 days), the

Fig. 1 One week after surgery that created the bilateral calvarial defect at 4 months of age, experimental rats (ACC) received oscillatory low-level accelerations for 20 minutes per day, 5 days per week, whereas control rats (CTR) were subjected to sham treatment. Neither group of rats received any treatment between 4 weeks and 8 weeks of the experimental protocol.

animals also received subcutaneous injections of buprenorphine (50 µg/kg; Bedford Laboratories, Bedford, OH). Two rats of the control group died from dural damage during surgery, reducing the number of evaluated rats to six in the control group.

Starting 1 week after surgery, we anesthetized (isoflurane) all animals for 20 minutes per day, 5 days per week for 3 weeks, and placed them in a supine position on either a vertically oscillating plate (experimental rats) or an inactive plate (control rats). The 1-week postsurgery delay was incorporated to allow healing of soft tissue, stabilization of the scaffold, and initiation of a natural healing response to optimize the healing outcome [13, 47]. The physical signal was applied as a sinusoidal motion at a frequency of 45 Hz and peak accelerations of 0.4 g, resulting in total (peak-to-peak) vertical displacements of the plate of 100 µm. Locally applied accelerations, rather than whole body vibrations, were used as the high-frequency mechanical stimulus because they can be applied readily to specific sites and accurately controlled without dampening by the axial skeleton. To focus the vibratory stimulus on the cranium, soft padding was placed underneath the body of the rat to dampen the vibration perceived by the rest of the body. Thus, the occipital region of the cranium was the only direct contact point between the rat and the vibrating plate, enabling transmission of the oscillatory accelerations to the calvarial defects located at least 1 cm away from the plate contact point (Fig. 2).

After the 3 weeks of oscillatory acceleration or sham treatment, animals were not subjected to any treatment and were allowed unrestricted cage activities for 4 weeks to allow for additional healing of the defect (Fig. 1). This schedule was chosen to facilitate comparisons of outcome measures between the stimulation and subsequent rest period and to assess the postmortem outcome variables at a time at which a substantial (re)modeling response in the defect had occurred. Eight weeks after surgery, all animals were euthanized by CO₂ inhalation and the calvarial bone was harvested.

Immediately after surgery and 4 weeks thereafter, an *in vivo* µCT of the rat cranium (38 µm spatial resolution)

Fig. 2 While anesthetized for 20 minutes per day, each experimental rat was exposed to extremely low-level accelerations induced by a vertically oscillating plate. The sinusoidal accelerations were transferred from the plate directly into the cranium while exposure of the rest of the body to the vibrations was minimized by soft padding that dampened the stimulus.

was constructed with the animals under anesthesia (VivaCT40; Scanco Medical, Bruettisellen, Switzerland). After sacrifice, *ex vivo* µCT scans (µCT40; Scanco Medical) were taken at a spatial resolution of 15 µm with the sample submerged in 70% EtOH. For each defect, an algorithm that identified the plane in the circular defect isolated the primary volume of interest (VOI) that contained new bone forming inside the defect border (Fig. 3). To evaluate the amount of spreading of new bone into the circular defect, we quantified the fraction of bone area covering the original defect surface in two dimensions (BA; new bone area/original defect area). The volume of the new bone, its thickness, and its tissue material density (TD) were computed with Scanco software that is capable of morphologically describing irregular structures. The secondary VOI contained a 0.4-mm thick ring of bone outside the defect to determine whether the physical stimulus affected TD of the bone near the defect (Fig. 3).

After fixation in 10% formalin, the calvarial bone was decalcified in EDTA (7%, pH ¼ 7.0) for 3 to 4 days, dehydrated in 70% ethanol, and embedded in paraffin. Paraffin sections were cut perpendicular through the center of the calvarial defect, fixed for 10 minutes with xylene, and stained with Masson's trichrome (MT). Digital images were captured under transmitting and polarized light on a microscope (BX51; Olympus Corporation, Tokyo, Japan). For immunohistochemical analysis, the deparaffinized

Fig. 3 Microcomputed tomography was used to assess morphologic features of bone and density inside and outside the rat calvarial defect. The primary µCT analyses focused on quantifying the volume, thickness, area, and mineral density of the newly forming bone within the 5-mm circular defect. As secondary analysis, tissue mineral density was measured in a ring that was 0.4 mm wide with an outer diameter of 7 mm.

sections were treated with an undiluted serum solution and incubated with primary antibodies specific for VEGF (1:100; R & D Systems, Inc, Minneapolis, MN) and osteocalcin (1:100; Takara Bio Inc, Shiga, Japan) according to the manufacturer's instructions. Immunostaining was detected colorimetrically by DAB (Vecta Laboratories, Burlingame, CA) and hematoxylin.

At 4 week and 8 weeks, we compared morphologic features of bone defects and density (bone area, volume, thickness, and tissue density) between control and experimental rats with unpaired t-tests (two-tailed) to infer whether the mechanical signal promoted osteogenesis in scaffolded and nonscaffolded defects. We used paired t-tests (two-tailed) to identify differences in morphologic features of bone and density between the two times for any given treatment group. Unpaired t-tests also were used to compare the 4-week with 8-week changes in bone defect variables between control and experimental rats. The interaction of the factors treatment (control versus experimental) and scaffold (absence versus presence), as computed by a multivariate general linear model, established whether the effect of treatment on the morphologic features (bone area, volume, and thickness) was different between scaffolded and nonscaffolded defects. All data were presented as mean $\pm$ standard deviation. Parametric distribution of the data was confirmed qualitatively. Statistical tests were performed using SPSS 14.0 (SPSS Inc, Chicago, IL).

Results

Four weeks postsurgery, evaluation of the morphologic features of the regenerating bone showed that the application of high-frequency accelerations to the occipital region of the cranium for 3 weeks, 20 minutes per day, was particularly effective in defects that contained a collagen scaffold (Fig. 4). The fraction of the circular defect area covered with bone (BA) was 257% greater ($p = 0.002$) in scaffolded defects of accelerated rats than in scaffolded defects of control rats (Fig. 5A). For scaffolded and nonscaffolded defects, all other morphologic variables were similar to those for controls (Fig. 5A–C).

At completion of the 8-week experimental period, a greater number of morphologic defect variables were different between control and experimental rats for scaffolded and nonscaffolded defects than at 4 weeks. In defects without a scaffold, short durations of very small amplitude oscillatory motions led to an 84% greater ($p = 0.03$) bone volume and a 33% greater ($p = 0.05$) bone thickness (Fig. 5B–C). The effect of the physical stimulus on osteogenesis was even more evident in defects comprising a scaffold with 181% greater

($p = 0.003$) bone fractional area, 137% greater ($p = 0.02$) bone volume, and 53% greater ($p = 0.02$) bone thickness in oscillated than in control rats (Fig. 5A–C). Not only were these outcome measures different between the two groups at 8 weeks, but the continued increase in bone formation between 4 weeks and 8 weeks (Table 1) also was affected by the biomechanical treatment. During the final 4 weeks of the protocol during which neither group received any treatment, changes in several variables were considerably greater in experimental than in control rats. Specifically, in defects without a scaffold, the increase in tissue volume was 85% greater ($p = 0.03$) in experimental than in control rats. In defects containing a scaffold, acquisition of bone volume (194%, $p = 0.002$) and bone thickness (244%, $p = 0.007$) during the last 4 weeks of the protocol was greater in oscillated than in control defects (Table 1).

Exposing a circular calvarial defect to short durations of low-level accelerations altered the healing process in scaffolded and nonscaffolded defects. Across the two times, we observed an interaction ($p = 0.001$) between the factors treatment and scaffold on morphologic features of defects suggesting the effect of the low-level stimulus on morphologic features of bone was different in scaffolded than in nonscaffolded defects. Stratified by the two different times, the probability values for interaction between treatment and scaffold on morphologic features of bone were 0.01 at 4 weeks and 0.07 at 8 weeks.

Except for the 4-week time at which nonscaffolded regenerating bone was 5% less ($p = 0.04$) mineralized in oscillated than in control rats, no tissue density differences were detected between the experimental and control groups (Fig. 5D). The qualitative histologic evaluations of the MT-stained sections showed there was no inflammatory reaction around and in the scaffold. In stimulated rats, the central zone in the defect space was filled and bridged with new bone, whereas in control rats, bridges consisting of connective soft tissue were more apparent (Fig. 6). Similarly, evaluation of the immunohistochemical sections stained with antibodies for either osteocalcin or VEGF did not reveal a quality defect in regenerating bone of mechanically stimulated rats (data not shown). Tissue material density in the secondary VOI contained a 0.4-mm thick ring of bone outside the defect, as a measure of systemic effects of the oscillatory stimulus, and similar between the control and experimental rats (data not shown).

Discussion

In regenerative medicine, improving the limited or delayed bone-forming ability of osteoconductive bone materials is a

Fig. 4A–L Microcomputed tomographs of calvarial defects of a control (CTR) and experimental (ACC) rat immediately after creating the defect and 4 weeks and 8 weeks after surgery are shown. Defects **G** through **L** contained a collagen scaffold, whereas defects **A** through **F** were left empty. Tissue in light gray in the circular defect represents newly formed bone, indicating the efficacy of high-frequency acceleration to promote bone formation in the presence and absence of a pre-existing collagen matrix.

major concern in orthopaedic or maxillofacial surgeries [16, 44]. Extremely low-magnitude, high-frequency mechanical stimuli can stimulate bone cells to enhance bone formation and decrease resorption [20, 36, 48] and, we hypothesized these signals can increase the quantity of the regenerating bone in scaffolded and nonscaffolded rat calvarial defects 4 weeks postsurgery. Furthermore, we asked whether a treatment effect remains during a subsequent 4-week rest period, whether the presence of a scaffold alters efficacy of the mechanical signal, and whether there is evidence that the stimulus produces a systemic response or negatively affects the quality of the regenerating bone.

The relatively small samples sizes used in this study had sufficient power to show proof of principle for using low-level accelerations to enhance bone regeneration and to suggest that the presence of a collagen scaffold potentiates this effect. Nevertheless, more detailed studies with larger sample sizes are needed to delineate the precise temporal and spatial effects of this low-level physical signal on tissue forming in a defect. Furthermore, it is likely the body of the animal also received at least a certain component of the physical signal despite efforts to primarily target the cranium. As in previous studies [6, 14, 15, 37], systemic perturbations were not evident but cannot be excluded as a factor that influenced the results. Finally, transmissibility

Fig. 5A–D Mean ($\pm$ standard deviation) (A) bone fractional area, (B) bone volume, (C) bone thickness, and (D) tissue mineral density of new bone in the defect area of control rats (CTR) and rats subjected to low-level accelerations (ACC) are shown for scaffolded and nonscaffolded defects 4 weeks and 8 weeks into the protocol. The treatment effect was greater at 8 weeks than at 4 weeks and greater in scaffolded defects than in non-scaffolded defects.

Table 1. Changes (mean $\pm$ standard deviation) between 4 and 8 weeks

Scaffold	Group	BA (%)	BV (mm ³)	TH (mm)
No scaffold	CTR	8.5 $\pm$ 6.5 (p = 0.02)	1.26 $\pm$ 0.51 (p = 0.02)	0.03 $\pm$ 0.03 (p = 0.06)
	ACC	11.4 $\pm$ 7.5 (p = 0.004)	2.33 $\pm$ 1.06 (p < 0.001)	0.06 $\pm$ 0.07 (p = 0.06)
	p*	0.46	0.03	0.34
Scaffold	CTR	11.4 $\pm$ 9.0 (p = 0.03)	1.24 $\pm$ 0.97 (p = 0.03)	0.02 $\pm$ 0.01 (p = 0.007)
	ACC	18.7 $\pm$ 10.4 (p = 0.001)	3.64 $\pm$ 1.32 (p < 0.001)	0.08 $\pm$ 0.05 (p = 0.001)
	p*	0.18	0.002	0.007

p values in parentheses reflect the differences between 4 weeks and 8 weeks for a given group; * differences in the 4-week to 8-week changes between CTR (control rats) and ACC (experimental rats); BA = bone fractional area; BV = bone volume; TH = thickness; TD = tissue density.

from the plate into the cranium was confirmed only qualitatively and it is possible the matrix and cells around and in the defect experienced acceleration magnitudes that were substantially different from those generated by the vibrating plate. Although this limitation has no bearing on the conclusions of this study, transmissibility and the mechanical environment induced in the matrix/cells need to be investigated to optimize the stimulus and explore the underlying physical and cellular mechanisms.

During the last decade, evidence for the anabolic and antiresorptive effects of high-frequency, low-level mechanical signals has accumulated [6, 17, 21]. These signals are most commonly applied through foot-based whole body vibrations [34] but retain their efficacy when applied as high-frequency oscillatory motions [15, 31]. Our data suggest this stimulus applied to the cranium also can enhance regeneration of new bone in a rat calvarial defect. This effect was observed in defects with or without a

Fig. 6A–B Histologic sections of calvarial defects (40× magnification), stained with Masson trichrome (MT), of (A) a control (CTR) rat and (B) an experimental (ACC) rat showed that healing in defects subjected to the oscillatory accelerations proceeded normally.

* = calvarial bone at defect margin; NB = new bone inside of defect margin; FT = fibrous tissue; SDT = subdermal tissue; V = vessel.

supporting collagen scaffold. In the quiescent adult skeleton, primarily trabecular bone responds to high-frequency mechanical signals, whereas cortical bone is unresponsive [31, 37]. Consistent with the high sensitivity of the regenerative processes in the cortical calvarial defect to the high-frequency signal, even cortical bone can respond to this mechanical signal if the skeleton is undergoing modeling [17, 49]. Together with recent data suggesting mechanical stimuli may accelerate differentiation and/or proliferation of cells in general [19, 25], and encourage differentiation of mesenchymal stem cells toward the osteogenic cell lineage [9, 38], this may suggest the presence of specific stem cells and osteogenic cell populations are critical for efficacy of high-frequency mechanical signals.

The benefit of the applied mechanical signal continued even during the 4-week rest period, perhaps suggesting the additional bone and vessels formed during the treatment period acted as a secondary resource for osteogenic and stem cells or the mechanism triggered by the signal remained active even after the stimulus had subsided [42]. The robust osteogenic effects of this noninvasive treatment observed after the 8-week experimental period also emphasizes some advantages over tissue-engineering approaches that rely on stem cells and/or physical signals such as ultrasound, electrical currents, or electromagnetic fields [1, 11, 28, 43]. Not only is application of the physical signal noninvasive, simple, and inexpensive, it also does not require incubation time, bears no substantial risk of transmitting infections and diseases, and is unlikely to promote development of tumors from electrical waves. Furthermore, current conventional treatments using bone grafts or tissue-engineered bone substitutes may be associated with disuse atrophy during the immobilization period ranging from several weeks to several months [26].

The promotion of scaffolds or osteoconductive bone substitutes to enhance osseous regeneration in bone defects is well known [3, 26, 29]. In the current study, the extremely low-magnitude, high-frequency mechanical stimulus caused greater increases in bone area, volume, and thickness in scaffolded than in nonscaffolded defects. The large increase in bone fractional area in scaffolded defects that received oscillatory accelerations indicates the primary effect of the signal was the promotion of cells spreading across the defect. In defects without a supporting scaffold, the opportunity of cells to spread was inherently limited and the small-amplitude oscillations served to increase bone thickness rather than to increase bone area. Detailed μ CT-based studies were performed to evaluate the influence of scaffold pore structure on bone healing [32] or to describe healing patterns after guided bone regeneration [45] in the rat calvarial defect model. However, differences in the experimental design, including the absence of a nonscaffolded control group, precluded direct comparisons to our study and contrasting the regenerative effects of mechanical stimuli in bone defects to other bone-promoting agents requires further study.

Extremely low-magnitude, high-frequency accelerations applied for 20 minutes per day enhanced bone regeneration in an osseous defect in rats, particularly in the presence of an existing collagen scaffold that allowed for effective osteoconduction. The treatment effect was not limited to the 3-week period during which the stimulus was applied but extended into the subsequent 4-week rest period. Because no negative effects were observed and the extremely low-level physical signal can be applied to most skeletal segments in a safe manner [2] without requiring cell culture, growth factors, or stem cells, this method of promoting osseous regeneration has the potential to

overcome some disadvantages associated with current tissue-engineering approaches or exogenous devices.

Acknowledgments We thank Andrea Trinward for assistance in preparing the figures.

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