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J DENT RES 2012 91: 413 originally published online 14 February 2012

DOI: 10.1177/0022034512438590

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RESEARCH REPORTS

Biological

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J Dent Res 91(4):413-419, 2012

ABSTRACT

Mechanical stimulation contributes to the health of alveolar bone, but no therapy using the osteogenic effects of these stimuli to increase alveolar bone formation has been developed. We propose that the application of high-frequency acceleration to teeth in the absence of significant loading is osteogenic. Sprague-Dawley rats were divided among control, sham, and experimental groups. The experimental group underwent localized accelerations at different frequencies for 5 min/day on the occlusal surface of the maxillary right first molar at a very low magnitude of loading (4 $\mu\epsilon$). Sham rats received a similar load in the absence of acceleration or frequency. The alveolar bone of the maxilla was evaluated by micro-computed tomography (μ CT), histology, fluorescence microscopy, scanning electron microscopy (SEM), Fourier Transform Infrared Spectroscopy (FTIR imaging), and RT-PCR for osteogenic genes. Results demonstrate that application of high-frequency acceleration significantly increased alveolar bone formation. These effects were not restricted to the area of application, and loading could be replaced by frequency and acceleration. These studies propose a simple mechanical therapy that may play a significant role in alveolar bone formation and maintenance.

KEY WORDS: acceleration, high frequency, bone formation, gene expression, strain, mineralization.

DOI: 10.1177/0022034512438590

Received November 23, 2011; Last revision January 16, 2012; Accepted January 18, 2012

A supplemental appendix to this article is published electronically only at <http://jdr.sagepub.com/supplemental>.

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INTRODUCTION

The alveolar process of the jaw supports teeth during function. The loss of this bone has significant effects on the survival of teeth, as observed in millions of patients with periodontal disease. Significant resorption of the alveolar bone also has great impact on clinical dentistry, including the stability of removable prostheses and the success of dental implants. The combination of surgical and pharmaceutical methods for the maintenance or repair of alveolar bone has been suggested, but these techniques are invasive, costly, and have limited application. Therefore, a significant demand exists for a safe and non-invasive treatment for the preservation or increase of alveolar bone. In the medical field, the same demand to find safe and non-invasive treatment for bone loss led researchers to switch from pharmacotherapy, which has many side-effects (Mashiba *et al.*, 2000; Lacey *et al.*, 2002), to alternative treatment, such as mechanical stimulation (Rubin *et al.*, 2004).

Mechanical treatments rely on the ability of the skeleton to adapt to altered levels and patterns of mechanical loading. Studies on the effects of exercise and loading show an anabolic effect on weight-bearing bones (Honda *et al.*, 2001; Tanaka *et al.*, 2003). Similarly, jaws are exposed to significant mechanical stimulation (Herring, 2007), which plays an important role in the health of alveolar bone. The replacement of a regular diet with a soft diet (Bresin *et al.*, 1999; Mavropoulos *et al.*, 2004) or the lack of function due to missing teeth is accompanied by significant alterations in alveolar bone density or resorption (Cardaropoli *et al.*, 2003; Araujo and Lindhe, 2005). However, which mechanical stimulation has an osteogenic effect in alveolar bone is not known.

The osteogenic effects of mechanical stimulation in long bones have been related to the magnitude of the strain (matrix deformation) (Mosley *et al.*, 1997), strain-related derivatives (*e.g.*, strain rate) (O'Connor *et al.*, 1982), streaming potential and fluid flow (Qin *et al.*, 2003; Malone *et al.*, 2007), the frequency of the applied load (Rubin *et al.*, 2001a), and acceleration (Garman *et al.*, 2007).

Although studies on weight-bearing bones have provided fundamental information on the bone responses to different components of mechanical stimulation, caution on the generalization of similar conclusions for non-weight-bearing bones, such as jaws, is recommended. The embryonic origin of weight-bearing bones is different from that of craniofacial bones. Weight-bearing bones have

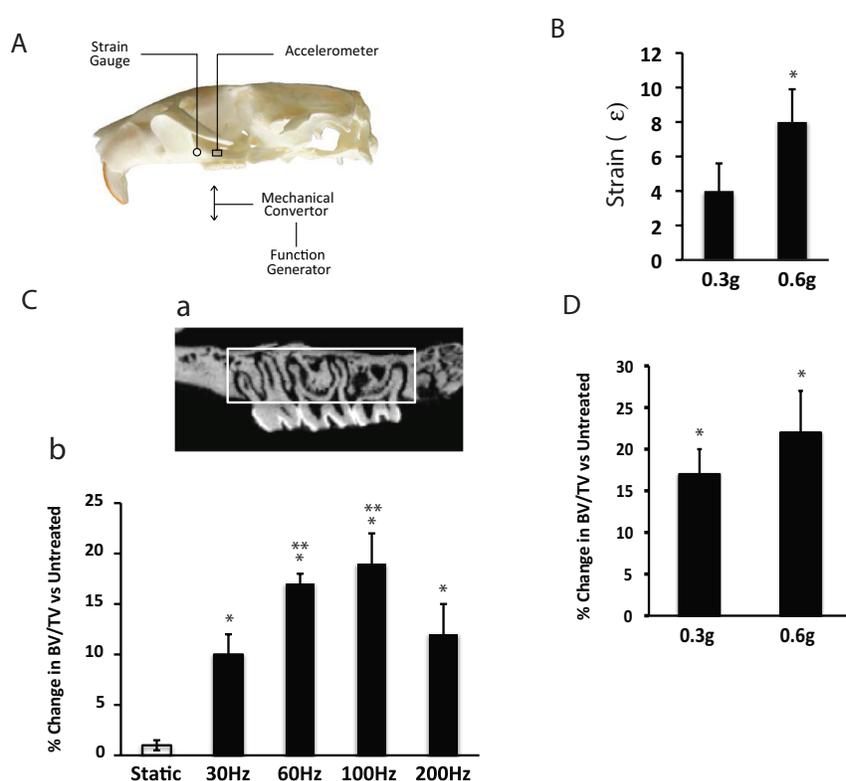


Figure 1. High-frequency accelerations increase alveolar bone volume. **(A)** Schematic of vibration application to the occlusal surface of the maxillary right first molar. **(B)** Average peak strain (mean \pm SEM) in buccal and palatal surfaces of the alveolar bone surrounding the upper first molar in response to 0.3 or 0.6 g acceleration with a set frequency of 60 Hz. *Significantly different from 0.3 g. **(C)** a: Schematic of the area of analysis using μ CT. b: Change in BV/TV from μ CT analyses of maxillae exposed to different frequencies at peak accelerations of 0.3 g and a peak strain of 4 $\mu\epsilon$ compared with untreated animals after 28 days. *Significantly different from untreated and static animals. **Significantly different from 30 Hz and 200 Hz. **(D)** Percentage change in BV/TV from μ CT analysis of maxillae exposed to different accelerations at a set frequency of 60 Hz compared with untreated animals after 28 days. Each value represents the mean \pm SEM of 4 samples. *Significantly different from untreated animals.

endochondral origin, which enables growth to occur under heavy mechanical loads. Most craniofacial bones are not exposed to heavy loads and form directly from mesenchymal cells (intra-membranous origin) (Teixeira *et al.*, 2010b). In addition, weight-bearing bones are exposed to direct loading, but alveolar processes are exposed to indirect loading *via* teeth, which produces a complex pattern of strain distribution due to periodontal ligaments. Therefore, a mechanical treatment regimen that is specifically suited to the alveolar bone is required. These stimuli should be applied through the teeth with a minimum load to minimize tooth damage.

MATERIALS & METHODS

Animal Model and Study Design

Adult male Sprague-Dawley rats ($n = 85$, average weight 400 g, 120 days of age) were treated according to a protocol approved by the New York University Institutional Animal Care and Use Committee. Animals were randomly divided into three groups: untreated (control), sham, and experimental. The experimental group received different high-frequency accelerations (vibration)

that produced a strain of 4 $\mu\epsilon$ (microstrain) on alveolar bone. The sham group received 4 $\mu\epsilon$ of static load, and the control group did not receive any intervention. All stimuli were applied to the occlusal surface of the right first maxillary molar for 5 min/day for 28 days under the influence of 3% isoflurane. Animals were sacrificed by CO₂ narcosis, and the hemimaxillae were collected for different studies [4 animals *per* condition for μ CT analysis (7 \times 4 = 28)]; these same animals were used for fluorescence microscopy and FTIR analysis, 3 animals for paraffin embedding (3 \times 3 = 9), 5 animals for RT-PCR at 3 time-points (3 \times 3 \times 5 = 45), and 3 animals for acceleration and strain measurements. Bone labeling was performed by means of an intraperitoneal injection of calcein (15 mg/kg) on days 0 and 26.

Acceleration and Strain Measurements

Devices for mechanical stimulation in the 30-, 60-, 100-, and 200-Hz frequency range and accelerations of 0.3 g and 0.6 g were prepared and calibrated at the Mechanical Engineering Department of the Polytechnic Institute of Viseu-Portugal. Device calibration was performed with a sensor (OMRON – E2E – X7D1-N 23304; OMRON Electronics Iberia SAU, Lisbon, Portugal) that was connected to an oscilloscope (Metrix OX 803B 40 MHz, Metrix Electronics, Hampshire, United Kingdom) and a Digital Tachometer (Lutron DT 2236, Lutron Electronic Enterprise, Taipei, Taiwan). Strain gauges (UFLK-1-11-1L, 1 mm gauge length, 120 Ω , TML Gages, Texas Measurements, College Station, TX, USA) were attached (cyanoacrylate) to the palatal and buccal sides of the alveolar bone near the first maxillary molar on fresh and dry skulls. Strain signals were amplified by a low-noise amplifier (SX500, Beacon Dynamics, Byram Township, NJ, USA). Data acquisition and analyses were performed with the SPIDER 8 system and Catman 4.5 software (HBM, Darmstadt, Germany), respectively. Acceleration was measured with a piezoelectric sensor-Bimorph vibration element, 4 V 5 mm (Allied Electronics, Fort Worth, TX, USA), and a MotionNode 3-DOF inertial measurement unit (GLI Interactive, Seattle, WA, USA).

FTIR Analysis and Fluorescence Microscopy

Specimens were fixed in formalin, washed overnight, dehydrated in an alcohol series, cleared with xylene, and embedded in methylmethacrylate (Erben, 1997). The samples were sectioned at a 2- μ m thickness on a SM 2500 Leica microtome and placed on BaF₂ windows (Spectral Systems, Hopewell Junction,

NY, USA). FTIR images were acquired with a Spectrum Spotlight 100 imaging system (Perkin-Elmer Instruments, Waltman, MA, USA) in transmission mode at a spectral resolution of 4 cm and pixel size of 6.25 x 6.25 μm. All FTIR images were processed with ISYS Chemical Imaging software (Spectral Dimensions Inc., Olney, MD, USA). Some samples were sectioned at a 5 to 7 mm thickness and viewed under fluorescent microscopy to detect calcein labeling (Nikon Microscopy, NIS-Elements software, Tokyo, Japan). Histology and μCT analysis were performed as described previously (Teixeira *et al.*, 2010a) and in Appendix 1.

Reverse Transcriptase-Polymerase Chain-reaction Analysis

Five randomly selected animals from each group were sacrificed on days 0, 3, and 14, and the hemimaxillae were immediately dissected and frozen in liquid nitrogen. After mRNA isolation, gene expression was evaluated as described previously (Teixeira *et al.*, 2010a). Each mRNA specimen was tested 3 times. Relative levels of mRNA were calculated and normalized to the mRNA levels of GAPDH and acidic ribosomal protein (Teixeira *et al.*, 2010a).

Statistical Analysis

Significant differences between test groups and controls were assessed by analysis of variance (ANOVA). Pairwise multiple comparison analysis was performed by Tukey's *post hoc* test. Two-tailed p-values were calculated, and p < 0.05 was set as the level of statistical significance.

RESULTS

High-frequency Accelerations in the Absence of a Significant Force Are Osteogenic

A device was developed to deliver vibration to the upper right first molar along its longitudinal axis (Fig. 1A and Appendix 2). An average peak strain of 4 με was induced in the buccal and palatal plates of the alveolar bone in the proximity of the upper right first molar. The doubling of the peak acceleration to 0.6 g doubled the strains to 8 με (Fig. 1B). Bone density

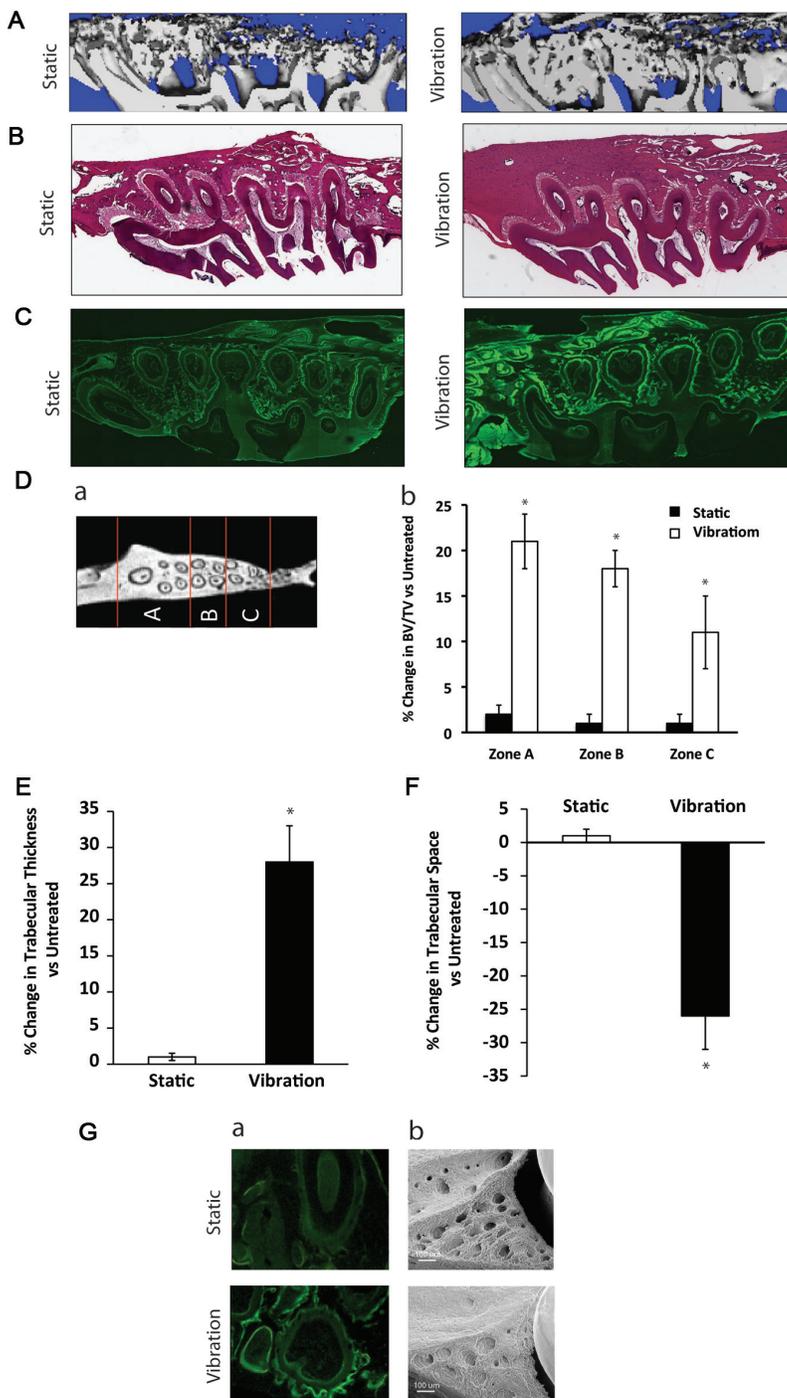


Figure 2. Osteogenic effect of high-frequency acceleration is not limited to the point of application. Sagittal sections from maxillary alveolar bone of the vibration (60 Hz, 0.3 g, 4 με) group and the static group (4 με) 28 days post-treatment. (A) μCT 3D reconstruction of alveolar bone showing changes in trabecular spacing and thickness. (B) Photomicrographs of the entire alveolar bone stained with H&E. (C) Fluorescence microscopy of sections showing calcein labeling. The increased intensity of the label in most of the trabecular surface in the vibration group is indicative of extensive bone modeling. (D) a: Schematic indicating the coronal sections (A, B, C) used in the analysis. b: Bone volume fraction in different zones of alveolar bone in the vibration (60 Hz, 0.3 g, 4 με) and static groups (4 με) at 28 days post-treatment. (E) Average trabecular thickness (F) and trabecular spacing changes in Zone A of the alveolar bone in the vibration and static groups compared with untreated animals after 28 days. Each value represents the mean ± SEM of 4 samples. *Significantly different from untreated and static animals. (G) a: Fluorescence microscopy and b: SEM images of the cortical bone around the mesiobuccal root of the maxillary right first molar reveal the bone modeling activity and changes in the appearance of cortical bone.

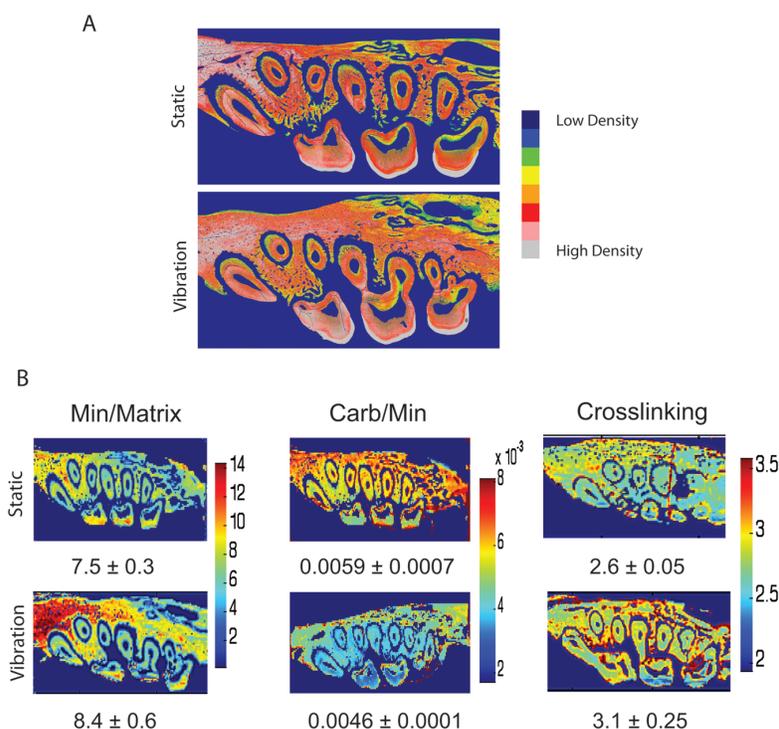


Figure 3. High-frequency acceleration changes the bone mineral content of alveolar bone. Longitudinal sections through the rat right alveolar bone of the vibration (60 Hz, 0.3 g, 4 $\mu\epsilon$) and static groups (4 $\mu\epsilon$) after 28 days of mechanical stimulation. **(A)** SEM images color-coded for visualization of the differences in mineral density. **(B)** FTIR images of the static (top row images) and the vibration group (bottom row images) alveolar bone showing *in situ* changes in the mineral-to-matrix ratio (min/mat), carbonate-to-mineral ratio (carb/min), and collagen crosslinking (crosslinking). The color scale is included for easier visualization of quantitative differences. The mean values \pm SD are also included. All FTIR data show significant differences between static and vibration animals.

was measured in an area shown in Fig. 1C.a. Four different frequencies were tested (Fig. 1C.b). A 28-day application of a 4- $\mu\epsilon$ static force was not osteogenic ($p > 0.05$), but the application of a similar strain at a peak acceleration of 0.3 g and frequencies of 30, 60, 100, and 200 Hz increased BV/TV in alveolar bone 10%, 17%, 19%, and 12%, respectively, compared with untreated animals ($p < 0.05$) (Appendix 3). Bone formation in response to 60 Hz and 100 Hz was statistically higher than that in response to 30 Hz and 200 Hz ($p < 0.05$), but there was no significant difference between accelerations of 60 Hz and 100 Hz. Therefore, experiments at 60 Hz were continued. An increase in acceleration from 0.3 g to 0.6 g increased BV/TV from 17% to 21%, respectively, at 60 Hz (Fig. 1D and Appendix 3). This effect may be the result of both increase in acceleration and higher strain.

Osteogenic Effect of High-frequency Acceleration Is Not Limited to the Area of Application

The μ CT scans of hemimaxillae, light microscopy (H&E sections), and fluorescence microscopy (Figs. 2 A, B, C) showed no differences between the untreated and static force groups, while the osteogenic effect of vibration extended beyond the point of application to adjacent bone. The alveolar bone was divided into 3 zones (A, B, and C) that corresponded to the bone

surrounding the upper right first, second, and third molars, for investigation of whether the osteogenic effect of vibration is equal in all these areas (Fig. 2D.a). Increases in BV/TV of 21%, 18%, and 11% were observed in zones A, B, and C, respectively, compared with untreated animals ($p < 0.05$ for all zones). These results demonstrated that the osteogenic effect of vibration had a gradient response that was greater near the point of application (Fig. 2D.b and Appendix 3). A detailed analysis of the μ CT in Zone A revealed that the increase in BV/TV occurred primarily through an increase in trabecular thickness (27%) (Fig. 2E) and a consequent decrease in trabecular spacing (26%) (Fig. 2F) ($p < 0.05$).

Predominant effects were observed in trabecular bone, but bone formation was not limited to this area. Fluorescence microscopy and SEM showed similar effects in cortical bone adjacent to PDL (Fig. 2G.a) and at the alveolar crest (Fig. 2G.b).

High-frequency Acceleration Alters the Alveolar Bone Mineral Content

SEM images of alveolar bone at 28 days post-treatment demonstrated a higher mineral density in the vibration (60 Hz, 0.3 g, 4 $\mu\epsilon$) group compared with the static group (4 $\mu\epsilon$) (Fig. 3A). FTIR imaging also demonstrated a higher mineral density in response to vibration (Fig. 3B). The carbonate content decreased, which may lead to decreased solubility. Collagen crosslinking, which is a measurement of collagen maturity, increased. Overall, analysis of these data demonstrated a higher rate of mineralization in alveolar bone, confirming μ CT data (not shown).

High-frequency Acceleration Induces the Expression of Bone Markers and Regulators

The expression of 92 different osteogenic-related genes was studied by RT-PCR at 0, 3, and 14 days after vibration application (Fig. 4). The expression of 26 genes in the rats that received vibration (0.3 g, 60 Hz) was significantly higher ($p < 0.05$) on day 14 compared with that in the static force group. The expression of 6 growth factors (Fig. 4A), 6 growth factor receptors (Fig. 4B), and 5 transcription factors, which play an important role in osteoblast differentiation (Fig. 4C), increased 2- to 3.5-fold. This increase was accompanied by a 2.5- to 6-fold increase in the expression of extracellular matrix proteins (Fig. 4D) and a 2.5- to 3.5-fold increase in the expression of mineralization proteins (Fig. 4E). At day 3, the expression of EGF, FGF2, Collagen I, Runx2, Smad3, and COMP expression increased 2- to 3-fold ($p < 0.05$). No differences were observed in the static group between 3 and 14 days.

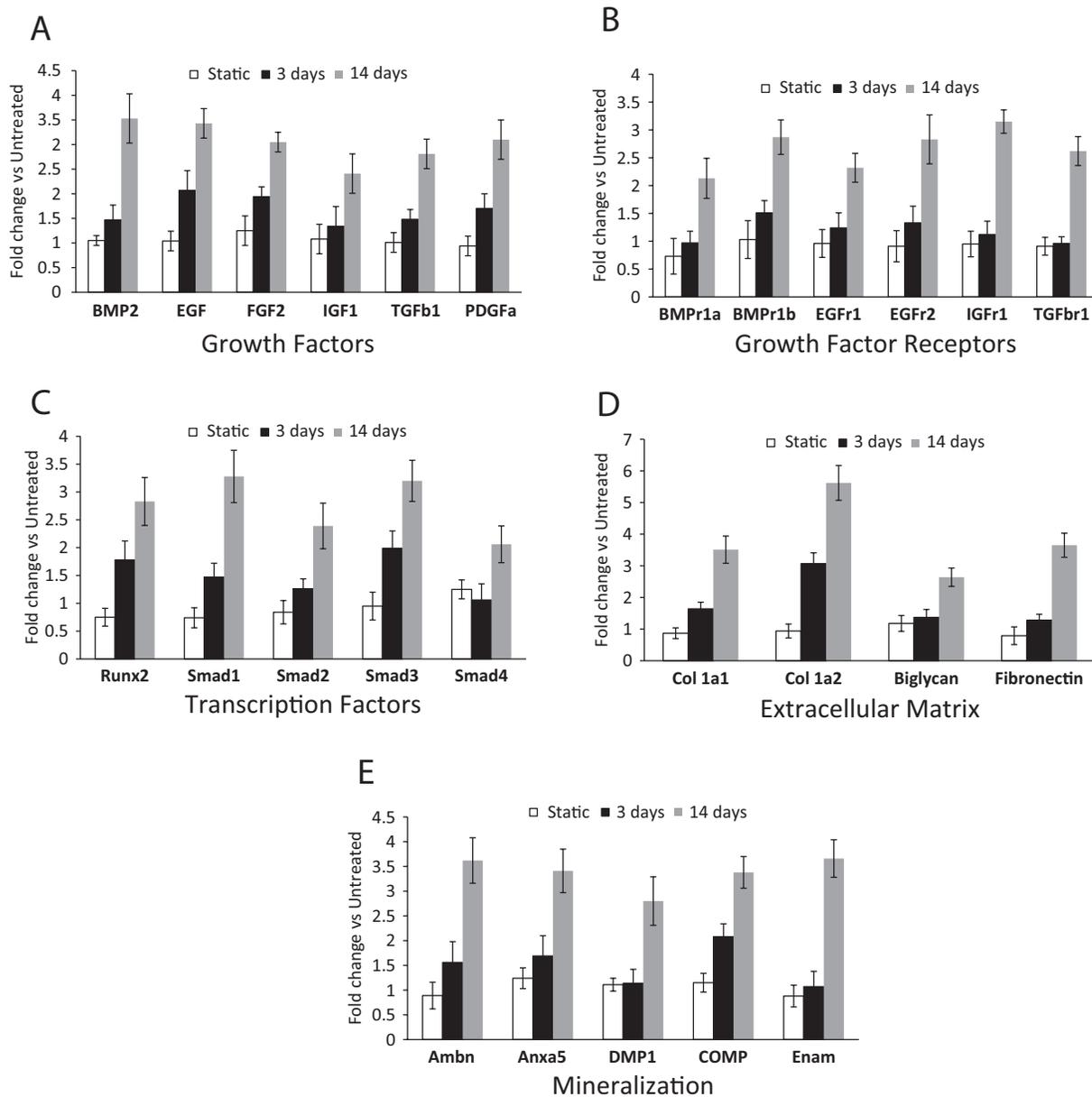


Figure 4. High-frequency acceleration induces the expression of bone markers and regulators. Mean “-fold” increases in the expression of osteogenic growth factors (A), growth factor receptors (B), transcription factors (C), extracellular matrix proteins (D), and proteins involved in matrix mineralization (E) are shown for the static group (after 14 days) and the vibration (60 Hz, 0.3 g, 4 μ s) group at days 3 and 14. Data are shown as a fold-change in gene expression compared with the untreated group. All data from the 14-day vibration group are significantly different from those of the static group at 14 days and the vibration group at 3 days.

DISCUSSION

The current study investigated the components of mechanical stimulation that are osteogenic in alveolar bone and safe for application through teeth. Our results suggest a possible interaction among the magnitude of the strain, frequency, and acceleration in which a decrease in one factor should be compensated by an increase in other factors for a signal to be osteogenic.

The magnitude of the applied load was minimized to a level far below the osteogenic threshold to separate the osteogenic effect of the strain magnitude from the osteogenic effects of frequency and acceleration (Turner *et al.*, 1994). Our experiments

demonstrated that, in the absence of significant load, it is possible to increase bone formation by increasing both frequency and acceleration. These results are consistent with those from previous studies that have shown that small oscillatory accelerations independent of matrix deformation can enhance bone formation in weight-bearing bones (Garman *et al.*, 2007).

Higher accelerations are usually accompanied by higher strains, which limit the application of higher acceleration as the osteogenic source in the mouth. Changes in frequency are a safe compensation for this shortcoming. Our experiments demonstrated that constant loading and acceleration produced higher levels of bone formation in response to higher frequencies. This

is in agreement with previous studies showing that high-frequency low-magnitude forces are osteogenic in weight-bearing bones (Rubin *et al.*, 2001b). This effect is important in clinical situations, such as periodontal disease or newly placed implants, in which mechanical stimulation that relies on the application of a large load may be infeasible because of the fragility of the area.

The highest osteogenic effect of vibration was near the application point, which is consistent with previous studies showing that the osteogenic effect of mechanical stimulation is site-specific (Judex *et al.*, 1997). The osteogenic effect of vibration in our study exhibited a gradient response, demonstrating an anabolic effect on adjacent alveolar bone that is distant from the point of application. This is clinically significant, because this procedure permits an increase in bone formation in fragile areas with vibration application on teeth away from those areas.

The mechanism behind these changes is unclear. Our gene expression studies suggest that the increase in trabecular thickness was due to an increase in osteoblast activity rather than cellular proliferation, because the number of osteoblasts *per mm*³ was not different (data not shown), while the expression of type I collagen and other non-collagenous matrix proteins increased. This new bone matrix had increased collagen cross-linking, which suggested an acceleration of bone deposition and maturation by resident osteoblasts. Studies in long bones did not produce similar results, which can be related to differences in mechanical stimulation regimen, time-points and genes studied, and different types of bones (Judex *et al.*, 2005; Kotiya *et al.*, 2011). Our results also support an important role for vibration during the mineralization process. The expression of proteins that are responsible for initial crystal formation, such as annexin 5 and biglycan, and crystal growth and organization, such as enamel and DMP1, significantly increased in response to vibration, which is consistent with the FTIR findings.

Both the trabecular and cortical bones responded to vibration, but the higher response in trabecular bone suggested that other factors play a role in the regulation of cellular activity, such as the surface-to-volume ratio between the trabecular and cortical bones. The effect of vibration on osteoclast activation was not investigated in this study. Changes in the activity or number of osteoclasts may play a role in the long-term effect of vibration on alveolar bone density. However, it is unlikely that osteoclasts were the main target of the vibration effect on bone volume, due to the short duration of our study.

Other therapeutic modalities, such as ultrasound (Duarte, 1983; El-Bialy *et al.*, 2002), electric fields (Bassett *et al.*, 1964), and magnetic fields (Yan *et al.*, 1998; Xu *et al.*, 2001), have been suggested to increase bone formation. Unfortunately, the cost and complexity of these approaches have limited their application to alveolar bone. Our studies suggest a simple mechanical therapy that may play a significant role in alveolar bone formation and maintenance.

ACKNOWLEDGMENTS

This investigation was supported by Grant 5K08DE017426 and AR046121 from National Institutes of Health, Bethesda, MD 20892. The work described in the article provides the basis for the following patent held by New York University (US Patent application No. 12/555,964).

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