Strategic Use of Ultrasonic Frequencies for Targeted Bone Biomodification Following Piezoelectric Bone Surgery in Rats. Part I: Early Phase

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Piezoelectric surgery utilizes selective piezoelectric vibrations for cutting bone tissues without damaging soft tissues. The piezoelectric knife was developed for atraumatic bone surgery using three-dimensional (3D) ultrasonic vibrations as an alternative to the mechanical and electrical instruments used conventionally, and it has selective cutting capabilities for different ultrasonic frequencies. The knife was introduced in 1988 by Tomaso Vercellotti as a unique treatment method for “sinus lift” surgery. The piezoelectric surgery units typically used ultrasonic frequencies ranging from 28 to 36 kHz to cut osseous tissues while safely avoiding damage to soft tissues in the operative field. Nowadays, the use of the piezoelectric knife has expanded to the fields of maxillofacial, craniofacial, periodontal, endodontic, orthopedic, and neural surgeries. In 2009, Piezocision was introduced as a new minimally invasive procedure for surgically facilitated orthodontics. This technique combines micro-incisions and piezoelectric bone decortications with selective tunneling that allows for hard or soft tissue grafting. Its clinical benefits have proven to be substantial. In a randomized control trial, Charavet et al demonstrated a significant reduction in patients’ orthodontic treatment time when

Piezocision can set in motion a cascade of physiologic events that lead to accelerated orthodontics, but do all ultrasonic frequencies generate the same effects on bone? Two different Piezotome modulation frequencies (10 and 30 Hz) were tested on the rat maxilla. The animals were sacrificed at days 1, 3, 7, 14, 28, and 70, and MRI, histologic, and biochemical analyses were performed. The results indicated that at 30 Hz, the demineralization process started at day 1 and peaked at day 7, and was initiated by osteocyte apoptosis. The process was different in the two groups, with bone demineralization increasing significantly in the 30-Hz group compared to the 10-Hz group (P < .05). These results could indicate that bone biomodification is frequency-dependent.


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using Piezocision-assisted vs conventional orthodontics (43% time reduction). Piezocision is based on the judicious use of the Regional Acceleratory Phenomenon (RAP) following bone injury to facilitate tooth movement, and it is bimodal in nature with a demineralization phase (early phase) and a remineralization phase (later phase), of which the exact cellular mechanism is still partially unknown at this time.

The authors designed the present study to shed some light on the various cells’ roles and interactions during both processes as well as to expand the understanding of frequency-related bone biomodification when using the piezoelectric knife. Two modulation frequencies (or pulse repetition rates) of 10 and 30 Hz were used with the Piezotome 2 (Acteon). The early biologic effects of Piezosurgery-induced bone resorption were the focus of the first part of the study, including the activation, resorption, and initiation of the reversal phase. The depth of penetration was standardized to reach the medullary bone for proper RAP activation. Information on this early phase could lead to a better understanding of its use during orthodontic tooth movement (the bone being more “pliable”). The second part of this study was devoted to the same aims but during the remineralization phase, to see if the information gathered could be used to enhance new bone formation during guided bone regeneration or pre-implant preparation of the bone site.

Materials and Methods

All animal procedures were approved by the Boston University Medical Center Institutional Animal Care and Use Committee (BUMC IACUC), protocol #AN-15335. Nine-week-old male Sprague Dawley rats (N = 93), each weighing approximately 300 g, were purchased from Charles River Laboratories International. The animals were acclimatized in the animal care facility for at least 48 hours prior to surgery. To monitor systemic health, postoperative weight was measured and hematologic analyses were performed. The piezoelectric knife (BS1 insert) of the Piezotome 2 was used with its fixed power setting and a modulation frequency of 30 Hz (group 1) or 10 Hz (group 2). Tissue responses were studied at days 1, 3, 7, 14, 28, and 70. Five animals in each group were euthanized at each time point. Five untreated controls that did not undergo surgery were also euthanized at each time point (group 3). Samples were obtained at day 0 as a baseline (n = 3).

Surgical Procedure

The animals were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). Two vertical cuts were placed on the maxillary palatal gingiva, one mesial and one distal to the right first molar. Decortication was carried out to reach the medullary bone (approximate depth: 0.5 mm) using the Piezotome 2 and an amplitude of either 10 or 30 Hz, with 60-mL/minute sterilized saline irrigation. After euthanization by carbon dioxide, the maxilla was removed and split from the midpalatal suture for analysis. One half was taken for morphometric and histologic evaluation and immediately fixed with 10% paraformaldehyde. The other half was immersed in liquid nitrogen for snap freezing, then kept at –80°C for future immunologic or molecular analysis.

MRI Analysis

The Bruker 500-Hz magnetic resonance imager is specifically designed to image small rodents and tissues. The magnetic resonance imaging (MRI) analysis was performed at Boston University MRI/NMR High Field Imaging Core facility.

Isolation of Serum

Serum was isolated from each rat following protocol previously described. After centrifugation, serum was apportioned into 0.5-mL aliquots and stored at –80°C.

Hematologic analysis: Hemavet 950 FS auto blood analyzer (Drew Scientific) was used. Blood was collected following protocol previously described, and the sample was analyzed within 2 hours after collection.

Serum protein assay: Total protein concentration was determined by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific), with bovine serum albumin as the standard (2 mg/mL).
Serum calcium assay: The calcium assay utilizes an optimized variant of the well-established o-Cresolphthalein-calcium reaction.

Rat serum C-terminal telopeptide of collagen Type I (CTX-I) assay: The RatLaps (CTX-I) EIA kit (Immunodiagnostic Systems) was used to measure the C-telopeptide degradation products from type I collagen released in the peripheral blood. This measurement is more specific for bone resorption than other measurements.9

Histologic Analysis

For histologic analysis, the maxilla was decalcified by immersion in a volume of 5% ethylenediaminetetraacetic acid (EDTA). Decalcified tissue samples were embedded in paraffin and stained with hematoxylin-eosin (h&e).7 Samples were also stained with DAPI (4',6-diamidino-2-phenylindole) for deoxyribonucleic acid (DNA) damage screening in osteocytes associated with micro-damages. TUNEL (Terminal deoxyribonucleotidyl transferase dUTP nick end labeling) staining was used to assess early osteocyte apoptosis. Digital images of stained cells were captured using an immunofluorescent microscope.

Tartrate-resistant acid phosphatase (TRAP) staining was used to identify osteoclast activity.10 Masson’s trichrome, reticular fiber, and picro-sirius red staining analyses were used to identify morphologic changes in the collagen structure (new collagen deposition and/or new bone formation).11 Reticular fibers are composed of type III collagen secreted by reticular cells. Reticular fiber staining was used for collagen fiber orientation.12

Statistical Analyses

Statistical analyses used one-way analysis of variance (ANOVA; JMP Pro 13). If F values were significant (F < 0.05), Tukey honestly significant difference (HSD) test was used (P < .05 as significant) between each group.

Results

Morphologic Change After Piezoelectric Bone Surgery (Post-Piezosurgery) in Rats

The MRI analysis helped quantify the demineralization and remineralization time-dependent morphologic changes in alveolar bone following Piezosurgery (Fig 1). Compared to the 10-Hz group, the 30-Hz frequency group resulted in significant post-Piezosurgery morphologic changes. The alveolar bone demineralization process/resorption phase started at day 1 and peaked at day 7. The remineralization process/formation phase began at day 14 and continued until complete healing at day 70.

Postoperative Weight Gain

In the early postoperative phase, both the 10- and 30-Hz groups showed weight loss due to discomfort/difficulty in chewing (ANOVA: probability of F < 0.01; Tukey HSD: P < .05). This was later self-corrected with total body weight gains in both groups as of day 14 (Fig 2a).

Hematologic Analysis

Hematologic analysis was performed to assess the systemic impact by Piezosurgery (Table 1). Each group’s postoperative white blood cell population was assessed on days 0, 1, 3, 7, 14, 28, and 70. Differential white blood cell counts indicated a relative percentage of each type of white blood cell. In peripheral blood, the number of PMN white blood cells significantly increased in the 10- and 30-Hz groups at day 3 and returned to normal range values by day 7, remaining there throughout the experiment (Table 1). There was no significant difference between the control group and the experimental groups for monocytes and lymphocytes (Table 1).

Serum Total Protein Analysis

Based on morphologic changes shown by the MRI analysis, the authors hypothesized that serum protein would change during the healing process. The 30-Hz stimulus significantly increased total serum protein levels from day 7 to day 70 compared to the control group (Fig 2b). Serum proteins in the 10-Hz group did not change significantly during the experiment.
Serum Calcium Level

The MRI and histologic data suggested that Piezosurgery induced alveolar bone degradation. Thus, the authors expected that the released calcium would result in significantly increased calcium levels in the peripheral blood. This occurred only at day 1 in both the 10- and 30-Hz groups (Fig 2c).

Collagen Fiber Destruction in the Early Bone Demineralization Phase

In the 30-Hz group, the histologic analysis using Masson’s trichrome collagen and reticulin stain indicated that alveolar bone degradation was evident from days 1 to 7, and bone regeneration started occurring from day 14 (Fig 3).

Nucleus Staining in the Early Phase of Post-Piezelectric Bone Injury

The first hypothesis was that piezo-electric bone injury might create microdamage and induce osteocyte apoptosis in the early postoperative phase by activation of RAP. Histologic samples were stained with DAPI to observe DNA damage in osteocytes (Fig 4a). DAPI-positive osteocytes were slightly reduced at day 1, and no positive cells were detected at day 3. Ultrasonic microdamage in bone is associated with osteocyte death by apoptosis, which signals target osteoclasts to initiate remodeling at a damaged site. Under normal conditions, osteocytes express high amounts of transforming growth factor beta (TGF-β) and thus repress bone resorption. However, when bone is old or damaged, bone resorption is accelerated by decreased TGF-β expression and increased expression of osteoclast-stimulatory factors, such as receptor activator of nuclear factor kappa-B ligand (RANKL) and macrophage colony-stimulating factor. The MRI and histologic analyses indicated that alveolar bone degradation already began at day 1, was induced by osteocyte apoptosis, and was significantly higher in the 30-Hz group (Fig 4b).
Piezoelectric Bone Injury–Enhanced Osteoclast Activity in a Time-Dependent Manner

The second hypothesis was that piezoelectric bone injury would increase osteoclastic activity above baseline levels in the resorption phase by activation of the RAP. TRAP staining was used to evaluate osteoclast activity (Fig 5a). TRAP activity increased significantly in the 30-Hz group—peaking at day 3 and decreasing at day 14—compared to the control group.

Serum CTX Level

CTX-1 is a common diagnostic laboratory test used to assess bone resorption in patients with metabolic bone diseases. In the 30-Hz group, the serum CTX-1 levels gradually increased from day 1 to day 3, peaked at day 7, then returned to and maintained initial levels afterwards (Fig 5b). There was no significant difference between the 10-Hz and control groups.

Discussion

The clinical significance of accelerating orthodontic tooth movement has been conceptualized for almost a century, and a number of surgical and physical approaches have been used, in conjunction with conventional orthodontics, to this effect. Burs, the piezoelectric knife, screws, and lasers have been used to create a bone injury (corticotomies) that will initiate the RAP. It is well known that osteocytes are the major mechano-sensors in bone, responsible for

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Table 1 Hematologic Analyses of the Study Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Systemic variable</th>
<th>Postoperation (d)</th>
<th>0</th>
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<th>3</th>
<th>7</th>
<th>14</th>
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<td>Control, n (%)</td>
<td>NE</td>
<td>5.3–38.1</td>
<td>34.82 (1.44)</td>
<td>36.88 (1.28)</td>
<td>34.79 (3.04)</td>
<td>29.88 (5.30)</td>
<td>25.37 (7.53)</td>
<td>26.19 (7.95)</td>
<td>34.62 (2.48)</td>
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<td>LY</td>
<td>56.7–93.1</td>
<td>60.48 (1.36)</td>
<td>56.47 (0.85)</td>
<td>60.02 (3.18)</td>
<td>64.39 (5.90)</td>
<td>69.84 (8.99)</td>
<td>67.74 (8.42)</td>
<td>58.95 (2.68)</td>
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<td>MO</td>
<td>0.0–7.7</td>
<td>4.34 (0.27)</td>
<td>6.42 (1.06)</td>
<td>4.54 (0.44)</td>
<td>5.18 (1.24)</td>
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<td>EO</td>
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<td>0.34 (0.22)</td>
<td>0.20 (0.06)</td>
<td>0.59 (0.42)</td>
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<td>0.02 (0.02)</td>
<td>0.03 (0.02)</td>
<td>0.05 (0.03)</td>
<td>0.12 (0.00)</td>
<td>0.20 (0.17)</td>
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<td>10-Hz Piezosurgery, n (%)</td>
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<td>45.57 (4.18)</td>
<td>42.30 (6.68)</td>
<td>29.99 (6.54)</td>
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<td>23.02 (7.94)</td>
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<tr>
<td></td>
<td>LY</td>
<td>56.7–93.1</td>
<td>48.34 (3.85)</td>
<td>51.68 (7.47)</td>
<td>65.78 (6.18)</td>
<td>61.74 (3.72)</td>
<td>62.24 (5.53)</td>
<td>72.46 (8.25)</td>
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<td>5.25 (1.04)</td>
<td>3.88 (1.29)</td>
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<td>5.99 (0.14)</td>
<td>4.37 (0.46)</td>
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<td>0.02 (0.02)</td>
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<td>30-Hz Piezosurgery, n (%)</td>
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<td>37.27 (13.27)</td>
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<td></td>
<td>LY</td>
<td>56.7–93.1</td>
<td>72.69 (11.33)</td>
<td>45.38 (3.40)</td>
<td>62.78 (8.11)</td>
<td>54.89 (14.18)</td>
<td>55.45 (11.69)</td>
<td>73.90 (6.59)</td>
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<td></td>
<td>MO</td>
<td>0.0–7.7</td>
<td>3.75 (1.22)</td>
<td>5.71 (0.89)</td>
<td>4.41 (0.47)</td>
<td>7.39 (1.86)</td>
<td>6.39 (0.73)</td>
<td>3.74 (0.47)</td>
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<td></td>
<td>EO</td>
<td>0.0–3.4</td>
<td>0.31 (0.15)</td>
<td>0.54 (0.28)</td>
<td>0.47 (0.21)</td>
<td>0.39 (0.21)</td>
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<td>0.0–0.4</td>
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NE = neutrophils; LY = lymphocytes; MO = monocytes; EO = eosinophils; BA = basophils.

In peripheral blood, the number of PMNs significantly increased in both groups at day 3 (P < .05), then returned to normal range values by day 7 and remained stable throughout the experiment (bolded numbers are values outside the normal range).

There was no significant difference between the control group and the experimental groups for monocytes and lymphocytes.
sending signals that influence general bone remodeling through induction of osteoclastic bone resorption or osteoblastic bone formation.\(^{15,16}\)

But are all corticotomies equal?

In a previous publication, the authors compared various corticotomy methods on calvarial bone.\(^{17}\) Corticotomies done with the piezoelectric knife, the high-speed bur, and the hand-held screw device were tested for similarities or differences. The Piezotome 2–driven corticotomy (D1 setting, BS1 insert) appeared to generate, for the same surgical injury, a much greater degree of demineralization when compared to corticotomies forged by burr or screw perforation. This could be due to the additive effect of the osteocyte response to the low frequency vibrations from the Piezotome, which couples with and amplifies the natural response to surgical injury. Similarly, in the second phase of this bimodal response, bone apposition seemed to be enhanced when using the Piezotome compared to the other two devices. The logical next step for the authors was to investigate this phenomenon further by comparing two different Piezotome modulation frequency settings (10

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**Fig 2** (a) Postoperative weight loss in the early phase. Both groups show significant weight loss until day 7. In the mid-postoperative phase (day 14), there are no significant differences between each group. In the late phase, there is a similar slope gradient from day 28 to day 70 in each group. (b) Postoperative total serum protein counts. Compared to the control group, the 30-Hz stimulus significantly increased total serum protein levels from day 7 to day 70. Serum proteins in the 10-Hz group did not change significantly during the experiment. (c) Postoperative serum calcium levels. There was a significant increase in blood calcium level in the peripheral blood only at postoperative day 1 in both groups. *P < .05, compared with each time point control group. The MRI and histologic data suggested that Piezosurgery induced alveolar bone degradation. Thus, it was expected that the released calcium would result in significantly increased calcium levels in the peripheral blood. This occurred only at day 1 in both groups.
and 30 Hz) and their effect on bone biology. The final frequency is made of the initial frequency given by the generator to the Piezotome (preset) + an additional frequency that can be adjusted, to allow the modulation of this initial frequency. The usual commercial setting for the Piezotome 2 at D1 is 32 kHz (preset) + 60 Hz modulation frequency.

Early Phase of Piezoelectric Bone Injury

Tissue injury may cause acute inflammation in which leukocytes (PMNs and eosinophils) migrate to the damaged site by sensing chemo-attractants, such as leukotrienes, cytokines, chemokines, and exogenous chemo-attractants, released by damaged tissues. Neutrophils are recruited to the inflammatory site to engulf damaged tissues; the neutrophils are then cleared from inflammatory sites by either returning to the systemic circulation or undergoing apoptosis and macrophage clearance. Once phagocytosis is complete, macrophages are cleared by lymphatic drainage. Osteocytes die as a consequence of senescence, degeneration/necrosis, apoptosis (programmed cell death), and/or osteoclastic engulfment. The percentage of dead osteocytes in bone increases from less than 1% at birth to 75% after age 80. Osteocyte apoptosis is related to decreased mechanical transduction, which can possibly lead to the development of osteoporosis. Apoptotic osteocytes release apoptotic bodies expressing RANKL to recruit osteoclasts. Mechanical loading increases osteocyte viability in vitro and contributes to solute transport through the lacuno-canalicular system in bone, which enhances oxygen and nutrient exchange and diffusion to osteocytes. Skeletal unloading induces osteocyte hypoxia in vivo, causing osteocytes to undergo apoptosis and

Fig 3  Collagen fiber destruction in the early bone demineralization phase. In the 30-Hz group, the histologic analysis indicated that alveolar bone degradation was evident as early as day 1 and peaked at day 7, and bone regeneration started at day 14. Masson's trichrome collagen stain: bone (blue); collagen fiber type 1 (light blue); cytoplasm and muscles (red). Reticulin stain: reticular fiber (black, silver stain).
recruit osteoclasts to resorb bone. In bone, this occurs as the result of repetitive events of cyclic loading and is associated with osteocyte death by apoptosis, which signals osteoclasts to initiate remodeling at a damaged site. Mechanical stimulation of osteocytes results in the opening of hemi-channels to release prostaglandin E2 and adenosine triphosphate, among other biochemical-signaling molecules. These play a crucial role in maintaining the balance between bone formation and resorption. Osteocyte cell death can occur in association with pathologic conditions, such as osteoporosis and osteoarthritis, that lead to increased skeletal fragility linked to the loss of ability to sense microdamage and/or signal repair. Oxygen deprivation that occurs as the result of immobilization (bed rest), glucocorticoid treatment, and/or withdrawal of oxygen have been shown to promote osteocyte apoptosis. The present results show that piezoelectric bone injury at 30 Hz rapidly induces osteocyte apoptosis (within 1 day) as a result of 3D-vibration microdamage in bone lacunae (Fig 4). Thus, the RAP may occur in the early postoperative phase through osteocyte apoptosis, and it may be a more immediate and sudden reaction than what was originally believed. Apoptotic osteocytes can secrete molecules that recruit and differentiate pre-

Fig 4 (a) Morphologic analysis of nuclei in the early bone demineralization phase. Cell apoptosis is visualized using the nucleic-acid stain DAPI and fluorescent microscopy. There were a significantly decreased number of DAPI-positive osteocytes at postoperative day 1 and no positive cells at day 3. (b) Osteocyte apoptosis from piezoelectric injury at day 1. Increased apoptotic cells (TUNEL-positive, green) were observed in the alveolar bone area at day 1 (early phase) in the 30-Hz group. Actin fibers were stained with phalloidin (red), and nuclei were counterstained with DAPI (blue). Green: early apoptotic nucleus; red: actin fiber (blood vessels); blue: nucleus (non-apoptotic cells). Alveolar bone degradation started at day 1, was induced by osteocyte apoptosis, and was significantly higher in the 30-Hz group.
osteoclasts, such as RANKL and/or MCS-F (macrophage colony stimulating factor), which may lead to osteoclast activation and the resorption phase. Furthermore, piezoelectric bone injury may enhance osteoclastic activity in the resorption phase by increasing RANK-RANKL interaction following
osteocyte apoptosis, which may intensify the activation of RAP and magnify bone demineralization (Fig 6). Indeed, TRAP activity was significantly higher in the 30-Hz group compared to the control group: It peaked at day 3 and started decreasing at day 14 (Fig 5a). It is tempting to surmise that using the Piezotome D1 commercial setting (60 Hz) would induce even stronger bone biomodification than this experimental setting of 30 Hz; further research is being conducted in this area in the authors’ laboratory.

Conclusions

It was observed that the scope of bone biomodification was frequency-dependent when using the piezoelectric knife. Frequency is a key factor in generating the initiation and intensity of the RAP. In the rat, the RAP begins as early as day 1 and is driven by the osteocyte apoptosis following micro–bone damage. The demineralization that follows due to the osteoclast activation peaks from day 7 to day 14 before slowly decreasing and allowing space for the initiation of the regeneration phase.

Acknowledgments

The authors declare no conflicts of interest.

References


