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Apparent elastic modulus of ex vivo trabecular bovine bone increases with dynamic loading

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Abstract

Although it is widely known that bone tissue responds to mechanical stimuli, the underlying biological control is still not completely understood. The purpose of this study was to validate required methods necessary to maintain active osteocytes and minimize bone tissue injury in an ex vivo three-dimensional model that could mimic in vivo cellular function. The response of 22 bovine trabecular bone cores to uniaxial compressive load was investigated by using the ZETOS bone loading and bioreactor system while perfused with culture medium for 21 days. Two groups were formed, the "treatment" group (n = 12) was stimulated with a physiological compressive strain (4000 $\mu\varepsilon$) in the form of a "jump" wave, while the "control" group (n = 10) was loaded only during three measurements for apparent elastic modulus on days 3, 10, and 21. At the end of the experiment, apoptosis and active osteocytes were quantified with histological analysis, and bone formation was identified by means of the calcium-binding dye, calcein. It was demonstrated that the treatment group increased the elastic modulus by 61%, whereas the control group increased by 28% ($p < 0.05$). Of the total osteocytes observed at the end of 21 days, 1.7% ($\pm$0.3%) stained positive for apoptosis in the loaded group, whereas 2.7% ($\pm$0.4%) stained positive in the control group. Apoptosis in the center of the bone cores of both groups at the end of 21 days was similar to that observed in vivo. Therefore, the three-dimensional model used in this research permitted the investigation of physiological responses to mechanical loads on morphology adaptation of trabecular bone in a controlled defined load and chemical environment.

Keywords

Bone, culture, histology, bioreactor, mechanical test

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Introduction

Bone is a dynamic tissue, adapting morphologically to changes in its external and internal environments. The fact that bone adapts to the strain induced by the environment has been known for more than a century.[1] However, the physiological mechanisms that control the specific mineralization distribution of these morphological adaptive responses are not clearly understood. This lack of understanding is due to the limited delineation of the basic interrelationship of the three major bone cells: the osteoblast, the osteoclast, and the osteocytes that respond to both biochemical and mechanical stimuli in their three-dimensional (3D) in vivo environment. The interrelationship of the osteoblast, the osteoclast, and the osteocytes is key in the modeling and remodeling processes. All three cells reside in a 3D matrix that makes investigations of their interrelationships difficult. The osteocytes are the most abundant bone cell of the skeleton making up 95% of the cells. The osteocyte has been shown to function both in mechanosensing and in mechanotransduction

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in response to mechanical stimuli.[2–4] These two responses of the osteocytes have shown significant impact in the control of bone modeling and bone remodeling and thus bone matrix adaptation. The osteocyte is well suited as a sensor in that the osteocyte density, depending on the species and age, ranges from 14,000 to 93,000 osteocytes per cubic millimeter.[5]

In order to clarify cellular response to loads that results in an increase in bone matrix and thus skeletal adaptive strength, the underlying control mechanism must be delineated in a 3D model where the distribution of the adaptive mineralization along lines of strain may be quantified. This is very difficult to do in vivo[6–10] and limited in the two-dimensional (2D) cell culture model. To this end, a number of researchers have developed ex vivo models that attempt to mimic the 3D interconnectedness of the osteocytes in the skeletal system. The ex vivo models eliminate the systemic neural and hormonal control and fill the gap between the animal model and the 2D cell culture model. For example, El Haj et al.[11] developed an ex vivo model in which they used canine trabecular bone cores 10 mm in diameter and 10 mm in height. The marrow was removed from the bone cores and then perfused with culture medium at a rate of 1 mL/min and loaded in uniaxial compression to a maximum bulk strain of 5000 $\mu\varepsilon$. Takai et al.[12] developed an ex vivo model in which they used trabecular bone cores 5 mm in diameter and 4 mm in height harvested from the metacarpal bones of 3- to 4-month-old calves. The bone cores, after the bone marrow was removed, were loaded to a peak pressure of 3 MPa at 0.33 Hz with a triangular waveform for 1 h/day. All experiments and culture environments were maintained at 37 °C. This system was able to maintain viable osteocytes in bone cores for approximately 8 days. Jones et al.[13] developed an ex vivo model using a system called ZETOS, a bone loading and bioreactor system, in which trabecular bone cores, with intact bone marrow, were perfused with culture medium. The diffusion of culture medium into the core maintained viable cells for 49 days.[14] The ex vivo loading system provides a controlled environment to investigate mechanisms that control the morphological response of bone to environmental strain and biochemical stimuli while maintaining an in vivo-like environment with the 3D connectivity of osteocytes.[13,15,16] Maintaining intact bone cores with marrow during perfusion and loading retains the stem cells for both osteoclasts and osteoblasts necessary for bone modeling and remodeling.

In order to mimic an *in vivo* environment, the *ex vivo* model must demonstrate that: (a) osteoblast, osteoclast, and osteocytes have a clear viability for at least 21 days, an adequate period of time is require for bone formation activity[15]; (b) there is a measurable mineral apposition rate (MAR) similar to *in vivo*; (c) the level of apoptosis is similar to the *in vivo* rate for a similar age;and, (d) the model responds to both mechanical and chemical stimuli similarly to the *in vivo* response. Given the sensitivity of bone tissue to mechanical loading, it is reasonable to consider the use of controlled mechanical and biochemical environments as a means to enhance our understanding of bone modeling and remodeling. Thus, the purpose of this study was to validate required methods necessary to maintain active osteocytes and minimize bone tissue injury in an ex vivo 3D model that could mimic in vivo cellular function. This study stimulated and monitored changes of apparent elastic modulus, tissue injury, and apoptosis of bovine trabecular bone specimens for a duration of 21 days. It was hypothesized that bone specimens that were stimulated with a physiological load would have less osteocyte apoptosis and demonstrate an increase in elastic modulus over the duration of the experiment greater than bone specimens that were similarly prepared but not stimulated. A secondary purpose of this study was to demonstrate an experimental apparatus and tissue preparation procedure with minimal tissue injury for investigating the response of ex vivo trabecular bone to load.

Materials and methods

Ex vivo bone loading and bioreactor system

ZETOS bone loading and bioreactor system developed by Smith and Jones[17] was used to maintain viable bone cores and to apply controlled uniaxial compressive loading. The bioreactor bone chamber permits the loading of live trabecular bone cores 5 mm in height and 10 mm in diameter with a deformation accuracy of $\pm 3\%$ in the micrometer range of strains.[13] The chambers were continually perfused with culture medium, permitting nutrients to diffuse into the bone cores. Bone cores in the bioreactors have been maintained viable for up to 49 days.[14] The active tissue may be evaluated for structural and cellular responses to either mechanical or biochemical stimuli.[13,14,18] The details of experimental methods and equipment have been described elsewhere.[18,19] Briefly, uniaxial compressive displacement is applied via a piezoelectric actuator (PZA; model P-239.30, Physik Instrumente, Karlsruhe, Germany) that expands according to applied voltage. Strain gages on the PZA, mounted in a Wheatstone bridge configuration for temperature compensation, measure its expansion, and a load cell (type 9011A, Kistler, Winterthur, Switzerland) measures the reaction force. The deformation and force data are recorded during the bone core loading periods and used to calculate stiffness or apparent elastic modulus. The maximum force the ZETOS bone loading system applied was limited by the control system software to 1500 N, and the maximum displacement was limited by the maximum expansion of the unloaded PZA to 70 μm. More recently, our research group has developed a validated calibration procedure for the ZETOS system with reference bodies of known properties in a working range of 0.915–29.2 N/μm, which is equivalent to an

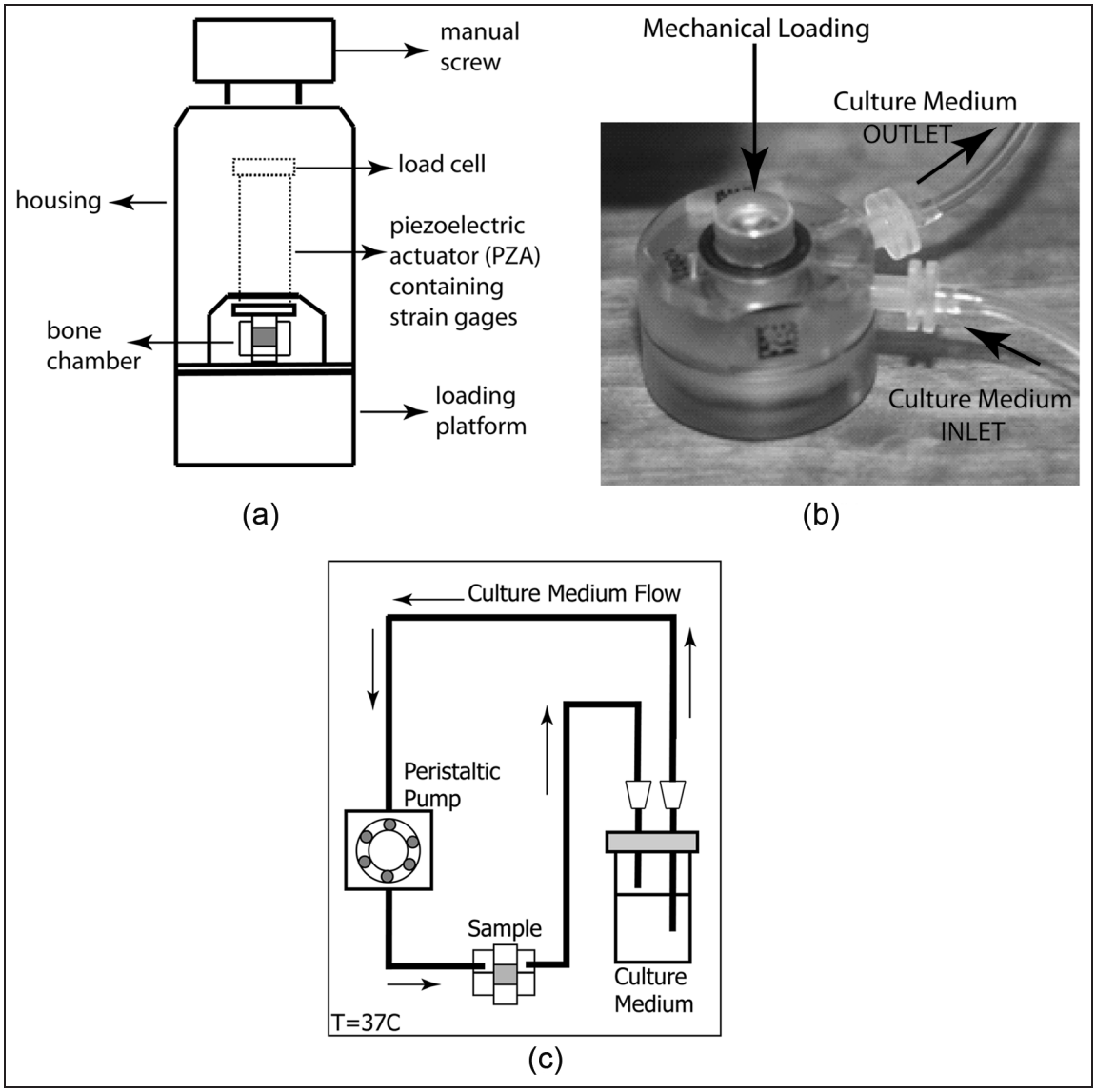

Figure 1. (a) Schematic representation of the ZETOS loading unit, including the load cell, piezoelectric actuator, and bone chamber; (b) ZETOS bone loading and bioreactor chamber, bone cores placed inside are perfused and loaded under physiological conditions; and (c) schematic diagram of the ZETOS perfusion system. Each sample was held in an individual chamber through which culture medium was delivered by a peristaltic pump at a rate of 6.6 mL/h. The ZETOS bone loading and bioreactor system was maintained at 37 °C.

apparent elastic modulus of 58.3 MPa to 1.86 GPa for the range of trabecular bone.[20] Thus, the system is appropriate to measure stiffness in the range of trabecular bone while applying loads in the physiological strain range to bone cores 5 mm in height and 10 mm in diameter. A schematic diagram of this bone loading and bioreactor system is shown in Figure 1.

Sample preparation

A trabecular bovine sternum bone was obtained from an 18- to 20-month-old animal from a local slaughter house for this experiment. Previous research has been conducted using similar low-weight-bearing bone.[15] The animal was free of disease and processed within 45 min from the time of slaughter. The bovine sternum was prepared under sterile conditions creating slices of trabecular bone 7 mm thick using a diamond band saw (Exakt, Norderstedt, Germany) while being continuously irrigated with 0.9% sterile sodium chloride solution at 4 °C. A custom-made diamond-tipped coring bit was constructed to excise the 10-mm-diameter bone cores. While coring and milling, the bone cores were submerged in a 0.9% sterile sodium chloride solution at 4 °C to limit heat-generated injury to the peripheral tissue cells during preparation, which is essential in minimizing tissue injury and osteocyte cell death on the cut surfaces. Once the cores were excised from the slices, their ends were milled using a six-fluted endmill to obtain flat and parallel surfaces and a core height of 5 mm. Great care was taken to ensure that the coring bits and endmills were sharp and that the cutting speed

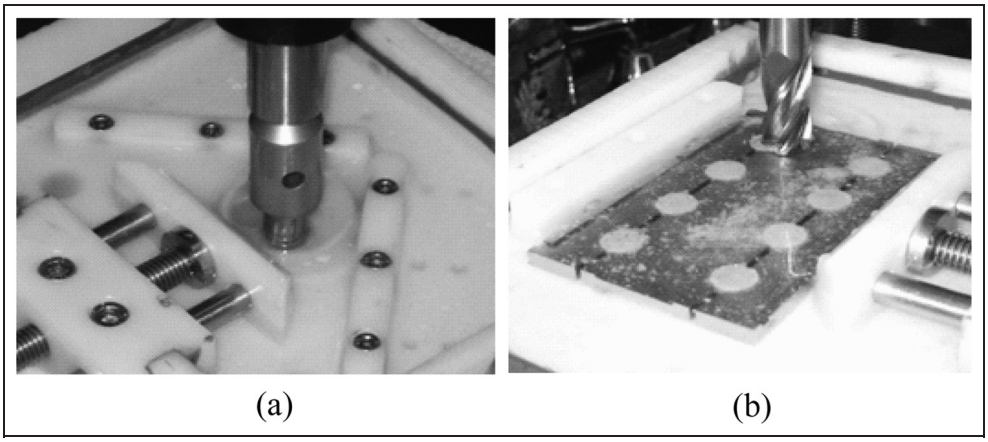

Figure 2. (a) Delrin® holder and example bone slice, fixed to the bench of the drill press for excision of bone cores with a diamond-tipped coring bit. (b) Delrin holder with the stainless steel milling fixture filled with cores and a six-fluted carbide milling bit on the milling machine.

during coring and milling were slow to minimize heat generated by the process. A total of 22 bovine bone cores were machined and washed twice with culture medium to be used in the study. The washing procedure accomplished two things: (1) it aided in the removal of machining residue without disturbing the marrow and bone lining cells and (2) the antibiotics in the culture medium helped reduce the potential for specimen contamination. Figure 2 shows the coring and endmilling processes.

Culture medium and flow rate

Each trabecular bone core was placed inside a bone loading chamber, maintained at 37 °C, and supplied culture medium for the duration of the 21-day experiment. The chambers were perfused with culture medium at 6.6 mL/h using a 24-channel peristaltic pump (ISMATEC SA, Glattbrugg, Switzerland). The culture medium was Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS), 2 mM glutamine, streptomycin, and penicillin G at 50,000 U/L each, 10 μg/mL of vitamin C, 0.12 g/L of $NaHCO_3$, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). A test tube was assigned to each bone loading chamber, containing 5 mL of culture medium that was maintained at 37 °C and a pH of 7.2–7.3 throughout the 21 days of perfusion. The culture medium was changed at 24-h intervals, and pH was measured every other day. During the 21 days of study, no contamination was observed in any bone core specimen. A single calcein (60 μg/mL of medium) label was added to the culture medium on day 13 in order to observe mineral deposition within the bone matrix.

Loading

After preparation, 22 bone cores were randomly divided into two groups, "load" (treatment N = 12) and "no load" (control N = 10), and allowed to equilibrate for 48 h before beginning with loading protocols. Two loading procedures were performed: (1) a quasi-static uniaxial compressive displacement applied on days 3, 10, and 21 to determine the apparent elastic modulus on both treatment and control groups and (2) a daily physiological dynamic load to stimulate bone cells in the treatment group. During the first loading procedure, a preload of 10 N followed by a gradually applied compressive displacement until a maximum of 20 μm, that is, 4000 με, was imposed. This quasi-static testing (~0.5 μm/s displacement rate) was performed in random order on all cores on days 3, 10, and 21 to monitor the apparent elastic modulus (E_{app}). For the second loading procedure, a waveform, which mimicked a physiological high-impact stimulus, specifically the vertical ground reaction force recorded during a vertical jump,[16,21] was applied in random order to each core of the treatment group. The "jump" waveform had a maximum bulk displacement of −20 μm or apparent strain of −4000 με repeated at 2 Hz for 100 cycles per day.

The force and displacement data recorded from ZETOS were processed using a custom-made software code (MATLAB; The MathWorks, Inc., Natick, MA). Apparent structural stiffness, K, was determined from the linear region of the force–displacement curve, defined as the slope of the fitted curve at the origin, or K = F/d, where "F" is the applied force and "d" is the compressive displacement. Using bulk core geometry of 5 mm height, h, and 10 mm diameter and assuming Hooke's law, E_{app} was determined, E_{app} = Kh/A, where A is the cross-sectional area (CSA) of the core. Mean percent changes and slope in E_{app} were calculated for each core to compare days 3 and 10, days 10 and 21, and days 3 and 21.

At the end of the 21 days, the bone cores were removed from the bioreactors, placed in 70% alcohol, dehydrated, embedded in methylmethacrylate, and sectioned at 5 and 8 μm for analysis. Sections were stained for osteoclasts with tartrate-resistant acid phosphatase

Table 1. Apparent elastic modulus (E_{app}) in MPa of treatment (n = 12) and control (n = 9) groups. Same tissues were measured at three evaluation days.

	Treatment			Control		
	Day 3	Day 10	Day 21	Day 3	Day 10	Day 21
Mean (SEM)	50.77 (±11.85)	72.27 (±13.79)	81.85 (±15.60)	30.09 (±7.70)	34.96 (±7.71)	38.41 (±8.76)

SEM: standard error of the mean.
Results expressed as mean (±SEM).

(TRAP) and for apoptosis (FragEL, DNA Fragmentation Detection Kit[22]). A 13 μm^2 area in the center of the core was measured to avoid any edge effects due to the initial core preparation and to assure we were assessing the effects of diffusion and loading on an intact structure and the level of apoptosis in the center of the core. Measurements included calcein single-label surface (sLS), total osteocyte (Ot.) number, apoptotic osteocyte (DAB positive, Ap.Ot.) number, osteoclast surface (Oc.S.), bone surface (BS), bone area (BA), bone volume (BV), and tissue volume (TV) and calculated indices included trabecular thickness (Tr.Th.) and trabecular number (Tr.N.).[23] Data were represented as ratios of total Ot. number, BS, or BV.

Statistical analysis

All analyses were conducted using the statistic package MiniTab 14 (MiniTab Inc., State College, PA, USA). Statistical differences between groups were analyzed using one-way repeated measures analysis of variance (ANOVA) to evaluate the effect of the load application on the change in apparent elastic modulus (E_{app}). Tukey's honestly significant difference (HSD) post hoc was performed to investigate the effect of time (three levels: 3, 10, and 21 days) on the rate of change of E_{app}. Difference on the bone histomorphometric measurements between the two groups were analyzed by independent sample t-test at the end of the experiment. Differences with p-values <0.05 were considered significant. All results were expressed as means and standard errors of the mean (SEMs).

Results

Twelve treatment bone cores and nine control cores were included in the analysis: at day 16, one control bone core was sacrificed to test that the calcein label was observable. The culture medium was changed every 24 h, and its pH measured for each individual bone core over the 21-day period. There was no significant difference in the culture medium pH between control and treatment groups. The initial pH of the culture medium was 7.4, and after 24 h, the pH was in the range from 6.80 to 7.45 for all the samples. The mean values of the pH culture medium over the 21 days for the treatment group was 7.21 (±0.11) and for the control group was 7.16 (±0.17).

Table 1 exhibits the absolute values of E_{app} for both treatment and control groups on days 3, 10, and 21 with their respective SEM. A statistically significant overall difference (p-values <0.05) between groups was found. The p-values associated with the change in E_{app} between the treatment and control groups at the three evaluation points (days 3, 10, and 21) were 0.02, 0.21, and 0.043, respectively. Furthermore, during the first 7 days, the E_{app} in the control group increased 23% with an average rate of change of 0.7 MPa/day, whereas the treatment group increased about 51% at a rate of change of 3.1 MPa/day. In the next 11 days, the control group had an average rate of change of 0.31 MPa/day as E_{app} increased about 9%, whereas the treatment group had a rate of change of 0.87 MPa/day as E_{app} increased 13%. The absolute E_{app} change of the two groups was 31 MPa in the treatment and 8 MPa in the control groups (Figure 3). The greatest rate of change occurred in the first 7 days in the treatment group and was statistically different (p-value <0.05) from the control group. The rate of change of the treatment group in days 10–21 was less than the first 7 days (p-value <0.05) (Figure 4).

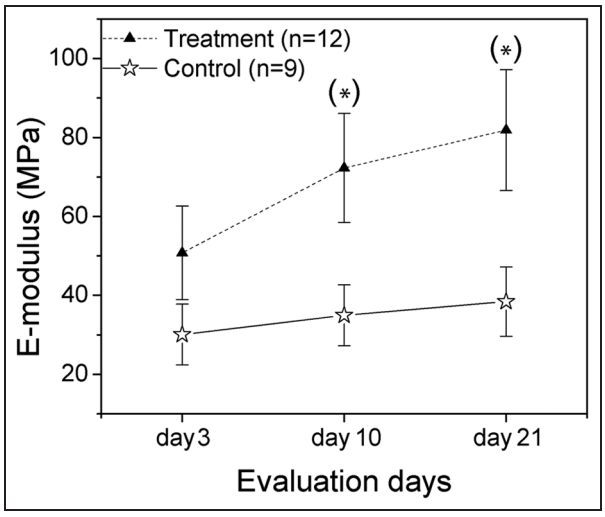

Figure 3. Apparent elastic modulus E_{app} evaluated at different periods of time. Values represent repeated measurements of the same tissue for treatment (n = 12) and control (n = 9) groups at different evaluation days. Errors bar are SEM. *p-values $<$ 0.05 were evaluated between treatment and control groups at each evaluation day, respectively.

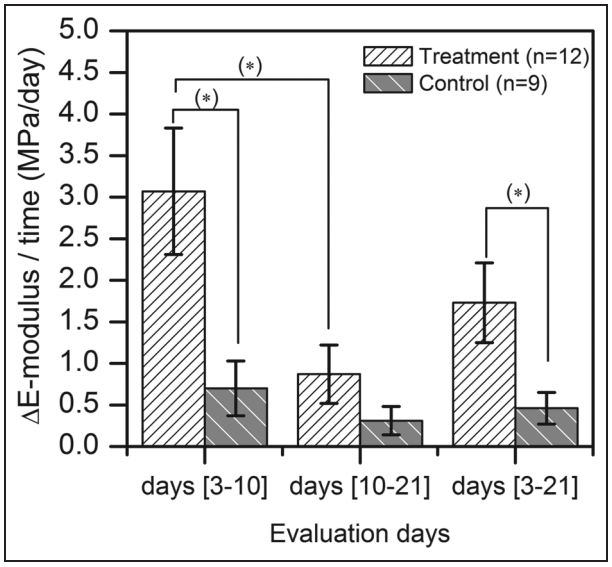

Figure 4. Rate of change of the apparent elastic modulus for different evaluation days. Errors bars are SEM. *p-value <0.05.

No statistical differences were found between the measured histomorphometry endpoints of the treatment and control groups (Table 2). In the middle of the cores, 9.5% (±1.2%) of the BS showed active bone formation with mineralization and 0.54% (±0.11%) of the surface was covered with osteoclasts. The cores averaged 119 (±5) osteocytes/mm^2 of BA. Of these osteocytes, in the control group 2.7% (±0.4%) stained positive for apoptosis and 1.7% (±0.3%) in the loaded group at 21 days. No statistical difference in the bone structure was found between groups by histomorphometry measurements. The BV averaged 19.0% (±0.7%) of the total area. The calculated average Tr.N. was 1.56 (0.05)/mm^2 of BA.

Tissue sections were also examined for edge effects to determine the extent of injury due to core preparation and potential impact on specimen viability. While the middle of the cores showed excellent osteocyte viability, surface bone formation, and osteoclast presence, there may be questions about edge effects. A preponderance of empty lacunae and apoptotic osteocyte nuclei within 100 μm of cut edges was found, but a normal distribution of osteocyte nuclei was found once past this 100-μm zone (Table 3).

Discussion

The treatment group that was dynamically loaded everyday increases in apparent elastic modulus by 61% over 21 days. Although the control samples were not loaded, the apparent elastic modulus increased by 28% over 21 days. A statistically significant overall difference in apparent elastic modulus (p-values <0.05) between control and treatment groups was found; however, no difference was found in the bone structure measurements. It has been shown that in vivo bone responds very rapidly to insults, such as an osteotomy. The bone core preparation is an osteotomy that induces injury to the bone cores. While prostaglandins (PGE_2) were not measured in this study, in a recent 21-day study using the same system and 48 bone cores from bovine sternal tissue,[24] PGE_2 was measured at three time points. In the treatment groups, PGE_2 concentration in the culture medium was elevated at 8 days post-osteotomy and remained elevated throughout the 21 days. On the other hand, in the control group, PGE_2 began to decrease on the 12th day and by the 15th day was 70% of treatment group.[24] Prostaglandin is essential in bone response to an injury like an osteotomy and in response to mechanical loading.[25] With the elevated level of PGE_2 as a response to the osteotomy, one would expect that bone formation would occur in the control bone cores to repair the injured tissue. Boppart et al.[26] have demonstrated that once osteoblasts are induced to form bone tissue, there is a lag time of about 6 days before formation decrease or the osteoblasts become quiescent. Thus, one might hypothesize that

Table 2. Bone core histomorphometry.

	sLS/BS (%)	Oc.S./BS (%)	BV/TV (%)	Tr.Th. (μm)	Tr.N. (number/mm^2)
Control	10.1 (±1.8)	0.52 (±0.11)	19.3 (±1.1)	121 (±6)	1.6 (±0.1)
Treatment	9.0 (±1.6)	0.57 (±0.11)	18.7 (±1.1)	123 (±6)	1.5 (±0.1)

Measurements included single-label surface (sLS), osteoclast surface (Oc.S.), bone volume (BV), tissue volume (TV), bone surface (BS), trabecular thickness (Tr.Th.), and trabecular number (Tr.N.). There was no statistically significant difference between groups. Results expressed as mean (±SEM).

Table 3. Morphological results expressed as mean (±SEM).

	Ot. number	Ap.Ot. number	BA (mm^2)	Ot./BA (number/mm^2)	Ap.Ot./BA (number/mm^2)	Ap.Ot./Ot. (%)
Treatment	253 (±17)	4.0 (±0.7)	2.1 (±0.1)	120 (±9)	1.9 (±0.3)	1.7 (±0.3)
Control	255 (±18)	6.9 (±1.9)	2.2 (±0.1)	118 (±6)	3.1 (±0.8)	2.7 (±0.7)

Ot.: osteocyte; Ap.: apoptotic; BA: bone area.
There was no statistically significant difference between groups.

the control group's response to the osteotomy increased bone formation up to about 18–19 days of the overall 21-day study accounting for the increased modulus. If this experiment was conducted for an additional 21 days, one would expect that the control group would show minimal changes in modulus and structural parameters and might even show a decline from the peak at 21 days. The rapid response of bone tissue to increase bone formation in response to an osteotomy has been demonstrated in a chick model. Clark et al. studied the response of radii osteotomy on 22-day-old chicks. The radius and ulna were measured daily using a microcomputed tomography (μ-CT) scanner to obtain bone mineral content (BMC) and CSA.[6] Clark reported that from days 5 to 10, BMC increased 93% in the radius and 17% in the ulna of the same growing animals; however, over the next 10 days, the rate of change in BMC and CSA slowed.

The increase in E_{app} observed in this study during the first 7 days parallels the higher alkaline phosphatase (AP) activity reported by David et al. in the first 10 days of their experiment with similar bone-testing conditions. The greater level of AP was consistent with increased osteoblast differentiation and bone formation stimulated by mechanical strain.[15] The increased E_{app} of the "load" group was also consistent with the increased number of rods and plates reported by David et al. in a similar bone core study using bovine sternum and μ-CT measurements before and after a 21-day study.

The calculation of E_{app} of trabecular cores is dependent on the core diameter. In every prepared trabecular core, there are "edge effect" artifacts that originate from interruption of the trabecular network along the sides of machined cores.[27] This effect creates an underestimation of the true (i.e. the in situ) apparent elastic modulus that has been evaluated in previous research using finite element analysis[27–29] and mechanical testing.[30–32] Early recommendations on core sizes of trabecular bone have proposed that the minimum core size needed to contain at least five trabeculae to satisfy the continuum assumption.[33] Although the core size for this research included at least five trabeculae, there was still an unavoidable experimental artifact that underestimated the E_{app} absolute stiffness by roughly 18%–20%.[31]

The density of osteocytes, measured at 119 osteocytes per mm^2, in the bovine bone ex vivo cores was consistent with the osteocyte density reported in iliac bone biopsies from human tissue.[7] The rate of apoptosis was low, and no statistical difference was found between groups. These measures of osteocyte viability along with bone formation and the presence of osteoclasts demonstrate sufficient tissue diffusion with nutrients to mimic baseline measures reported by Mann et al.[16] of 2.2% apoptosis and in vivo studies. These apoptotic values are essential if the ex vivo model is to be used to investigate osteocyte response to a variety of stimuli. The level of apoptosis between the "load" and "no load" groups was not significantly different, which indicates that tissue viability was not dependent on load by adequate level of diffusion.

In an earlier study, bone cores from human femoral heads were prepared and maintained for 49 days.[13] MAR was quantified using the day 34 calcein and day 44 alizarin labels in 16 bone cores. The MAR in the ex vivo bone cores of 0.48 ± 0.18 (μm/day) obtained from a 68-year-old male was similar to that of in vivo iliac biopsies measured by Recker et al. (0.477 ± 0.078 μm/day), obtained from 11 65- to 74-year-old females;[8] Dahl et al. (0.517 ± 0.075 μm/day), obtained from 27- to 52-year-old males;[9] and Clarke et al. (0.54 ± 0.07 μm/day), obtained from 60- to 69-year-old males.[10] In this study, we were not able to measure MAR due to technical problems with the second label. However, there was evidence that roughly 10% of the surface was undergoing active bone formation based upon the label incorporated at 13 days.

At 21 days, no statistical difference in the percentage of apoptosis between both groups was found, with values of 2.7% in the control group and 1.7% in the treatment group. These results were comparable to the values observed by Mann et al.[16] at the beginning (time 0) of a similar study in which they found a 1.88% of apoptosis.

For this study, all coring and milling operations were performed with the bone cores submerged under at least 1 mm of 0.9% sterile sodium chloride solution at 4 °C. This minimized the heating of the tissue and cells. Other precautions that were taken in this study included replacing the coring drill after 20 cuts and changing the culture medium every 24 h to minimize changes in pH (pH was maintained between 7.4 and 7.0). These methods of tissue preparation minimized tissue injury to the first 100 μm permitting the active osteocytes to facilitate the diffusion of culture medium to the center of the core with the marrow present. If there is high peripheral cell death, there may be secondary effects on local cell signaling and diffusion of culture medium toward the center of the core and increased potential for greater cell death in the core center. The preparation effects in this study appeared to be limited to the outer 100 μm, and it was noted that many healthy cells were found within that 100 μm as shown in Figure 5. The current investigation was limited to bone response due to a mechanical stimulus. With the ZETOS system, systematic osteogenic stimuli from the whole organism are not present. However, this model permits the isolation of specific stimuli in combination with mechanical stimuli that will help to understand the impact on various metabolic pathways. The results of this study clearly demonstrate that not only did the bone cores respond to mechanical loading similarly to that of animal models but also the level of apoptosis was maintained to the level reported in animal studies and that minimal cell death was observed past the outer 100 μm caused by

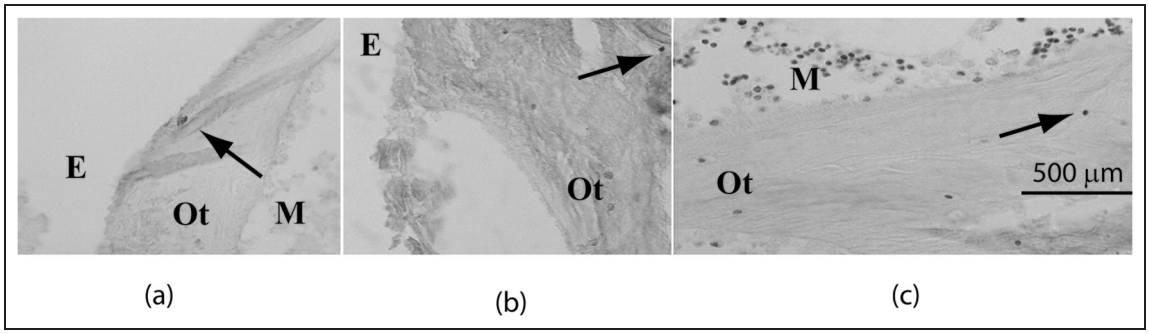

Figure 5. Tunnel-stained sections from (a, b) the core edge and (c) the center region of the sample. Tissue debris and cell death due to the initial core preparation were limited to the outer 100 μm of the sections. Cut edge (E), marrow (M), osteocyte in lacunae (Ot.), and apoptotic osteocyte (arrow).

tissue preparation. The sample preparation method of this study also allows keeping bone marrow that not only provides the stem cell source for osteoclasts and osteoblasts but also helps to eliminate a shear flow that is not present in vivo and may artificially activate lining cells. This ex vivo model also holds great potential in that the 3D structural environments of the osteocyte, the marrow, and marrow spaces are maintained along with normal apoptosis and a MAR similar to human in vivo measurements.

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Declaration of conflicting interests

No competing financial interests exist.

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References

1. Burr DB, Robling AG and Turner CH. Effects of biomechanical stress on bones in animals. *Bone* 2002; 30: 781–786.
2. Lanyon LE. Osteocytes, strain detection, bone modeling and remodeling. *Calcified Tissue Int* 1993; 53: S102–S107.
3. Bonewald LF and Johnson ML. Osteocytes, mechanosensing and Wnt signaling. *Bone* 2008; 42: 606–615.
4. Kamel MA, Picconi JL, Lara-Castillo N, et al. Activation of [beta]-catenin signaling in MLO-Y4 osteocytic cells versus 2T3 osteoblastic cells by fluid flow shear stress and PGE2: implications for the study of mechanosensation in bone. *Bone* 2010; 47: 872–881.
5. Schneider P, Meier M, Wepf R, et al. Towards quantitative 3D imaging of the osteocyte lacuno-canalicular network. *Bone* 47: 848–858.
6. Clark WD, Smith EL, Linn KA, et al. Use of peripheral quantitative computed tomography to monitor bone healing after radial osteotomy in three-week-old chickens (Gallus domesticus). *J Avian Med Surg* 2005; 19: 198–207.
7. Qiu S, Rao DS, Palnitkar S, et al. Differences in osteocyte and lacunar density between Black and White American women. *Bone* 2006; 38: 130–135.
8. Recker RR, Kimmel DB, Parfitt MA, et al. Static and tetracycline-based bone histomorphometric data from 34 normal postmenopausal females. *J Bone Miner Res* 1988; 3: 133–144.
9. Dahl E, Nordal KP, Halse J, et al. Histomorphometric analysis of normal bone from the iliac crest of Norwegian subjects. *Bone Miner* 1988; 3: 369–377.
10. Clarke BL, Ebeling PR, Jones JD, et al. Changes in quantitative bone histomorphometry in aging healthy men. *J Clin Endocr Metab* 1996; 81: 2264–2270.
11. El Haj AJ, Minter SL, Rawlinson SC, et al. Cellular responses to mechanical loading in vitro. *J Bone Miner Res* 1990; 5: 923–932.
12. Takai E, Mauck RL, Hung CT, et al. Osteocyte viability and regulation of osteoblast function in a 3D trabecular bone explant under dynamic hydrostatic pressure. *J Bone Miner Res* 2004; 19: 1403–1410.
13. Jones DB, Broeckmann E, Pohl T, et al. Development of a mechanical testing and loading system for trabecular bone studies for long term culture. *Eur Cells Mater* 2003; 5: 48–59.
14. Smith EL, Boudriot U, Daume B, et al. Long term perfusion loading of trabecular bone cores and formation rate. In: *American Society for Bone Mineral Research (ASBMR) 23rd Annual Meeting*. Phoenix, Arizona, USA. Vol. 16 Suppl 1, September 2001, pp S141.
15. David V, Guignandon A, Martin A, et al. Ex vivo bone formation in bovine trabecular bone cultured in a dynamic 3D bioreactor is enhanced by compressive mechanical strain. *Tissue Eng Pt A* 2008; 14: 117–126.
16. Mann V, Huber C, Kogianni G, et al. The influence of mechanical stimulation on osteocyte apoptosis and bone viability in human trabecular bone. *J Musculoskelet Neuronal Interact* 2006; 6: 408–417.
17. Smith EL and Jones D. *Combined perfusion and mechanical loading system for explanted bone*. Patent US6171812 B1, 2001.
18. Smith EL, Martens F, Koller K, et al. The effects of 20 days of mechanical loading plus PTH on the E-modulus

of cow trabecular bone. In: *American Society for Bone and Mineral Research (ASBMR) 22nd Annual Meeting*. Toronto, Ontario, Canada. Vol. 15 Suppl 1, September 2000, pp S247.
19. Davies CM, Jones DB, Stoddart MJ, et al. Mechanically loaded ex vivo bone culture system 'Zetos': systems and culture preparation. *Eur Cells Mater* 2006; 11: 57–75.
20. Garcia-Rodriguez S, Smith EL and Ploeg H-L. A calibration procedure for a bone loading system. *J Med Dev* 2008; 2: 011006.1-011006.6.
21. McNair PJ and Prapavessis H. Normative data of vertical ground reaction forces during landing from a jump. *J Sci Med Sport* 1999; 2: 86–88.
22. Weinstein RS, Nicholas RW and Manolagas SC. Apoptosis of osteocytes in glucocorticoid-induced osteonecrosis of the hip. *J Clin Endocr Metab* 2000; 85: 2907–2912.
23. Parfitt AM. Bone histomorphometry: standardization of nomenclature, symbols and units (summary of proposed system). *Bone* 1988; 9: 67–69.
24. Johnson MG, Meyer LA, Vivanco J, et al. Exposure to big endothlin-1 in bovine sternal cores mimics some aspects of mechanical loading. In: *American Society for Bone and Mineral Research. (ASBMR) 34th Annual Meeting*. Minneapolis, Minnesota, USA. Vol. 27, Suppl 1, October 2012.
25. Kohrt WM, Barry DW, Pelt REV, et al. Timing of ibuprofen use and bone mineral density adaptations to exercise training. *J Bone Miner Res* 2010; 25: 1415–1422.
26. Boppart MD, Kimmel DB, Yee JA, et al. Time course of osteoblast appearance after in vivo mechanical loading. *Bone* 1998; 23: 409–415.
27. Ün K, Bevill G and Keaveny TM. The effects of side-artifacts on the elastic modulus of trabecular bone. *J Biomech* 2006; 39: 1955–1963.
28. Harrison N and McHugh P. Comparison of trabecular bone behavior in core and whole bone samples using high-resolution modeling of a vertebral body. *Biomech Model Mechan* 2010; 9: 469–480.
29. Bevill G, Easley SK and Keaveny TM. Side-artifact errors in yield strength and elastic modulus for human trabecular bone and their dependence on bone volume fraction and anatomic site. *J Biomech* 2007; 40: 3381–3388.
30. Lievers WB, Waldman SD and Pilkey AK. Minimizing specimen length in elastic testing of end-constrained cancellous bone. *J Mech Behav Biomed* 2010; 3: 22–30.
31. Lievers WB, Petryshyn AC, Poljsak AS, et al. Specimen diameter and "side artifacts" in cancellous bone evaluated using end-constrained elastic tension. *Bone* 2010; 47: 371–377.
32. Keaveny TM, Pinilla TP, Crawford RP, et al. Systematic and random errors in compression testing of trabecular bone. *J Orthopaed Res* 1997; 15: 101–110.
33. Harrigan TP, Jasty M, Mann RW, et al. Limitations of the continuum assumption in cancellous bone. *J Biomech* 1988; 21: 269–275.