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Combined exposure to big endothelin-1 and mechanical loading in bovine sternal cores promotes osteogenesis

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ABSTRACT

Increased bone formation resulting from mechanical loading is well documented; however, the interactions of the mechanotransduction pathways are less well understood. Endothelin-1, a ubiquitous autocrine/paracrine signaling molecule promotes osteogenesis in metastatic disease. In the present study, it was hypothesized that exposure to big endothelin-1 (big ET1) and/or mechanical loading would promote osteogenesis in *ex vivo* trabecular bone cores. In a 2 × 2 factorial trial of daily mechanical loading (−2000 με, 120 cycles daily, "jump" waveform) and big ET1 (25 ng/mL), 48 bovine sternal trabecular bone cores were maintained in bioreactor chambers for 23 days. The bone cores' response to the treatment stimuli was assessed with percent change in core apparent elastic modulus (ΔE_{app}), static and dynamic histomorphometry, and prostaglandin E2 (PGE2) secretion. Two-way ANOVA with a post hoc Fisher's LSD test found no significant treatment effects on ΔE_{app} ($p = 0.25$ and 0.51 for load and big ET1, respectively). The ΔE_{app} in the "no load + big ET1" (CE, 13 ± 12.2%, $p = 0.56$), "load + no big ET1" (LC, 17 ± 3.9%, $p = 0.14$) and "load + big ET1" (LE, 19 ± 4.2%, $p = 0.13$) treatment groups were not statistically different than the control group (CC, 3.3% ± 8.6%). Mineralizing surface (MS/BS), mineral apposition (MAR) and bone formation rates (BFR/BS) were significantly greater in LE than CC ($p = 0.037$, 0.0040 and 0.019, respectively). While the histological bone formation markers in LC trended to be greater than CC ($p = 0.055$, 0.11 and 0.074, respectively) there was no difference between CE and CC ($p = 0.61$, 0.50 and 0.72, respectively). Cores in LE and LC had more than 50% greater MS/BS ($p = 0.037$, $p = 0.055$ respectively) and MAR ($p = 0.0040$, $p = 0.11$ respectively) than CC. The BFR/BS was more than two times greater in LE ($p = 0.019$) and LC ($p = 0.074$) than CC. The PGE2 levels were elevated at 8 days post-osteotomy in all groups and the treatment groups remained elevated compared to the CC group on days 15, 19 and 23. The data suggest that combined exposure to big ET1 and mechanical loading results in increased osteogenesis as measured in biomechanical, histomorphometric and biochemical responses.

Abbreviations: A, cross-sectional area, mm^2; BFR/BS, bone formation rate, $\mu m^3/\mu m^2$/year; big ET1, big endothelin-1; BS, bone surface, μm; BV/TV, bone volume, %; CC, "no load + no big ET1" control group; CE, "no load + big ET1" treatment group; δ, axial deformation, mm; ΔE_{app}, percent change in apparent elastic modulus, %; E_{app}, apparent elastic modulus, MPa; *Ece1*, endothelin converting enzyme 1; ET1, Endothelin-1; ET_A, endothelin receptor type A; ET_B, endothelin receptor type B; F, axial force, N; K_{axial}, axial stiffness, N/mm; L, core height, mm; LC, "load + no big ET1" treatment group; LE, "load + big ET1" treatment group; LRP5, lipoprotein receptor-related protein 5; MAR, mineral apposition rate, μm/day; MS/BS, mineralizing surface, %; PGE2, prostaglandin E2; PTH, parathyroid hormone; SOST, sclerostin; ZETOS, ZETOS Bone Loading and Bioreactor System.

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1. Introduction

Mechanical loading is a well-known stimulus in the process of bone modeling, remodeling and homeostasis; however, the interactions of the mechanotransduction pathways are less well understood [1–6]. Frost suggested that bone has an internal mechanical set point that controls bone's response to mechanical stimuli that he termed the mechanostat. He postulated the existence of a strain set point; with ambient strains below it eliciting bone resorption and ambient strains above it eliciting bone formation [7,8]. Studies by the authors and others on *ex vivo* cultured trabecular bone cores exposed to mechanical bulk strains of −2000 to −4000 με found, in comparison to controls, increased percent change in apparent elastic modulus (ΔE_{app}), histological and biological markers of bone formation, demonstrating that bone's response to load can be recapitulated in an organ culture system [9–14]. *Ex vivo* testing provides a controlled environment, without systemic effects, to investigate mechanotransduction in live bone.

Bone modeling and remodeling are affected by genetic factors in addition to mechanical stimulus. The discovery that low-density lipoprotein receptor-related protein 5 (LRP5) gene mutations alter skeletal mass provided a direct demonstration that skeletal mass is under genetic control. These findings demonstrate that the WNT signaling pathway occupies a central role in regulating mechanotransduction in bone [15–18]. In particular, the WNT inhibitor sclerostin (SOST) is expressed by mature osteocytes, and its expression is inhibited in response to mechanical loading [19]. In addition to the WNT pathway, mechanotransduction is also modulated by prostaglandins, parathyroid hormone (PTH) and a variety of identified and unidentified molecules (reviewed in [18,20,21]). If any of these signaling pathways are up- or down-regulated, bone formation and resorption are affected. Increased levels of prostaglandin E2 (PGE2) and PTH each decrease SOST gene expression [22,23].

Previously, we demonstrated that big endothelin-1 (big ET1) increased osteoblast mineralization *in vitro* and changed the expression of multiple miRNA known or hypothesized to affect expression of proteins that affect bone physiology, including SOST [24]. These results showed that big ET1 regulated SOST in a post-transcriptional manner [24].

A segment of mouse chromosome 4 harbors a gene or genes that modulate the magnitude of the bone modeling response to experimental loading [25]. We identified endothelin converting enzyme 1 (*Ece1*) as a candidate gene within the quantitative trait loci *bmd7* on mouse chromosome 4 which is responsible for 40% of the variation in bone size, strength and density between the recombinant congenic mice strains HCB8 and HCB23 [26–28].

The purpose of this study was to investigate the biomechanical, histomorphometric and biochemical responses of *ex vivo* cultured bovine trabecular bone cores to exposure to big ET1 in conjunction with regular mechanical loading. The authors have demonstrated in previous studies that big ET1 added to cell culture increased osteogenesis [24]; however, it is yet to be shown that the cell culture result translates to a similar finding in trabecular bone organ culture. In the current study it was hypothesized that exposure to big ET1 and/or mechanical loading would promote osteogenesis in bovine trabecular bone organ culture.

2. Materials and methods

In a 2 × 2 factorial trial of big ET1 (25 ng/mL) and daily mechanical loading (−2000 με, 120 cycles daily, "jump" waveform), 48 bovine sternal trabecular bone cores were maintained in bioreactor chambers for 23 days. The cores were equally allocated to four groups based on rank order of their apparent elastic modulus (E_{app}) so that each group had approximately the same average E_{app}: CC (no load + no big ET1), CE (no load + big ET1), LC (load + no big ET1), and LE (load + big ET1). Cores in the CE and LE treatment groups were given 25 ng/mL of big ET1 daily. The concentration of big ET1 used in the current experiment was approximately equivalent to the lowest published concentration of active ET1 used in cell culture experiments [29]. Cores in the LC and LE treatment groups were loaded (−2000 με, 120 cycles daily, "jump" waveform) through the bioreactors' sapphire pistons using ZETOS Bone Loading and Bioreactor System (ZETOS) [9–13,30–32]. Bone formation in the cores was assessed with ΔE_{app}, static and dynamic histomorphometry, and PGE2 secretion.

2.1. Preparation and culture of bovine trabecular bone cores

A bovine sternum was removed from one 13 ± 1 month old animal obtained from a local slaughterhouse. The animal was determined to be free of disease and infection. Within an hour of slaughter, sample preparation started under sterile conditions. Individual trabecular bone cores (10 mm diameter by 5 mm high) were excised from the sternum by machining according to the procedure developed by Smith and Jones [9–13,30,31]. The cylindrical axis for all cores was oriented along the cranial-caudal axis of the sternum. After machining, the cores were washed twice with culture medium to remove any residual bone left from the machining process and to minimize the risk of infection. A total of 52 bone cores were prepared from the single bovine sternum. The stiffest 48 cores were selected for the experiment.

After excision, machining, and washing, each bone core was inserted into a polycarbonate bioreactor chamber between two sapphire pistons. After preparation, the bone cores were allowed to recover for 48 h before initiating the load and big ET1 treatments on day 1 of the experiment. While in these chambers, the bone cores were supplied with circulating culture medium (6.6 mL/h) using two 24-channel peristaltic pumps (model Ismatec ISM939D, IDEX Health & Science SA, Glattburgg, Switzerland); maintained at 37 °C and a pH of 7.2–7.3 throughout the 23-day experiment. A separate sterile test tube containing 5 mL of the culture medium was allocated to each bioreactor and replaced every 24 h. The culture medium, standard Dulbecco's Modified Eagle Medium, contained: 10% fetal calf serum, 2 mM of glutamine, 50,000 U/L each of streptomycin and penicillin G, 10 μg/mL of Vitamin C, 0.12 g/L of sodium bicarbonate, and 10 mM of HEPES. Calcein dye (60 μg/mL) for double labeling was added to the culture medium on days 9 and 19 and allowed to perfuse for 24 h.

2.2. Load treatment and mechanical analysis

The loading regimen for each of the cores in the LC and LE treatment groups consisted of daily dynamic loading. Each core was cyclically loaded in compression to a maximum change in bulk strain of 2000 με every 0.5 s for 120 cycles for 23 days [9–13,30–32]. The dynamic loading implemented in the study mimicked a "jump" waveform, the shape of which was determined from normative human jump data [33]. The vertical ground reaction forces from the jump trials were used to define the shape of a "jump" waveform which is simulated in ZETOS (Fig. 1). In this study, the average time to achieve maximum change in bulk strain was 0.375 s; therefore, the average strain rate during this period was approximately −5000 με/s.

To monitor bone core response for ΔE_{app}, each specimen was tested quasi-statically at a rate of 0.04–0.08 με/s, with a maximal bulk compressive strain of 4000 με on days 1 and 23 of the study. The axial stiffness (K_{axial}) of the cores was measured with this quasi-static compression (Fig. 2) through the bioreactors' sapphire pistons using ZETOS [9–13,30–32,34]. To ensure contact between the loading piston and bone core, a preload of 10 N was applied before quasi-static testing and the dynamic loading stimulus. For each loading trial, force and deformation data were recorded. The bone cores' E_{app} was determined assuming Hooke's Law (Eq. 1) [35–38]; where, F is the measured axial force, L is the core height

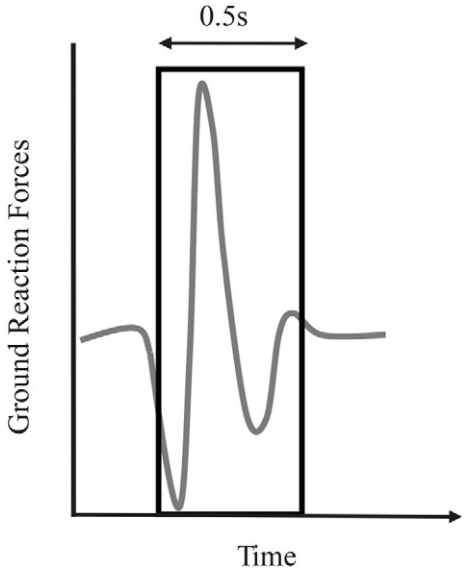

Fig. 1. "Jump" waveform as determined from normative data of vertical ground reaction forces from subject trials [33]. Highlighted portion shows specific waveform that was used in the study representative of landing after a jump.

(5 mm), δ is the axial deformation, and A is the cross-section area (78.5 mm^2).

$$E_{app} = \frac{FL}{\delta A} \quad (1)$$

The K_{axial} for each core was determined from the slope of the linear region of the F-δ curve (Fig. 2). A custom-made code in MATLAB (MathWorks Inc., Natick, MA) was used to determine the slope of the most linear region. An iterative algorithm performed linear regressions of the curve that gave both a coefficient of determination greater than 0.9 and maximized the number of data points in the regression analysis.

2.3. Histological and biochemical analyses

At the conclusion of the study, each bone core was extracted from its bioreactor and placed in 70% ethanol for static and dynamic histomorphometry using standard measures [39,40]. Over 14 days, the cores were dehydrated in graded ethanols and acetone, and then embedded individually in modified methylmethacrylate. The embedded cores were cut in half along the long axis (Leica SP1600 Saw Microtome, Buffalo Grove, IL). Thin sections (5 and 8 μm) were cut from the center of the core parallel to the direction of force application (Leica 2255 Rotary Microtome, Buffalo Grove, IL). One slide was examined without further staining under ultra-violet light; a second slide was stained with Goldner's trichrome stain. Each set of slides was given a random number to obscure specimen identity from the observer. Sections were analyzed using a light/epifluorescent microscope and a video camera interfaced with BIOQUANT TCW software (Bioquant Image Analysis Corp., Nashville, TN). The magnification for measurements was calibrated at the start of the study. Single label lengths were measured at 200× magnification; and, double label distances were measured at 400× magnification. The unstained specimen was used for assessing fluorochrome labeling, bone volume (BV/TV) and dynamic measurements of bone formation. The Goldner's stained section was used for static measurements of bone area. For data collection, a standard area (16 mm^2) was outlined in the central region of the slide containing only cancellous bone and marrow. Mineralizing surface (MS/BS, %) was calculated as 100 × (0.5 single labeled surface length + double labeled surface)/bone surface. The mineral apposition rate (MAR, μm/day) was calculated from distance between calcein labels over the interlabel time (10 days). Bone formation rate (BFR/BS, $\mu m^3/\mu m^2$/year) was calculated from MAR × (MS/BS), and extrapolated to a year by multiplying by 365 days/year. Bone specimens without double-labels were assigned a MAR of 0.3 μm/day [40]. Prostaglandin E2 secretions were measured in 5 mL of culture medium collected at 24 hour intervals and stored at −80 °C on days 0, 2, 4, 8, 12, 15, 19, and 23 using Prostaglandin E2 Parameter Assay Kit (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's protocol.

2.4. Statistical analysis

One-way analysis of variance (ANOVA) and two-tailed two-sample t-tests were used to test that there was no difference between groups' mean E_{app} on day 1, and groups' mean BV/TV measured at the end of the experiment. From the 48 bone cores, seven samples with E_{app} on day 1 less than 40 MPa (one from CC, one from CE, two from LC and three from LE) were not included in the statistical analyses as their stiffness was outside the calibration range (E_{app} = 40 to 1500 MPa) of ZETOS. Four additional bone cores (one each from CC and CE and two from LC) were not included in the histological analysis due to damage of the cores in tissue preparation. The percent change in E_{app} ($\Delta E_{app} = 100 \times (E_{app,day\ 23} - E_{app,day\ 1})/E_{app,day\ 1}$) was calculated for each core between days 1 and 23 and averaged for each group. Four cores, one from LE and three from CC groups, had ΔE_{app} outside two standard deviations of their group's mean and were excluded from the statistical analysis of ΔE_{app}. The effects of load and big ET1 on ΔE_{app}, MS/BS, MAR and BFR/BS were determined using two-way ANOVA and post hoc Fisher's least significant difference (LSD) test. Prostaglandin E2 measurements from all samples in a group were averaged to estimate the PGE2 secretion for time points 0, 2, 4, 8, 12, 17, 19 and 23 days. The effects of load and big ET1 on PGE2 secretion were determined using two-way ANOVA with time as a repeated measure (RM) and the Holm–Šídák test for multiple comparisons. A significance level, $\alpha = 0.05$, was used for all statistical analyses. Normal probability plots confirmed the normality of all data. All statistical analyses were performed with Minitab version 15 (Minitab Inc., State College, PA).

3. Results

The initial mean E_{app} and standard error of all 41 bones cores was $E_{app} = 136 \pm 12.8$ MPa with no statistical difference between groups (Table 1). Bone volume measured at the end of the experiment was also not different between groups (BV/TV = 16.8 ± 0.74%) (Table 1). Two-way ANOVA with a post hoc Fisher's LSD test found no significant treatment effects on ΔE_{app} ($p = 0.25$ and 0.51 for load and big ET1, respectively). The ΔE_{app} in the CE (13 ± 12.2%, $p = 0.56$), LC (17 ± 3.9%, $p = 0.14$) and LE (19 ± 4.2%, $p = 0.13$) treatment groups were not statistically different than the CC group (3.3% ± 8.6%) (Table 1 and Fig. 3A).

Two-way ANOVA with a post hoc Fisher's LSD test found no difference between CC groups and CE or LC treatment groups in all histological bone formation markers (Table 1 and Fig. 3B–D). However, greater MS/BS and BFR/BS in LC treatment group compared to CC group approached significance in ($p = 0.055$ and $p = 0.074$, respectively). The LE treatment group was significantly greater in MS/BS, MAR and BFR/BS than CC group ($p = 0.037$, 0.0040 and 0.019, respectively). Cores in LE and LC treatment groups had more than 50% greater MS/BS ($p = 0.037$, $p = 0.055$ respectively) and MAR ($p = 0.0040$, $p = 0.11$ respectively) than CC group. The BFR was more than two times greater in LE ($p = 0.019$) and LC ($p = 0.074$) treatment groups than CC group. The MAR and BFR of the cores in the LE treatment group were larger than the CE treatment group by 66% ($p = 0.021$) and 98%

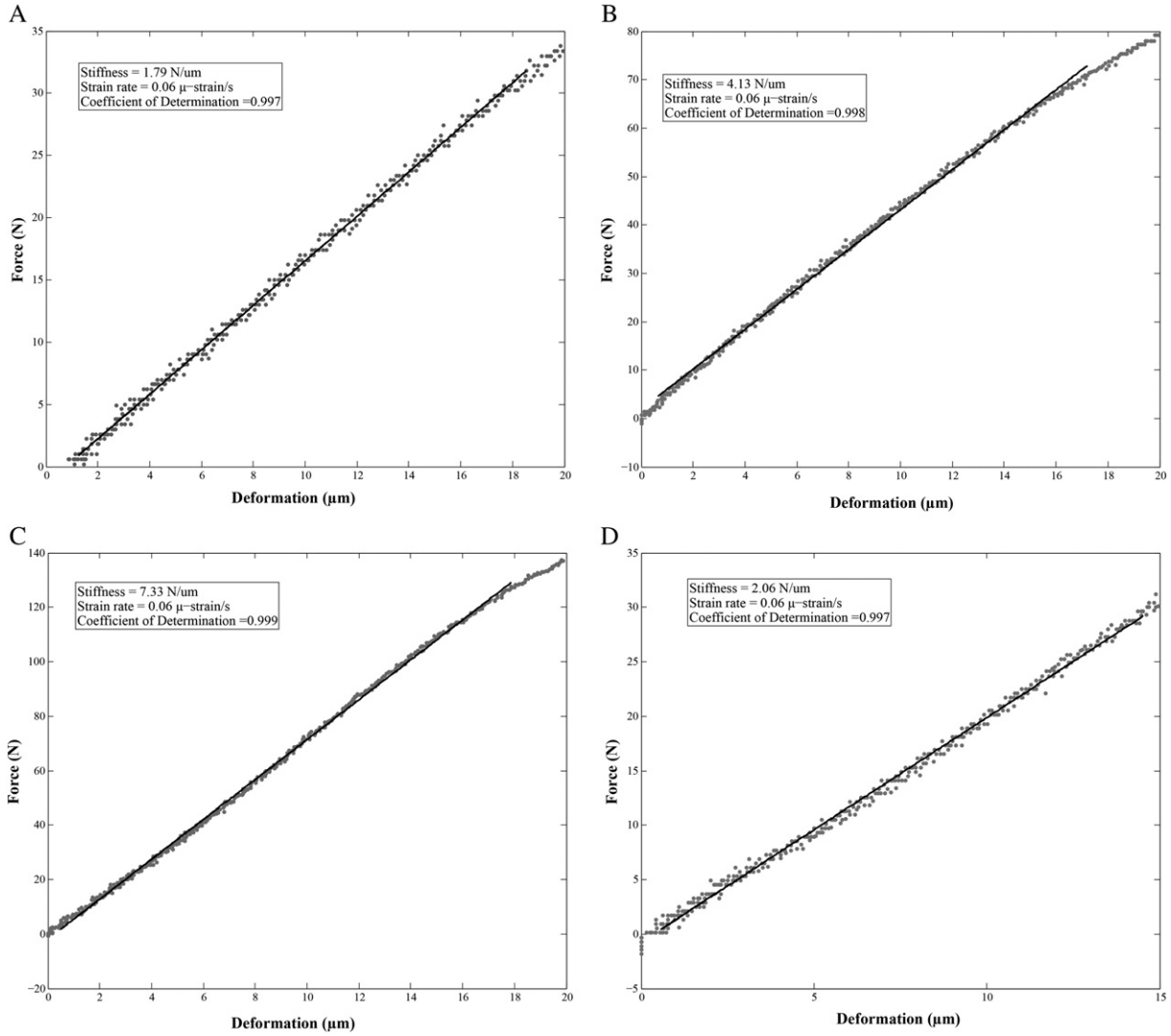

Fig. 2. Sample force versus deformation plots from quasi-static loading of representative bovine cores of groups CC, CE, LC and LE on day 23 of live bone study. Linear fit demonstrates axial stiffness to determine apparent elastic modulus. Groups are: CC (no load + no big ET1), CE (no load + big ET1), LC (load + no big ET1), and LE (load + big ET1).

($p = 0.044$), respectively. The LC and LE treatment groups were not different from each other for any of the bone formation markers. Two-way ANOVA with RM found PGE2 levels were elevated at 8 days post-osteotomy in all groups and the treatment groups remained elevated compared to the CC group on days 15, 19 and 23 (Fig. 4).

Table 1
Mean and standard error of the day 1 apparent elastic modulus (E_{app}), percent change in apparent elastic modulus (ΔE_{app}) from day 1 to 23, bone volume (BV/TV) mineralizing surface (MS/BS), mineral apposition rate (MAR), and bone formation (BFR/BS) for each group.

Measurement		CC	CE	LC	LE
E_{app} (MPa)	Day 1	123 (28)	132 (19)	144 (30)	149 (28)
	n	11	11	10	9
ΔE_{app} (%)	Days 1–23	3.3 (8.6)	13 (12.2)	17 (3.9)	19 (4.2)
	n	8	11	10	8
	p-Value	–	0.56	0.14	0.13
BV/TV (%)	Day 23	17.9 (1.6)	16.5 (1.6)	16.5 (0.86)	16.2 (1.8)
	n	10	10	8	9
MS/BS (%)	Days 9–19	14 (3.1)	16 (2.9)	22 (3.9)	23 (2.0)*
	p-Value	–	0.61	0.055	0.037
MAR (μm/day)	Days 9–19	0.89 (0.21)	1.08 (0.23)	1.4 (0.24)	1.8 (0.14)*,**
	p-Value	–	0.50	0.11	0.0040
	p-Value	0.50	–	0.33	0.021
BFR/BS ($\mu m^3/\mu m^2/yr$)	Days 9–19	63 (24)	76 (22)	131 (38)	151 (16)*,**
	p-Value	–	0.72	0.074	0.019
	p-Value	0.72	–	0.14	0.042

*Represents statistical difference with $\alpha = 5\%$ significance compared to CC; **represents statistical difference with $\alpha = 5\%$ significance compared to CE; where n is sample size; and, Fisher's LSD tests were used for pairwise comparisons. Groups are: CC (no load + no big ET1), CE (no load + big ET1), LC (load + no big ET1), and LE (load + big ET1).

4. Discussion

Bone modeling and remodeling in response to mechanical stimuli are controlled by the interactive relationship of stem cells, osteoblasts, osteocytes and osteoclasts, in differentiation, formation, maintenance and resorption in bone [21]. Recent studies have shown that bioreactor systems are advantageous for bridging the experimental gap between *in vitro* cell culture and animal or clinical studies [41,42]. *Ex vivo* organ culture allows investigations of whole tissues without disrupting the interactions among neighboring cells and the extracellular matrix [43]. The ZETOS Bone Loading and Bioreactor System (ZETOS) allows isolation and maintenance of trabecular bone cores with active and viable stem cells, osteoblasts, osteocytes, osteoclasts necessary to maintain bone homeostasis [10–14,30–32]. Bone formation in response to mechanical and biochemical stimuli during a multi-week experiment was assessed by measuring changes in axial stiffness of bone cores, using the ZETOS mechanical testing apparatus, and secretions of metabolic biomarkers [10–13,30–32]. Static and dynamic histomorphometry was used to trace morphological changes resulting from modeling and remodeling [10,13,30]. Contributors to mechanical stimulus are the magnitude of the load (με), the rate of load application (με/s), the number of loading cycles, and the rest periods between loads [1,2,6]. Previous *ex vivo* studies have investigated the impact of mechanical loading on trabecular bone including human, bovine, and ovine and found that these species respond similarly to mechanical load [9–11,13].

Endothelin-1 (ET1) was first recognized as a highly potent vasoconstrictive agent [44]. The active molecule consists of 21 amino acids and is produced by cleavage of the 34 amino acid, inactive big ET1, by a variety of proteases [45,46]. The ET1 signaling pathway is ancient and found, along with Notch [47], Hedgehog [48], and WNT [49] pathways, as far back in the evolutionary lineage as Hydra [50]. Like the other ancient pathways, the ET1 signaling axis is critical for development. Global ablation of any of the ET1 signaling axis genes leads to developmental lethality with ablation of ET1, the endothelin receptors type A (ET_A) and B (ET_B) type receptor, and *Ece1* leading to multiple abnormalities in neural crest-derived structures [51–53]. The ablation of ET_A causes

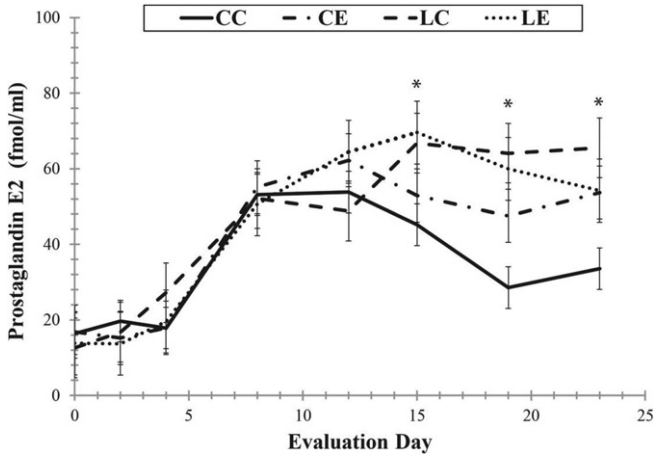

Fig. 4. Prostaglandin E2 secretion measured from culture medium sampled daily over duration of experiment. Data represent mean and standard error for each treatment group. Asterisks indicate statistically significant difference in all treatment groups from CC with α = 5% significance using Holm-Šídák test for multiple comparisons. Groups are: CC (no load + no big ET1), CE (no load + big ET1), LC (load + no big ET1), and LE (load + big ET1).

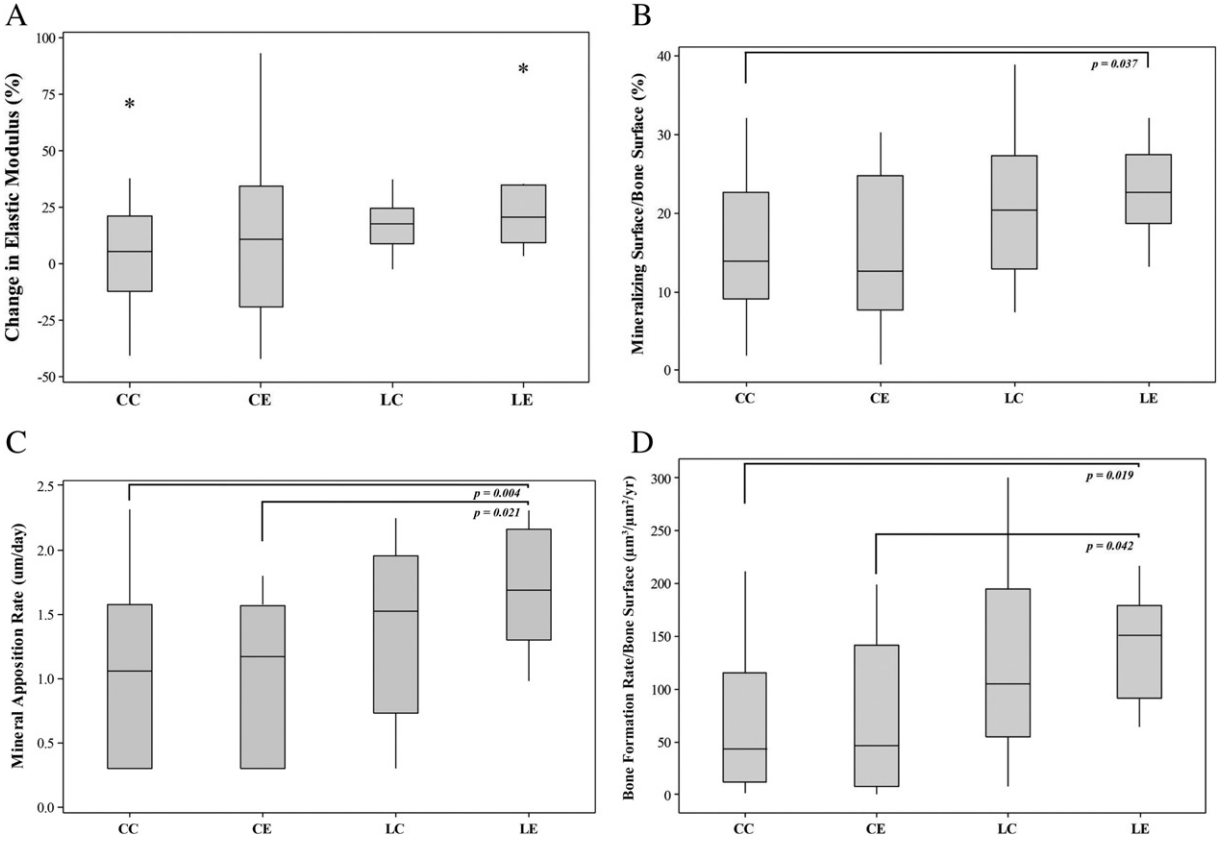

Fig. 3. A) Percent change in apparent elastic modulus (ΔE_{app}) between days 1 and 23; B) mineralizing surface (MS/BS); C) mineral apposition rate (MAR) between days 9 and 19; D) bone formation rate (BFR/BS) between days 9 and 19. Graphs represent medians, first and third quartiles, and the lowest/highest datum within 1.5 interquartile lower/higher range for each treatment group. Asterisks indicate outliers (CC outliers greater than 100% are not shown). Horizontal bars indicate statistically significant difference with α = 5% significance according to Fisher's LSD tests. Groups are: CC (no load + no big ET1), CE (no load + big ET1), LC (load + no big ET1), and LE (load + big ET1).

a decrease in trabecular volume in postnatal development [54]. The secretion of ET1 impacts local cell growth and differentiation and has been shown to promote mineralization by osteoblasts and disorganized bone growth in osteoblastic metastases of breast and prostate cancer [24,55–59].

This is the first study to explore the potential impact of big ET1 exposure and mechanical stimulus on osteogenesis in *ex vivo* cultured trabecular bone cores. The data suggest that combined exposure to big ET1 and mechanical loading results in increased osteogenesis as measured in biomechanical, biochemical and histomorphometric responses. Treatment with big ET1 and daily dynamic loading increased ΔE_{app}, MS/BS, MAR and BFR/BS in bovine trabecular bone cores in comparison to CC group. Also, the study results suggested that trabecular bone exposure to big ET1 evoked similar responses to mechanical loading, quantified by ΔE_{app} and PGE2 secretion in the treatment groups compared to the CC group.

Both treatments, big ET1 and load, significantly affected the secretion of PGE2 on days 15, 19, and 23. PGE2 levels were elevated at 8 days post-osteotomy in all groups and the treatment groups remained elevated compared to the CC group on days 15, 19 and 23. The initially elevated levels of PGE2 secretion were a reflection of the healing process induced by the osteotomy [60]. That the secretion of PGE2 remained elevated in all treatment groups, in comparison to the control group, implies that big ET1 and mechanical load are similar in their stimulation of trabecular bone [61–63]. The PGE2 secretion results support the ΔE_{app} and histological findings. While SOST could not be measured in this study because ELISAs for bovine SOST are not available, other studies have found elevated PGE2 decreased expression of SOST resulting in an increase in WNT signaling and an increase in bone formation [22,23]. We have also previously demonstrated that addition of exogenous big ET1 to mouse osteoblasts decreases SOST secretion [24]. Therefore the PGE2 findings are consistent with the ΔE_{app} and histological results from the current study.

Although all treatment groups showed an overall increase in E_{app} from day 1 to 23 of the study, the ΔE_{app} in the treatment groups compared to CC group were not statistically significant. The increase in E_{app} was reflected in the histological analysis. Bone formation was greater in LE treatment group compared to CC and CE groups, with LC treatment group trending to greater bone formation and no significant difference between the CE and CC groups.

Previous studies with a similar experimental setup and sample size to the current study reported a statistically significant difference in ΔE_{app} due to loading [9–11,13]. Type II error may be associated with some of the lack of statistical significance in the measured effects, a result of high variance and low sample sizes. Although 48 samples (twelve per group) may be sufficient to reduce type II error in *ex vivo* testing of trabecular bone cores [9–11,13], eleven samples (one to four per group) in the current study were not included in the statistical analyses of the ΔE_{app} and histological results. Variance in the data could have arisen from a number of sources including the inherent variability in trabecular tissue and loss of connectivity caused by excision [35–38]. In this study, the mechanical behavior (E_{app} and ΔE_{app}) of trabecular bone cores was assessed. It is important to keep in mind that the E_{app} of the cores is dependent on bone volume fraction and trabecular orientation. These parameters are highly variable in trabecular bone (Table 1) and could not be assessed at the start of the experiment without interfering with the sterility and viability of the specimens. The orientation of the trabecular structure relative to the tested load direction may have varied between cores depending on their *in situ* location in the sternum. The sternum is a non-weight bearing bone, where muscle pull may vary along the length of the sternum. However, use of a single bovine sternum enabled all cores to be harvested from one animal, avoiding variance due to genetics.

Potential sources of error in compression testing of trabecular bone include machine compliance, end artifacts and side artifacts [34,64–66]. Several steps in the mechanical testing procedure were designed to reduce these errors and sources of variance. The ZETOS loading system has been calibrated for E_{app} range of 40 to 1500 MPa [31,32]. Reference bodies tested within this range demonstrated a 3% accuracy in E_{app} [32]. Standard ZETOS specimen dimensions (10 mm diameter, 5 mm height) meet the main goal of maintaining core viability for several weeks [9,30], and are adequate for continuum assumptions [34,64–66] as required for equations of Hooke's Law. The aspect ratio of the bone cores (0.5) although favorable for maintaining viability could increase end artifacts in the measurement of E_{app} because of specimen-platen interface conditions and structural end artifacts [34,64–66]. The F-δ curves (Fig. 2) did not reveal a large "toe" region indicating machine compliance and possible end-artifact errors have been minimized.

Despite the study's limitations, the results of the current study align with those of previous loading studies. Increased secretion of PGE2 has been reported following mechanical stimulation of osteocytes [62,63] and bone [61], a similar result to the increased PGE2 secretion in all treatment groups in the current study. E*x vivo* experiments on bovine trabecular tissue under dynamic loading with a maximum bulk strain of −4000 με found an average increase in the E_{app} of approximately 50% over the course of 21 days [9]. The comparatively lower observed change in E_{app} in the present study (17–19%) may be due to the lower applied strains (−2000 με) or normal biological variation between different animals. Endres and co-workers experimented with the level of dynamic loading, finding a direct dose–response relationship for bulk strains between −1000 and −4000 με [13]. Similarly, comparative histological bone formation markers have been reported: MAR 0.94 ± 05 μm/day [10]; MAR 0.53 ± 0.08 μm/day [67]; and MS/BS 25 ± 5%, MAR 1.0 ± 0.3 μm/day, and BFR/BS 100 ± 11 μm/year [14].

Previous *in vivo* studies demonstrated that −1000 to −2500 με corresponds to physiological strain levels experienced during walking and other daily activities; however, compressive strains as high as 3000 to 5000 με have been measured during high intensity activities [2,68–70]. In these studies, strain was measured *in vivo* from strain gauges fixed to the outer surface of the cortex during physiological activities. In contrast, in the current study, strain was determined from the bulk compression of the trabecular bone core, including the initial nonlinear "toe" region of the force-deformation curve that remained beyond the 10 N preload. Therefore, the actual strain of the bulk of the bone core may be less than calculated. The loading of the trabeculae in the bone core is complex and depending on their morphology and orientation include tensile, compressive, bending and shear strains. A recent numerical study found 2.2–12% of total bone in bone cores, under −2000 με bulk strain, experienced local trabeculae strains between −1000 and −3000 με [14]. To determine the relationship between the bulk strain of the bone core and local strain in the trabeculae micro computed tomography based finite element analysis is required, and is the subject of a subsequent study.

5. Conclusion

This study successfully showed that combined exposure to big ET1 and mechanical loading promoted osteogenesis in *ex vivo* bovine trabecular bone. Specifically, MAR, BFR/BS and PGE2 secretion were higher in LE treatment group compared to CC group. The findings will be used to design studies on *ex vivo* human trabecular tissue with the goal of investigating mechanotransduction signaling pathways and the role of big ET1 in bone modeling and remodeling.

Conflict of interest

All authors declare that they have no conflict of interest.

Authors' roles

Study design: LAM, MGJ, JFV, RDB, HP, ELS. Study conduct and data collection: LAM, MGJ, JFV, ELS. Responsibility for the integrity of the

data analysis: LAM, MGJ, DCM, HP. Data interpretation, drafting, revising and approving final version of manuscript was performed by all authors.

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