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Does Vibrational Loading Modulate the Effects of Radiotherapy on Growing Bone?

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Does Vibrational Loading Modulate the Effects of Radiotherapy on Growing Bone?

A Capstone Project Submitted in Partial Fulfillment of the Requirements of the Renée Crown University Honors Program at Syracuse University

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Honors Capstone Project in Biophysical Science

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Abstract

Radiotherapy is an important part of cancer therapy, used in addition to surgery for treatment of patients with soft tissue sarcomas, and alternatively for treatment of patients with Ewing sarcoma of bone. Treating pediatric extremity tumors with radiotherapy has been shown to have harmful effects on the epiphyseal plate, resulting in permanent limb shortening and deformity when bone growth centers are exposed to radiation. Mechanical signals, specifically low-magnitude high-frequency vibrations (LMHFV), have been shown to be non-invasive and non-pharmacological growth factors in bone that have the potential to serve as a safe treatment for a number of clinical conditions. Thus, this study was aimed at evaluating the possible beneficial effects of low-magnitude high-frequency mechanical vibration (LMHFV) stimuli on growing irradiated bone and the possibility for restoration of function of the epiphyseal plate, a research topic that has never before been published in the literature.

Eighteen 3-week old weanling male Sprague-Dawley rats were subjected to a standard radiation dose of 17.5 Gray applied to right hind limbs, with the contralateral leg serving as a non-irradiated control. Then, the animals were divided into three groups: *A)* rats subjected to (LMHFV) only at 45 Hz, 0.3 g for 20 minutes once per day, 7 days/week, for 3 weeks, *B)* rats subjected the same conditions of LMHFV plus an injection of spermine NONOate, a nitric oxide donor that that has shown weak positive results as post-irradiation recovery agent, and *C)* rats subjected to sham LMHFV. After euthanizing the animals, skeletal growth was measured by x-ray analysis, marrow mesenchymal stem cell osteoblastic potential was measured by CFU-F analysis, and bone morphology was measured by micro-CT analysis.

X-ray and CFU-F analyses show statistically significant differences between right and left limbs in all groups. No statistical significance was observed between vibration versus control groups, but trends suggest there could be some positive effect of vibration, although not statistically significant. Micro-CT results show a clear difference between right and left limbs in all groups. Regarding vibration versus control groups, micro-CT results are ambiguous, but do suggest that vibration may have altered local growth characteristics and stimulated local shape changes in the 20% region from the distal end of the femur, just above the growth plate.

Despite the number of positive reports of LMHFV on bone, the present study did not reveal a clear, statistically significant effect on growth, structure or MSC colony formation. Thus, the effects of vibrational loading on irradiated growing bone are still unclear. Findings in this paper suggest that LMHFV may have a subtle positive effect, but this cannot be said with any statistical certainty. More studies on the effects of LMHFV on irradiated growing bone are needed to delineate the findings of this paper.

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Acknowledgements

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Brief Literature Review of Other Vibration Studies

Osteoporosis

Mechanical signals, specifically low-magnitude high-frequency vibrations (LMHFV), have been shown to be non-invasive and non-pharmacological growth factors in bone that have the potential to serve as a safe treatment for a number of clinical conditions[1]. Several studies have shown the potential enhancement properties of mechanical vibration stimuli when applied to bone in patients with osteoporosis. In the aging population, low-magnitude high-frequency vibration (LMHFV) has shown to have potential health benefits of improving coordination, strength, and movement speed[2], as well as improving balance and mobility in nursing home residents with limited functional dependency[3]. In postmenopausal women, vibration training was shown to improve muscle strength and significantly increase bone mineral density (BMD) and bone metabolism, suggesting use as a possible deterrent to osteoporosis in older women[2,4]. LMHFV was also shown to effectively inhibit bone loss in the spine and femur of postmenopausal women[5]. In an adult female sheep population, LMHFV was shown to improve both the quantity and quality of trabecular bone[6,7]. In addition, LMHFV has been shown to improve bone healing, strength and mass, as well as muscle strength, in ovariectomized rats[8-14], as well as rats treated with glucocorticoids[15]. LMHFV has been shown to promote fracture healing in

osteoporotic bone by enhancing callus formation, remodeling and mineralization in ovariectomized rats[16], as well as enhance bone-to-implant osseointegration in ovariectomized rats[17]. All of these findings provide a basis for use of mechanical vibration stimuli as a deterrent to osteoporosis in the elderly.

Other Bone-Related Healing

Additionally, LMHFV has been shown to accelerate fracture healing by enhancing bone remodeling and accelerating callus formation and mineralization, which have potential for improving fracture outcome clinically[16,18]. In an adult female mouse population, as well as an adult sheep population subjected to hindlimb unloading, application of LMHFV was shown to significantly increase the density of the spongy trabecular bone in the proximal femur[18,19]. Similarly, LMHFV has been found to preserve the marrow environment during disuse and enhance the initiation of tissue recovery upon reambulation[20,21]. In young women with low body mass density, LMHFV has been shown to increase bone and muscle mass in the axial skeleton and lower extremities[22]. In older men with age-related loss of skeletal muscle mass (sarcopenia), WBV training was shown to increase knee extension strength and muscle mass in the upper leg, with the potential to prevent or reverse sarcopenia[23]. In addition, LMHFV was found to restore anabolic bone cell activity inhibited by disuse by restricting increases in bone resorption, increasing bone formation, and reducing bone loss, with the potential to be applied to patients on bed rest or immobilized by several

degenerative conditions[24,25]. Another study showed that LMHFV can significantly increase the healing capacity of a bony lesion, even in non-weight bearing bone of the cranioskeleton[26]. In addition, LMHFV was found to stimulate peri-implant bone healing and osseointegration, with potential orthodontic benefits[27,28]. LMHFV enhances adaptive remodeling on condylar cartilage as well, which was evidenced by endochondral bone replacing hypertrophic cartilage[29]. These findings show that non-invasive vibrational stimulus may have potential for treating skeletal and muscle conditions.

Non-Bone-Related Healing

Interestingly, a study of the effects of LMHFV to tissues found that LMHFV was an anabolic stimulus to tendons, with similar effects demonstrated to its effects on bone and muscle, opening the potential that LMHFV may serve as a means to accelerate tendon healing[30]. LMHFV was also shown to enhance osseous regenerative processes, particularly in the presence of a supporting scaffold[31]. Thus, the anabolic properties of mechanical vibration stimuli can also be applied to tendon healing and connective tissue regeneration, in addition to osteoporosis deterrence, fracture healing, muscle strengthening, orthodontics and craniofacial repair.

Introduction

Radiotherapy

Radiotherapy is an important part of cancer therapy, used in addition to surgery for treatment of patients with soft tissue sarcomas, and alternatively for treatment of patients with Ewing sarcoma of bone. Treating pediatric extremity tumors with radiotherapy has been shown to have harmful effects on the epiphyseal plate, resulting in permanent limb shortening and deformity when bone growth centers are exposed to radiation[32-7].

Growth and Vibration

Children who undergo radiation treatment for cancer are at similar risk as adults with osteoporosis and stress fractures for a decrease in bone density, but at the same time, the epiphyseal plate is also affected, so not only is bone density effected, but also bone growth. Young mice exposed to extremely LMHFV were found to have improved quality in their musculoskeletal systems, with beneficial structural changes in trabecular bone, cortical bone, and muscle[38]. Also in the growing skeleton, short daily periods of extremely LMHFV were found to inhibit trabecular bone resorption, site specifically ease the declining levels of bone formation, and maintain a high level of matrix quality[39]. Children between the

ages of six to nine with motor disabilities subjected to daily high-frequency low-magnitude vibration witnessed improved bone mass and muscle strength with no side effects[40]. In post-pubertal disabled, ambulant children, high-frequency mechanical stimuli was found to be anabolic to trabecular bone growth as well[41]. Children with cerebral palsy, who have decreased strength, low bone mass and an increased propensity to fracture, were also found to benefit from LMHFV, specifically by increased cortical bone area and strength, which could translate into a decreased risk of long bone fractures in some patients[42]. These findings together all point to future implications for non-pharmacological and safe means to increase bone mass in children.

This Experiment

In an effort to reduce the stunting of normal growth that can accompany radiotherapy in children, the use of mechanical vibration stimuli is being explored. A thorough review of the literature has shown that vibration has never been studied in this capacity. Chondrocytes, the cells responsible for growth in the epiphyseal plate, are somewhat damaged by radiation, but continue to perform at a reduced level after radiation. Radiation damage of growth plate chondrocytes causes premature growth arrest and limb length shortening in children who undergo radiotherapy for malignant tumors[43-7]. It has been shown that mechanical loading regulates the proliferation and differentiation of growth plate chondrocytes[48]. Additionally, cyclic mechanical loading has been shown to

activate the cellular and biochemical responses of the cranial base growth plate (CBGP)[49]. Bone morphology, cellularity, growth plate height, and growth rate have all been shown to be negatively affected in irradiated animal models[43-7]. Thus, in this study, we examined the possible beneficial effects of low-magnitude high-frequency mechanical vibration stimuli on growing irradiated bone and the possibility for restoration of function of the epiphyseal plate.

Materials and Methods

Experimental Design

All experimental procedures were reviewed and approved by SUNY Upstate Medical University's Committee for the Human Use of Animals. Eighteen 3-week old weanling male Sprague-Dawley rats were obtained from Taconic Farms (Germantown, NY) and randomly divided into three groups: *A)* rats subjected to low-magnitude high-frequency vibration (LMHFV) only at 45 Hz, 0.3 g for 20 minutes once per day, 7 days/week, for 3 weeks, *B)* rats subjected to LMHFV with the same conditions as group A plus an injection of spermine NONOate, a nitric oxide donor that showed weak positive results as post-irradiation recovery agent in previous experiments by the Spadaro lab (not published) and others[50], and *C)* rats subjected to sham LMHFV, placed in cages used for vibration but with no stimulus applied.

After a 7-day quarantine period following delivery, a standard rat irradiation model was used on all eighteen SD weanling rats, with a radiation dose applied to the right hind limb and the contralateral leg serving as a non-irradiated control. Weanling rats were anesthetized using a Ketamine-Zylazine cocktail (80mg/kg). Then, the right hind limb was extended across a target area, such that the right knee joint crossed the middle of the target field, and legs were secured with masking tape. Lead shielding was placed around the rest of the animal. The

positioning plate was raised to a 30cm source-to-target distance and the light beam was collimated to approximate the 2cm x 6cm radiation field inscribed on the positioning plate. A single fraction 17.5 Gray (300kV, 10mA) radiation field was applied to include the distal half of the femur through the mid-tibia for 7 minutes, 25 seconds. Two animals were irradiated at once, and Plexiglas sheets 0.75 inches thick were placed under the thin Plexiglas to support standardized scatter. A warming pad was placed under the Plexiglas to help maintain animal body temperature during exposure. Yohobine reversal was used as needed to clear the anesthesia (0.05mL dose). For group B, two injections of spermine NONOate were given (2.4 mg/kg dose, 240µg/animal total), one an hour following radiation, and another three hours after the first. Spermine NONOate (A.G. Scientific) was given intending to stimulate cell survival during the early phase of recovery. Rats were housed three per cage and free access to a standard rodent chow and water administered by animal care technicians.

Once a day, rats were transferred to a Plexiglas cage without any bedding to prevent dampening of the mechanical signal. Sham LMHFV animals were transferred to identical cages as LMHFV groups for the same period daily, but the stimulus was not activated. Containers holding LMHFV rats were placed on a vibration platform (JUVENT) along with 20 pounds of weight to produce a vertical displacement of 50 micron (0.3 g) at a frequency of 45 Hz for 20 minutes per day for 3 weeks after radiation exposure. Two JUVENT Platforms were graciously loaned from Professor Ken McLeod at SUNY Binghamton. These platforms and perimeters have been used in many animal studies of mechanical

effects on bone and muscle and as a method of treating osteoporosis and bone loss in microgravity during spaceflight, but never to study bone growth[51-53]. Treated cages were alternated between the two platforms to be certain that they received the same stimulus on average during the experiment, and rats were allowed to freely roam the cages during these 20 minutes. No qualitative differences in behavior or activity patterns were observed between groups. Hair loss on the right leg was observed in all animals during week 5 after radiation, attributable to radiation. Body weights were recorded just prior to irradiation and weekly until at euthanasia at 6 weeks. Animals were euthanized using carbon dioxide narcosis, and death was verified by the absence of a cardiac pulse.

X-ray Analysis

Hind limbs were isolated by removal at the hip joint, and digital x-rays of both hind limbs were taken immediately using the Faxitron Model FX-20 as the x-ray source and then the Agfa CR-30RX digital plate system to record the images. Limbs were positioned with knee and ankle joints in 90° flexion, placed on clear film and upon the imager with lead identifiers for left and right limbs as well as calibration. Image-J software was used then used to open the x-ray images and measure femur and tibia lengths for all animals. Means, standard deviations (SD), and standard error of the means (SEM) were calculated in Excel, and paired two-tailed *t*-tests were performed between right and left femora and tibias of all groups as well as Anova test comparisons between mean femora and tibia lengths

of different groups using Prism software. All data is expressed here as means ± SD (n=6 for each group).

CFU-F Analysis

Following euthanasia and X-rays, bulk musculature was removed with clean handling. Then, three sets of femora from each group were placed in cold DMEM culture medium with added antimicotics and 10% calf serum, for short storage to preserve for colony-forming-unit-fibroblasts (CFU-F) analysis, and the other three sets of femora from each group were frozen for later micro-CT examination. A CFU-F assay measures the osteogenic potential of bone marrow mesenchymal stem cells (MSCs). Alkaline phosphatase (ALP) is an early marker that is necessary for osteoblast expression. After removing from preservation media, marrow from the femora preserved for CFU-F analysis was flushed in fresh medium by removing both bone ends of the femora. Then, cells were counted and diluted so that aliquots of 1 million cells each were added to 6 well plates (3 plates per specimen) in growth medium. After 9 days of incubation at 37°C, plates were assayed for ALP, and the portion of colonies expressing the osteoblastic phenotype (ALP+) were counted against those not expressing ALP (ALP-) by using the EPSON scanner to create images of the plates, open images with Image-J, and counting cells with the cell counter plug-in in order to determine the osteogenic potential of bone marrow cells derived from femora of the different experimental groups[54]. Means ± SD were expressed (n=3 for each

group) for ALP positive and negative colonies as well as total colonies for right versus left femora of different groups.

Micro-CT Analysis

Bone morphology of one set of femora from each group was reconstructed via micro-CT at a voxel size of 30 μm (Scanco-40). Transverse slices were made over the entire bone length, with sets of femur pairs scanned at the same time. Files were later transferred to a high capacity computer and analyzed using Image-J software with a Bone-J plug-in[55]. Slice Geometry measurements of cross-sectional area (CSA), second moment of area around major and minor axes (Imax, Imin), minimum and maximum diameters of the bone shaft (Min Diameter, Max Diameter), and perimeter (Perimeter) were taken. Second moment of area around major and minor axes (Imax, Imin) measure the strength of bending about the major and minor axes. Larger values translate to more resistance to bending and stronger bones. Slice numbers were normalized to percentages in order to compare different groups, with a focus on 50 slices at the regions 20% and 30% as measured from the distal end (n=1 for each group).

Results

Effect of LMHFV on Body Mass by Weight

The mean body weight of the three groups was ~89.2 g at the beginning of the study. All three groups gained similar amounts of body mass (315-335%, $P < 0.01$) to reach an average of 378 g at the end of the 7-week experimental period, with no significant differences detected between groups (Figure 1).

Skeletal Growth by X-ray

A consistent statistically significant difference of 14.2% ± 7.9% between right and left femora and tibias was observed in all groups ($P < 0.01$, Figure 2, Figure 3). No statistical significance was found between femora and tibias of different groups ($P > 0.05$), but there was possibly a difference, although not statistically significant, between right femora of different groups (A: 32.6±2.3 vs. B: 31.9±2.2 vs. C: 31.2±1.2, Figure 2), as well as total leg lengths of the femur plus the tibia between different groups (A: 66.4±2.4 vs. B: 65.3±2.9 vs. C: 64.4±2.1, Figure 4).

Marrow Mesenchymal Stem Cells Osteoblastic Potential by CFU-F

A consistent statistically significant difference of B: 63.6% ± 28% and C: 72.3% ± 34% was observed between ALP-Positive (ALP+) cell counts (CFU-F Assay) between right and left femora of groups B and C, with left femur counts much higher than right femur counts in both groups (B: $P < 0.05$, C: $P < 0.01$). ALP-Negative (ALP-) and total colony counts observed the same trend. No statistical significance was found between femora of different groups ($P > 0.05$), but there was possibly a difference, although not statistically significant, between the number of ALP+ colonies counted in samples from right femora of groups B and group C (3.0 ±2.6 vs. 2.11±2.1, Figure 5, Figure 6). Group A measurements are not detailed here because the assay did not work.

Bone Morphology by Micro-CT

Quantitative Measurements. NOTE: The following analysis is tentative and based on the bones of only one animal per group that could be analyzed. For 20% measurements from the distal end of the femur, right femur CSA measurements increased from group A to group C, with a 13.2% increase from group A to group B and a 12.3% increase from group B to group C (Figure 7). 20% Imax and Imin measurements observed the same trend, with a 17.0% increase from group A to group B and a 13.8% increase from group B to group C in Imax right femur measurements, a 5.35% increase from group A to group B and a 12.9% increase from group B to group C in Imin right femur measurements (Figure 9, Figure 11). Left femur CSA measurements at the 20% region were

notably lower than the right femur measurements in each group by an average of 21.0% (Figure 7). 20% Imax and Imin measurements observed the same trend, with left femur Imax measurements an average of 15.2% lower than right femur Imax measurements, and left femur Imin measurements an average of 23.2% lower than right femur Imin measurements (Figure 9, Figure 11). For the 30% CSA measurements, right and left femur measurements evened out (Figure 8).

For 30% Imax and Imin measurements, an inverse relationship to 20% measurements was observed, as right femur Imax measurements were notably lower than the right femur measurements in each group (from A to C) in the 30% region by an average of 21.5% in Imax measurements and an average of 22.0% in Imin measurements (Figure 10, Figure 12). For 20% Min Diameter and Perimeter measurements left femurs were slightly lower than right femurs in all groups, by an average of 8.90% in Min Diameter measurements and an average of 16.6% in Perimeter measurements (Figure 13, Figure 17). For 30% Min Diameter, Max Diameter and Perimeter measurements, the opposite trend was observed, as right femurs were slightly lower than left femurs by averages of 6.48%, 7.23%, and 8.19%, respectively, in all groups (Figure 14, Figure 16, Figure 18). Most notably of all measurements, for 20% Max Diameter measurements right and left femur diameters from groups A and B were much larger than right and left femur diameters from group C (A Left, Right: 5.92, 6.04, and B L,R: 6.05, 6.28 vs. C L,R: 4.58, 5.03, Figure 15). The difference between 20% Max Diameter measurements of groups A and B and group C are visibly significant.

Qualitative Image Observations. Clear differences can be see between right and left femurs of full bone images and graphs of left versus right variable analyses (CSA, Imax, Imin, Min Diameter, Max Diameter, Perimeter) normalized to percentages (Figure 19, Figure 20, Figure 21). Looking at 20% slices for left and right femora between all three groups shows much denser trabecular bone in right femora than left femora in all groups (Figure 22). Trabecular bone also appears to be coarser in texture in right femora of all groups than left femora. In addition, bone shape appears different between left and right femora at 20% cuts. Between different groups, trabecular bone density appears to increase from group A to group C proportionally in both left and right femora. Looking at 30% slices for left and right femora between all groups trabecular bone density is greatly decreased from the 20% cuts (Figure 23). Trabecular bone can still be seen in all right femora and only slightly in the Group C left femur. Shape of bone cuts are much more uniform in 30% slices than in 20% slices. 20% and 30% cut observations can also be seen in full bone images looking at 20% and 30% areas from the distal end (Figure 19, Figure 20, Figure 21).

Discussion

Overall Effects of LMHFV on Skeletal Growth and Marrow MSCs Osteoblastic Potential

Despite the number of positive reports of LMHFV on bone, the present study did not reveal a clear effect on growth, structure or MSC colony formation. Although no statistical significance was found in x-ray or CFU-F data between vibrated and non-vibrated groups, trends were observed in both measurements that suggest vibration may have had a small positive effect on skeletal growth and marrow MSCs osteoblastic potential that was simply not statistically significant. Given this finding, perhaps more animals (greater than n=6) were needed to see such subtle effects.

Overall Effects of LMHFV on Bone Morphology

Micro-CT data is much more dense and less clear than x-ray and CFU-F measurements. First of all, definite conclusions cannot be drawn because only one femur set from each group was analyzed using micro-CT. More sets of femora are needed from each group to substantiate initial findings. Another difficulty of the micro-CT analysis is exact anatomical positional matches between right and left femora were hard to make because of the complexity of growth that resulted from

radiated limbs versus non-irradiated limbs. This problem notwithstanding, data from right and left femora was normalized to percentages and tentative conclusions were drawn by comparing matched percentages of total bone length.

Looking at 20% slice CSA data analysis and 20% slices from left and right limbs of groups A, B and C, there is much more trabecular bone area in right femora, which most likely accounts for the greater CSA measured in the right limbs compared to the left limbs. Also looking at the 20% cut images, trabecular bone areas for left legs appear to be the same, so it appears that cortical bone area decreased from group C to group A, given that CSA measurements shows a decrease from group C femora to group A femora. Imin and Imax data are most influenced by cortical bone. Imax and Imin 20% measurements mirror the trends observed in CSA data, supporting the conclusion that cortical bone area is decreased in the vibration groups compared to the control. At 30% slices, trends observed in CSA, Imax and Imin are reversed – the left femora have greater Imax and Imin measurements than the right femora for all groups. These data suggests that radiation dominates in the 30% region, and thus, right limb measurements were smaller than left limb measurements. Given that 30% slices show that outer diameters of right bones are smaller than left bones, it makes sense that Imax and Imin measurements would be greater for left limbs than right limbs. At the same time, given that trabecular bone is much more apparent in right femora than left femora, it makes sense that 30% cuts of CSA are about the same between right and left limbs and all groups. Interestingly, 20% Max Diameter measurements show much higher diameters for both left and right femora in groups A and B

than group C, suggesting that vibration may have altered local growth characteristics and stimulated local shape changes by vibration. These changes may just be local and not reflected throughout the entire bone. Differences in perimeter measurements between left and right limbs at 20% and 30% slices suggest a shape change associated with radiation. More animals and more detailed micro-CT analysis is needed.

Other Studies in the Literature Show No Anabolic Properties Associated With Vibration

It is noteworthy that although the majority of the literature shows positive effects on bone associated with vibration therapies, a number of studies have shown no anabolic properties associated with mechanical vibration stimuli, and one study even concluded that whole body vibration (WBV) therapy is potentially harmful to the human body[56]. Low-amplitude WBV was shown to increase lower-leg bone mineral content (BMC) after 7 months but not after 22 months in mice, showing that the potential of WBV to enhance bone mass in age-related osteoporosis was not supported, but improvement of BMC was supported in younger animals[57]. Also, LMHFV was shown to be effective in improving musculoskeletal tissues in ovariectomized rats, but was not optimal for fracture healing[58]. Similarly, six weeks of LMHFV on ovariectomized rats was found to have no substantial effect on tibial bone microstructure and strength[59], and 12 months of WBV therapy did not alter BMD or bone structure in postmenopausal

women who received calcium and vitamin D supplementation[60]. In constrained tibial vibration (CTV) studies, high-frequency low-amplitude CTV loading of mice was not anabolic to bone in anesthetized, adult mice[61]. Parathyroid hormone (PTH) was studied in conjunction with WBV, and it was found that intermittent PTH treatment increased cortical bone volume and strength in adult mice, but daily exposure to low-magnitude WBV by itself did not improve skeletal properties[62]. Also, short-term low-strain vibration was shown to increase chemo-transport, but did not stimulate an increase in mechano-responsive, osteogenic gene expression, or cortical bone formation in tibias of adult mice[63]. It was also found that LMHFV did not enhance the osteogenic differentiation of mesenchymal stem cells (MSCs), but rather, inhibited matrix mineralization and decreased the mRNA level of a transcription factor necessary for osteoblast formation, showing that LMHFV may exert its anabolic effects in vivo via mechanosensing of a cell type other than MSCs[64].

Factors Influencing Effects of Vibration

These varying findings related to mechanical vibration stimuli are likely attributable to the fact that the ability of physical signals to influence bone morphology is strongly dependent on the signal's magnitude, frequency and duration[1]. All of the above-mentioned experiments were conducted within the perimeters of 30-90 Hz frequencies, 0.1-4 g magnitudes, and anywhere from an hour to a year of treatment. These varying signal and exposure characteristics are

likely the reason for discrepancies in findings. In vivo experiments excluded, the best results were overwhelmingly witnessed with a 30-60 Hz vibration frequency and a magnitude of 0.3 g[6,7,9,11,15,16,18,26-30,38,39,42,47,65,66]. As far as duration goes, experimental designs were varied, but for LMHFV studied with rat and mouse models over time, LMHFV exposure of 15-30 minutes per day, daily or 5 days per week for 4-8 weeks were uniform bounds for experiments that received positive results[2,8,10,13,15,16,18,30,42,49,57,59,66]. This experiment was in the beneficial range of such parameters. Thus, the results were surprising, despite the fact that LMHFV does not appear to have been tested previously in the case of irradiated bone.

A Note on Mechanisms of Mechanical Loading

An improved understanding of which components of bone's mechanical environment are anabolic, catabolic, or anti-catabolic will allow the development of biomechanical interventions in the areas, including orthodontics, craniofacial repair, osteoporosis and fracture healing. Much of the clinical evidence that mechanical forces are anabolic to bone has come from exercise studies performed in the last century[1]. Studies have shown that sporting activities of any kind cause the body to experience eternally applied forces, inducing vibrations and oscillations within the tissues of the body, whether it be impact shocks experienced through the leg during running when the heel hits the ground or more continuous tissue vibrations experienced for example through the legs during

skiing down a slope[67]. Studies have clearly shown that bone morphology can change strikingly in response to long-term exercise[1]. The effects of LMHFV in the body are thought to be analogous to the effects of exercise, but the mechanism by which mechanical signals become anabolic or anti-catabolic to bone are mostly unidentified. There is debate as to whether the mechanical input received by bones originates from ground reaction forces produced by the skeleton or from muscle activity.

Osteocytes are now thought to be the major mechanosensor in bone, responsible for sending signals to osteoblasts and osteoclasts, which carry out bone formation and resorption[64]. The prevailing view of bone mechanobiology is that osteocytes are responsible for detecting and responding to mechanical loading and initiating the bone adaptation process, but how osteocytes signal effector cells and initiate bone turnover is not well understood[68]. Osteocytes were found to sense LMHFV and respond by producing soluble factors that inhibit osteoclast formation[69]. Additional mechanical loading was shown to decrease the osteocyte's potential to induce osteoclast formation by direct cell-cell contact, and mechanically stimulated osteocytes to release soluble factors that can inhibit osteoclastogenesis induced by other supporting cells, including bone marrow stromal cells[68]. A study testing the effects of LMHFV on proliferation and osteodifferentiation of bone marrow-derived mesenchymal stromal cells (BMSCs) seeded on human bone-derived scaffolds found that microvibration promotes BMSC differentiation and increases bone formation of BMSCs by increasing their osteogenic lineage commitment and enhancing osteogenic gene expressions[70]. A

study of the changes in the mRNA levels of thirteen genes compared to altered indices of bone formation in the presence of LMHFV confirmed the complexity of the bone remodeling process, in terms of the number of genes involved, their interaction and coordination of resorptive and formative activity. More detailed analysis of the correlations between altered mRNA levels and tissue plasticity is needed to further delineate the molecules responsible for the control of bone mass and morphology[65].

Suggested Further Study

Given the findings of this paper, the effects of vibrational loading on irradiated growing bone are still unclear. Findings in this paper suggest that LMHFV may have a subtle positive effect, but this cannot be said with any statistical certainty. In future studies, more animals in each group are needed to determine whether vibrational loading can enhance bone growth in irradiated growing bone in a statistically significant manner. Also, more detailed micro-CT analysis including many more femora sets, and analysis of trabecular versus cortical bone areas are needed in order to delineate potential findings of this paper. Further study regarding the mechanism by which vibrational loading stimulates bone growth is also suggested in order to devise more standard, efficient experimental designs.

Supplemental Figures

Figure 1: Average Rat Weights

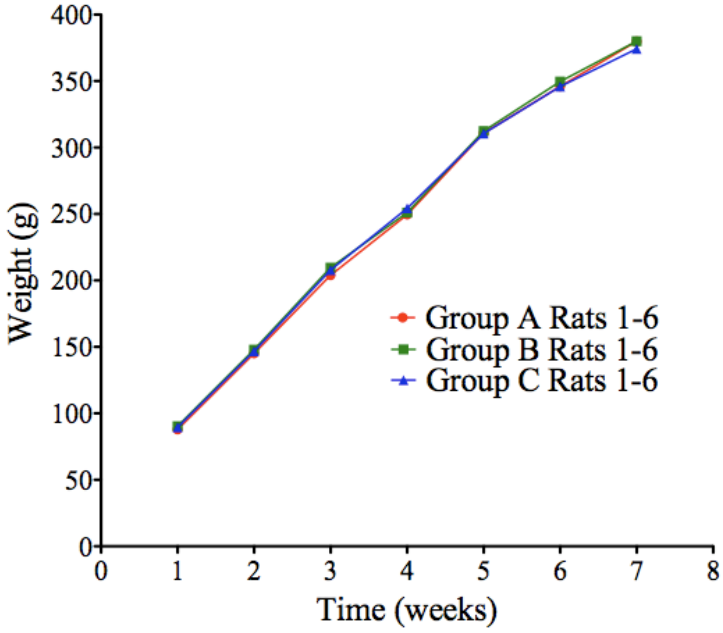


Figure 2: Left vs. Right Average Femur Lengths

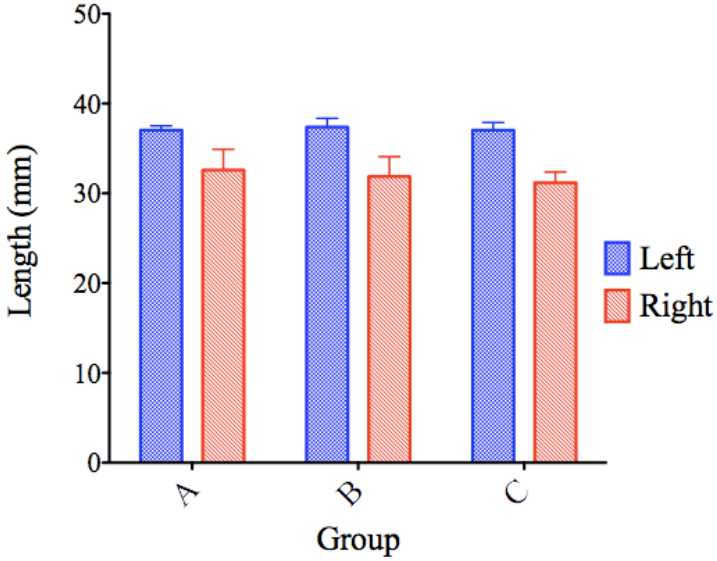


Figure 3: Left vs. Right Average Tibia Lengths

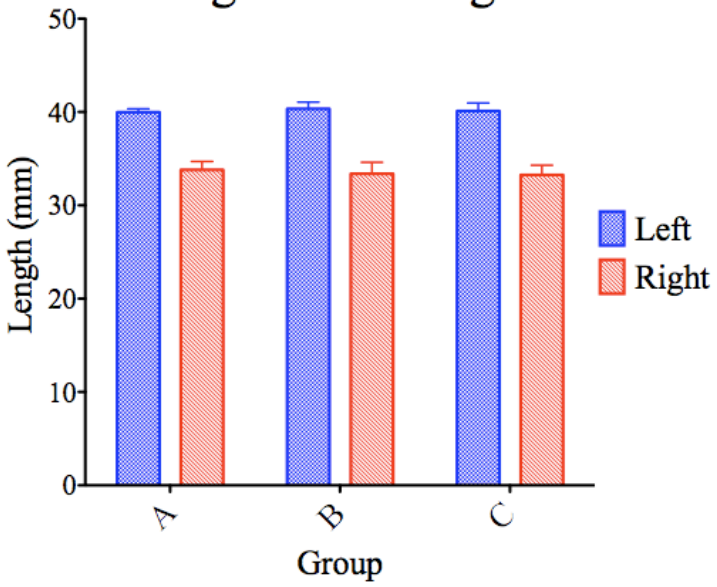


Figure 4: Left vs. Right Average Total (Femur + Tibia) Lengths

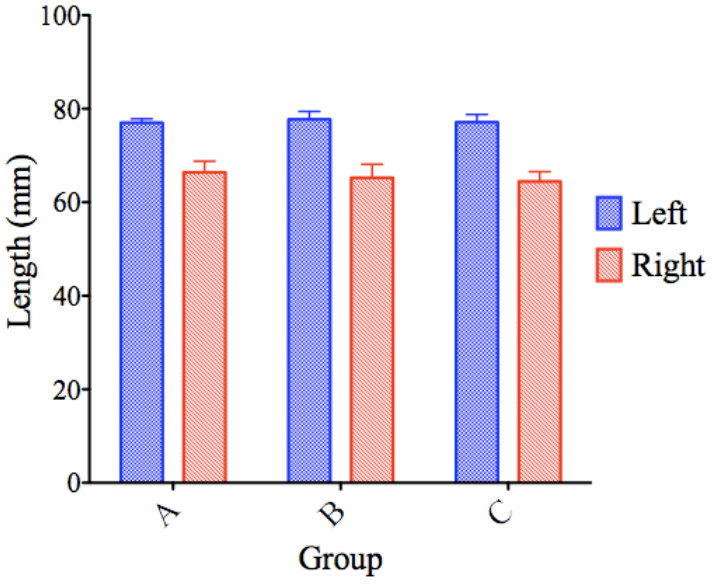


Figure 5: Group B Left vs. Right Average Femur Colony Counts

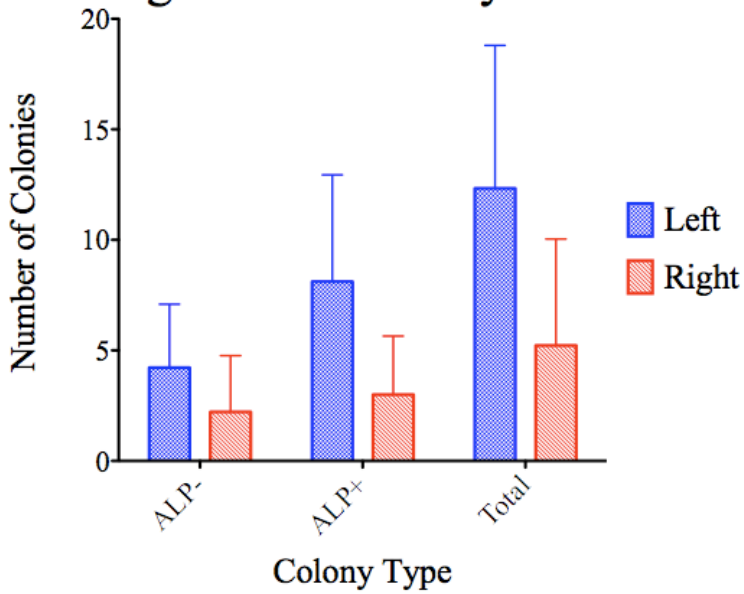


Figure 6: Group C Left. vs. Right Average Femur Colony Counts

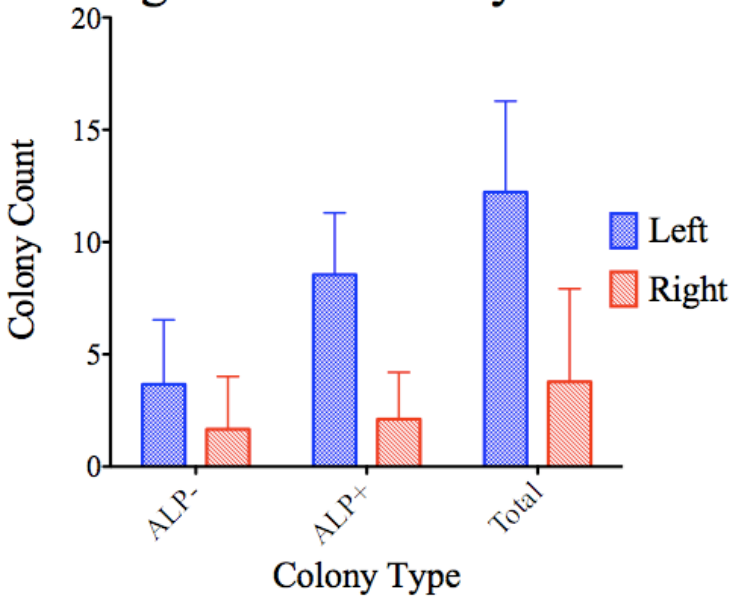


Figure 7: CSA 20% from Distal End

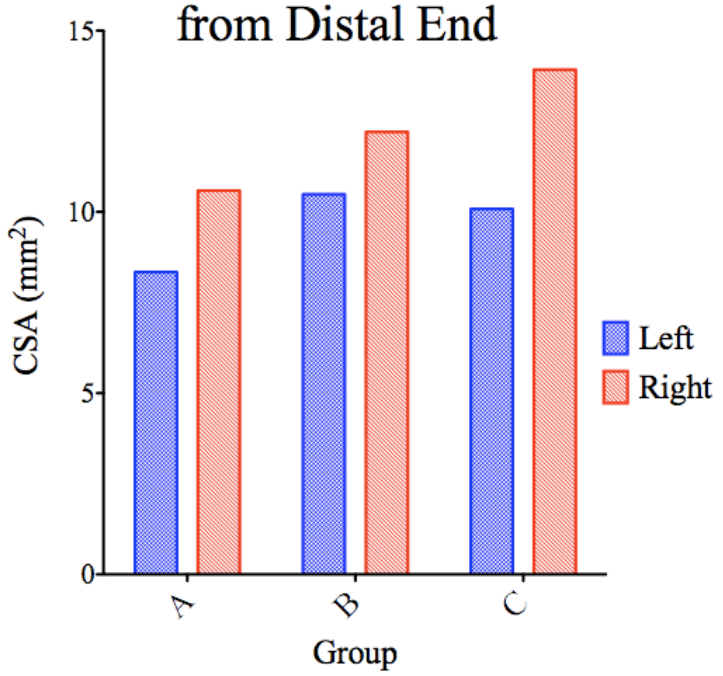


Figure 8: CSA 30% from Distal End

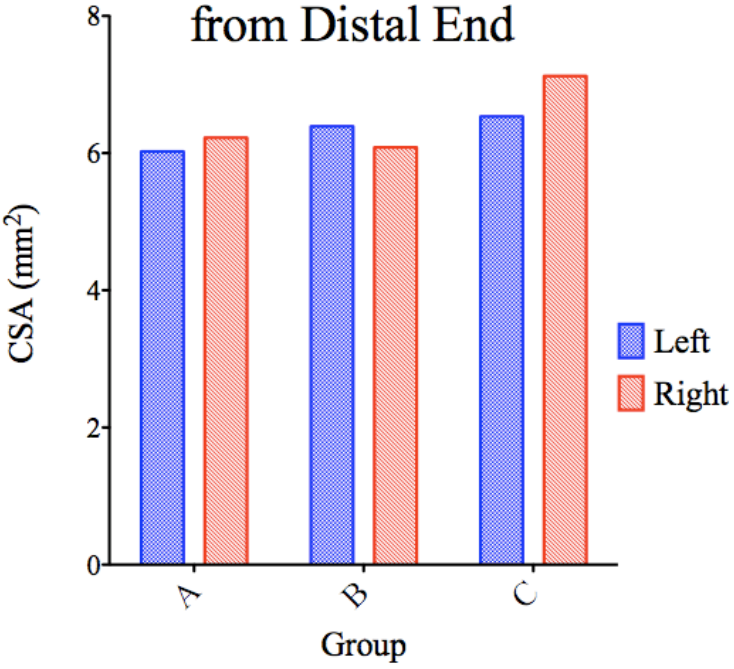


Figure 9: Imax 20% from Distal End

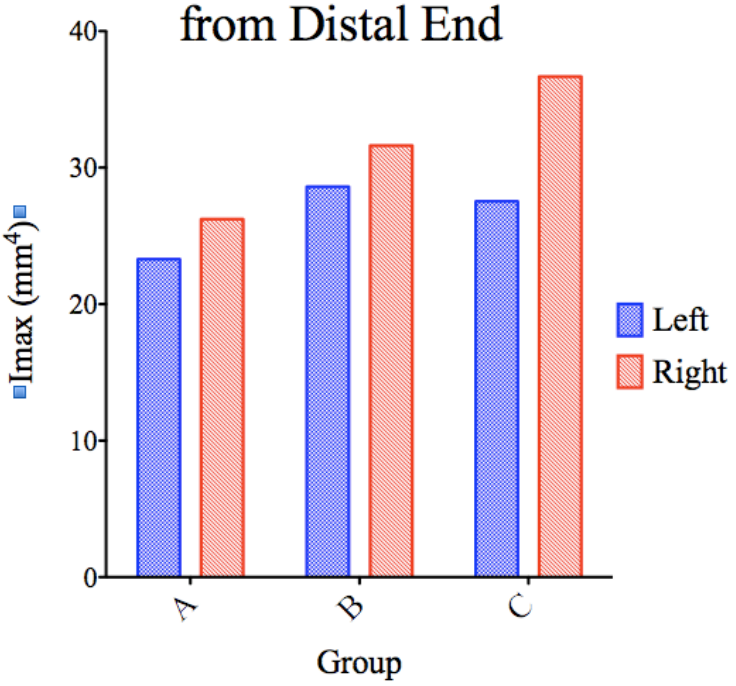


Figure 10: Imax 30% from Distal End

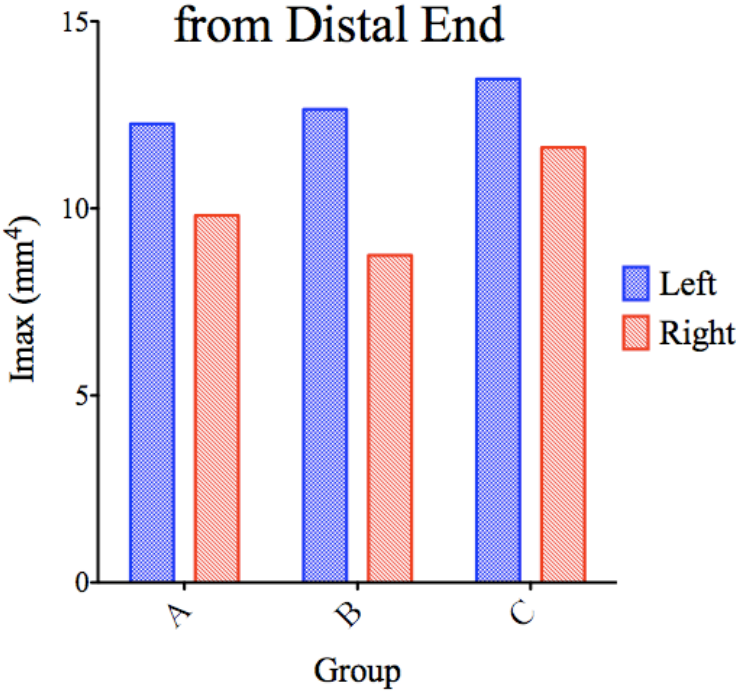


Figure 11: Imin 20% from Distal End

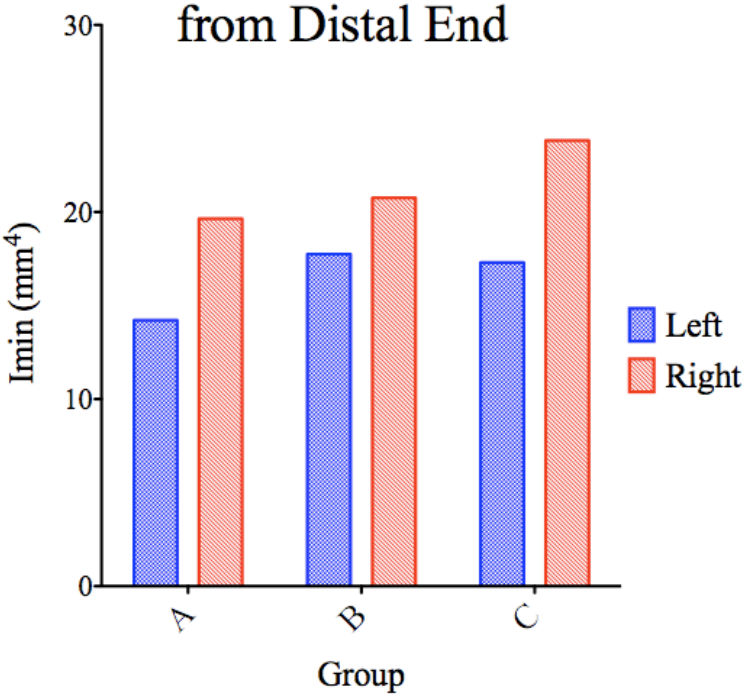


Figure 12: Imin 30% from Distal End

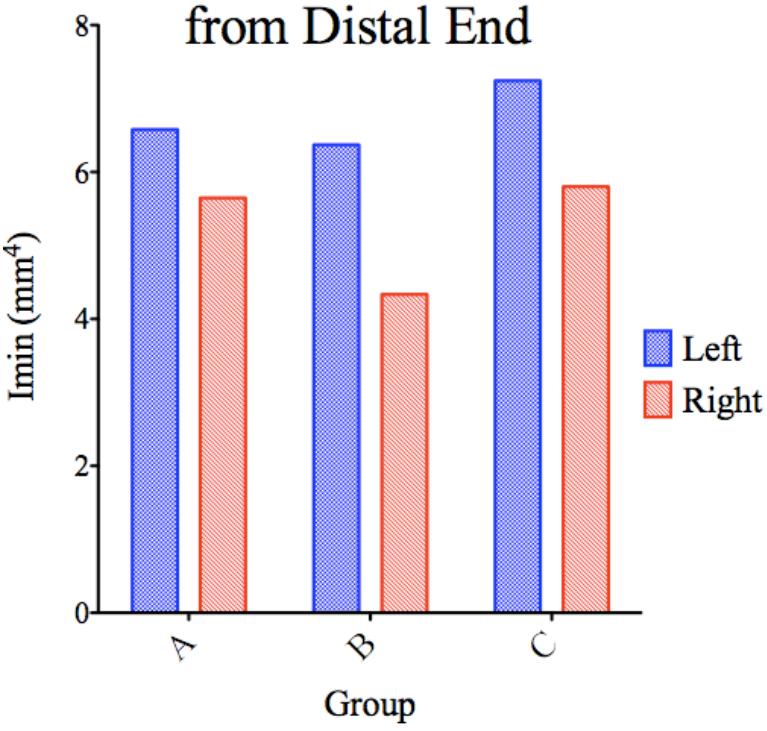


Figure 13: Min Diameter 20% from Distal End

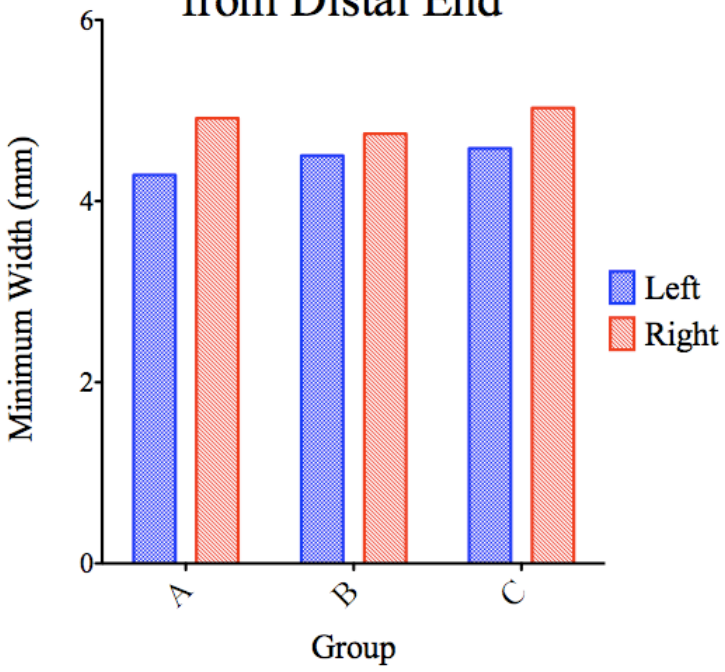


Figure 14: Min Diameter 30% from Distal End

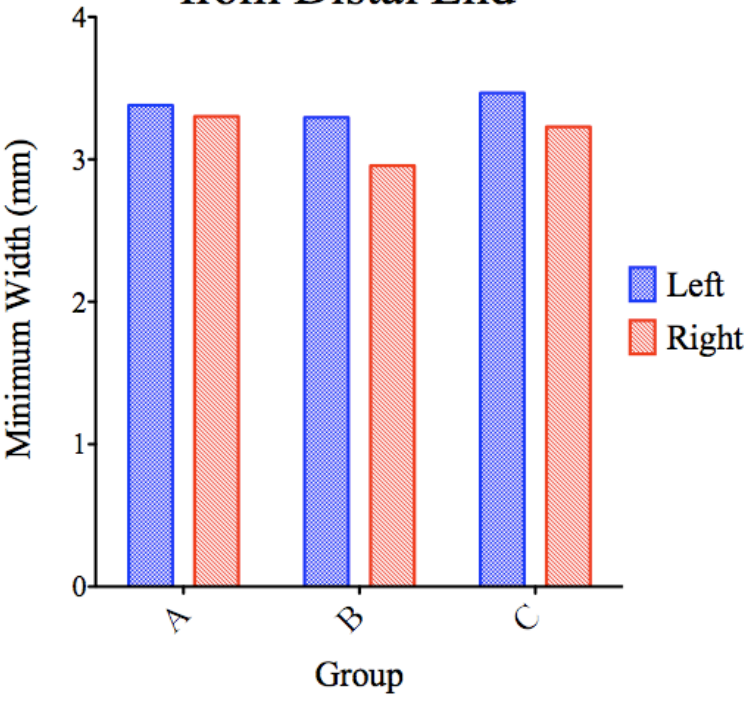


Figure 15: Max Diameter 20% from Distal End

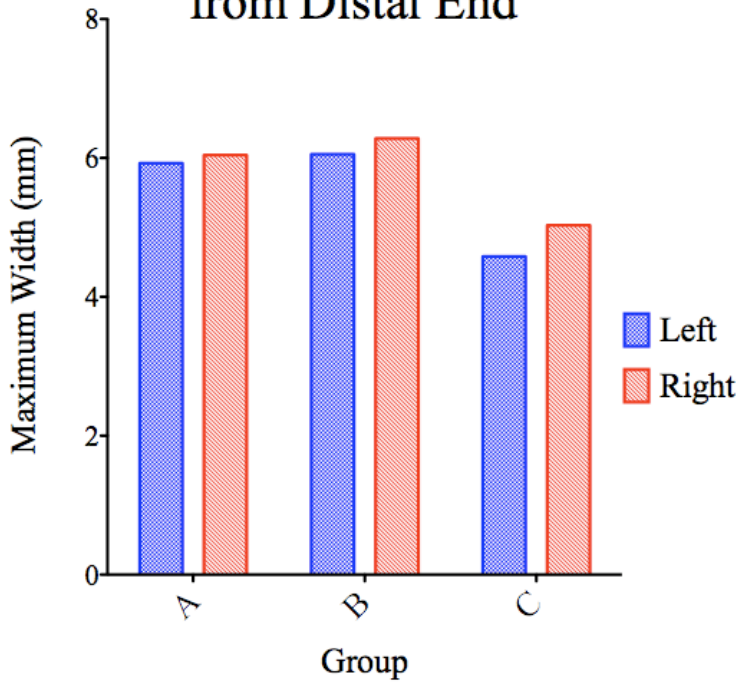


Figure 16: Max Diameter 30% from Distal End

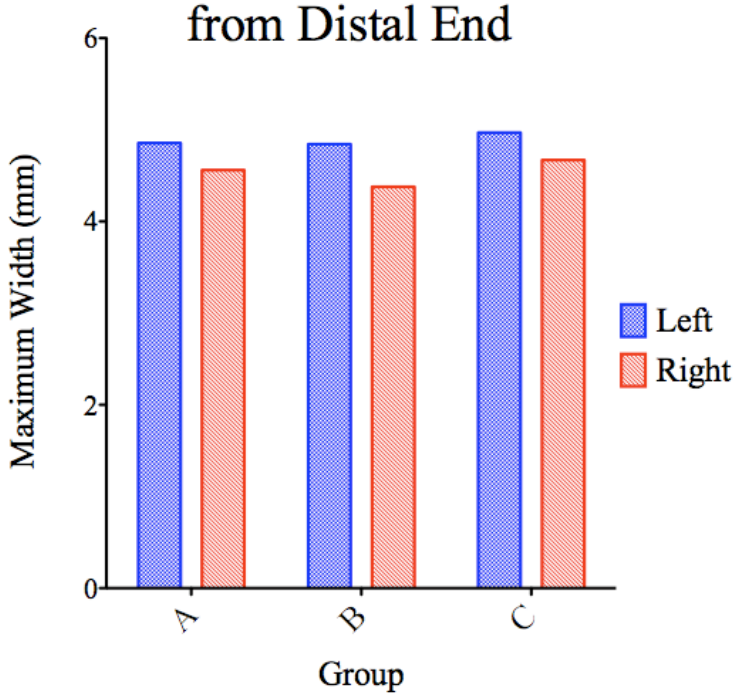


Figure 17: Perimeter 20% from Distal End

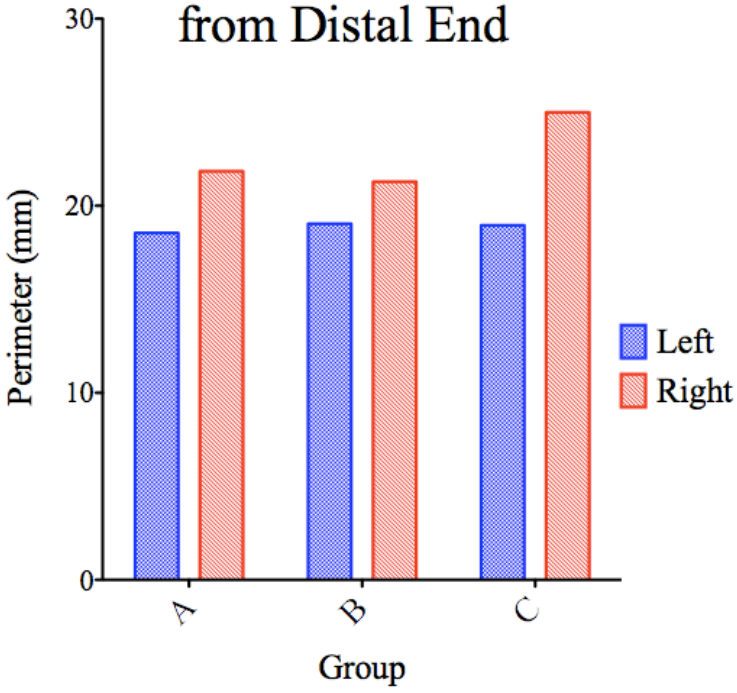


Figure 18: Perimeter 30% from Distal End

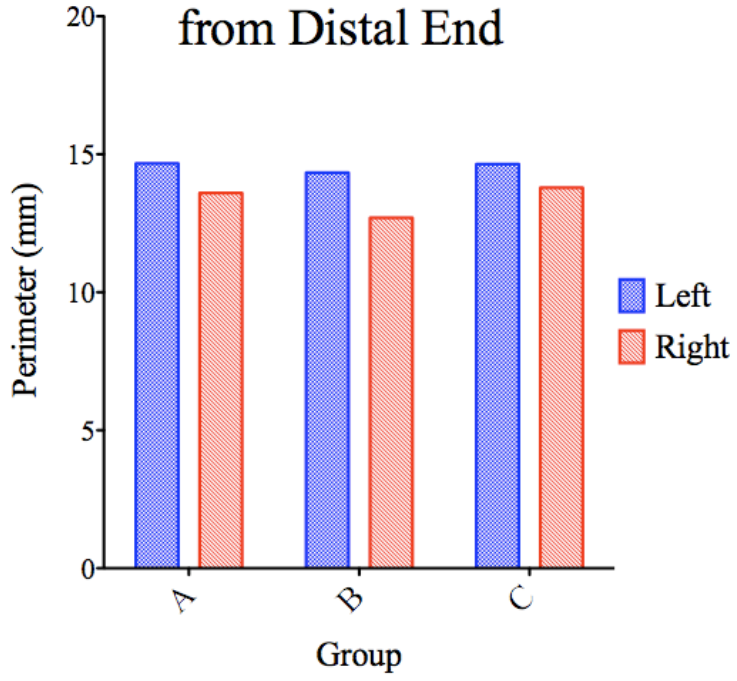
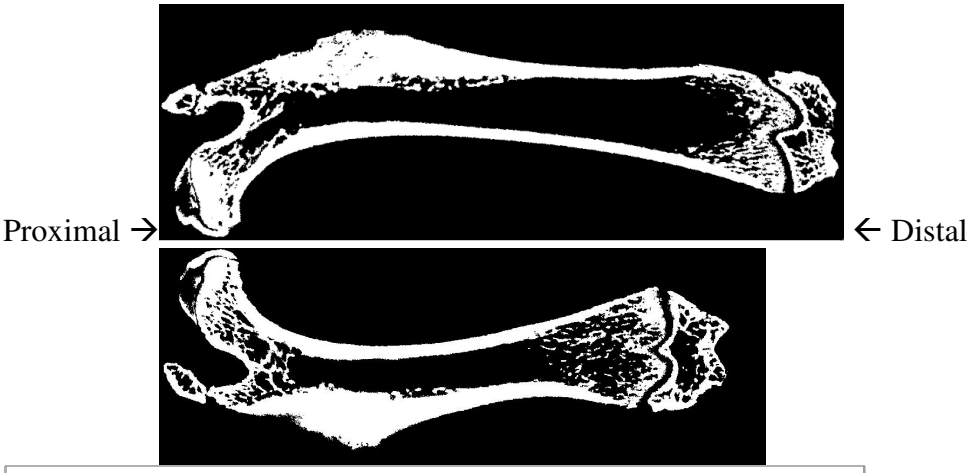
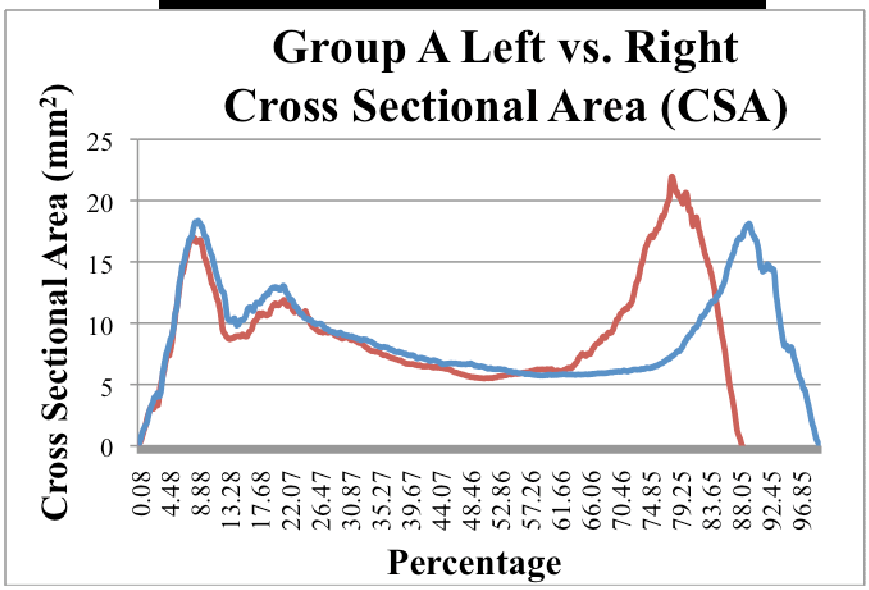


Figure 19: Images of Group A Left and Right Femurs Aligned With CSA Graph of Data Along the Bones.
Image 1: Group A Left Femur
Image 2: Group A Right Femur

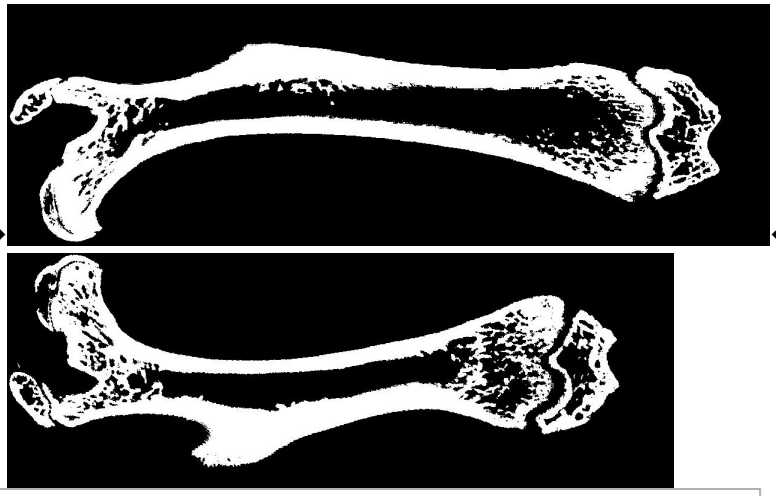


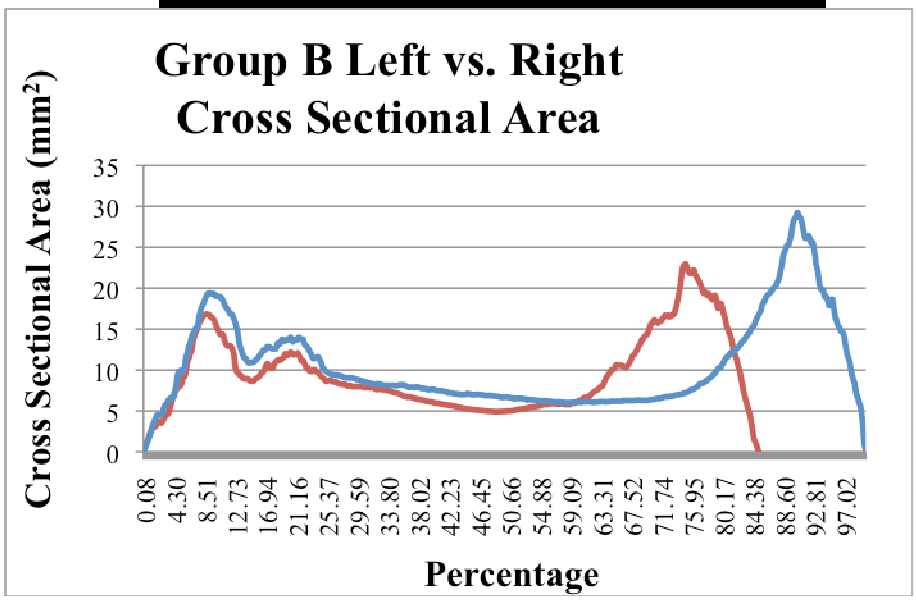


*Similar graphs were produced for Group A Imax, Imin, Min Diameter, Max Diameter and Perimeter measurements but are not shown here.

Figure 20: Images of Group B Left and Right Femurs Aligned With CSA Graph of Data Along the Bones.
Top Image: Group B Left Femur
Bottom Image: Group B Right Femur

Proximal → ← Distal

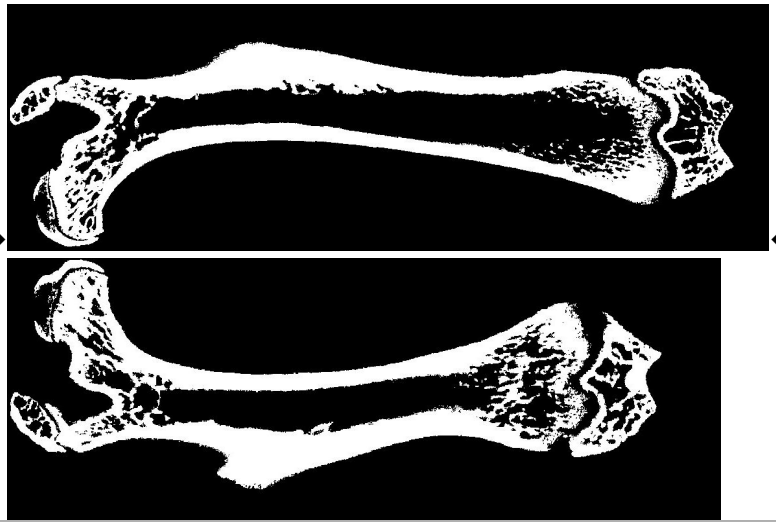


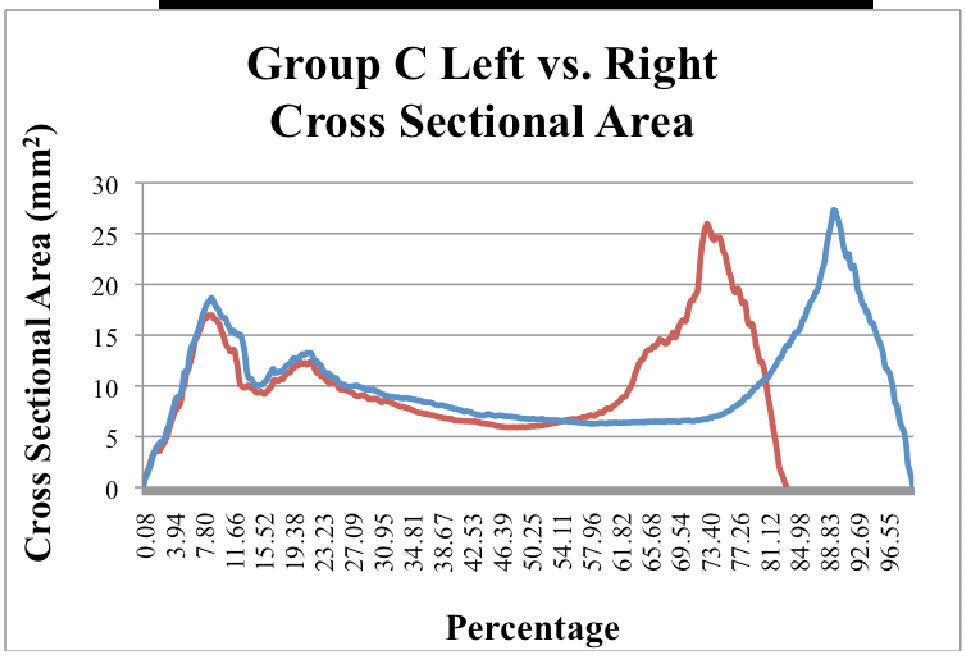


*Similar graphs were produced for Group B Imax, Imin, Min Diameter, Max Diameter and Perimeter measurements but are not shown here.

Figure 21: Images of Group C Left and Right Femurs Aligned With CSA Graph of Data Along the Bones.
Top Image: Group C Left Femur
Bottom Image: Group C Right Femur

Proximal → ← Distal





*Similar graphs were produced for Group C Imax, Imin, Min Diameter, Max Diameter and Perimeter measurements but are not shown here.

Figure 22: Images of 20% Bone Slices of Left and Right Femora from Groups A, B and C.
*Columns from left: Group A, Group B, Group C
**Rows from top: Left femur, Right femur

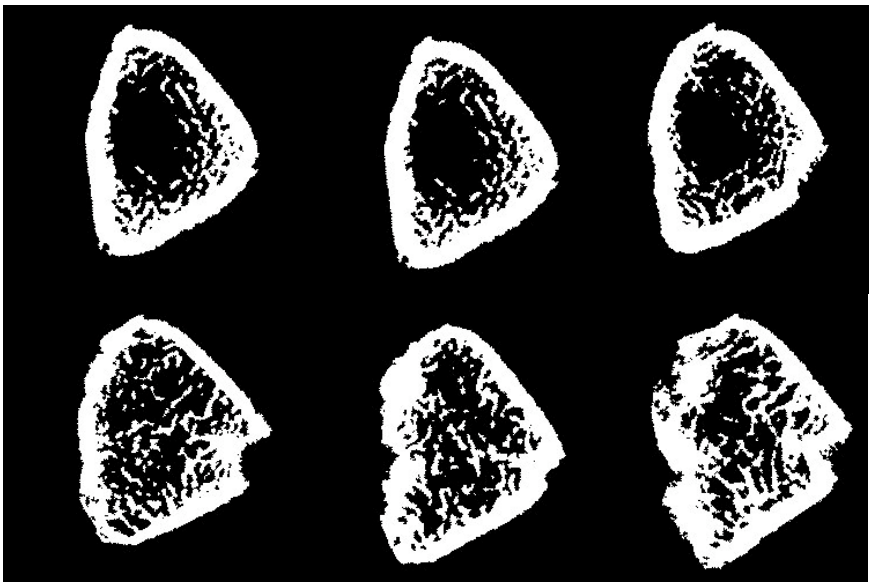
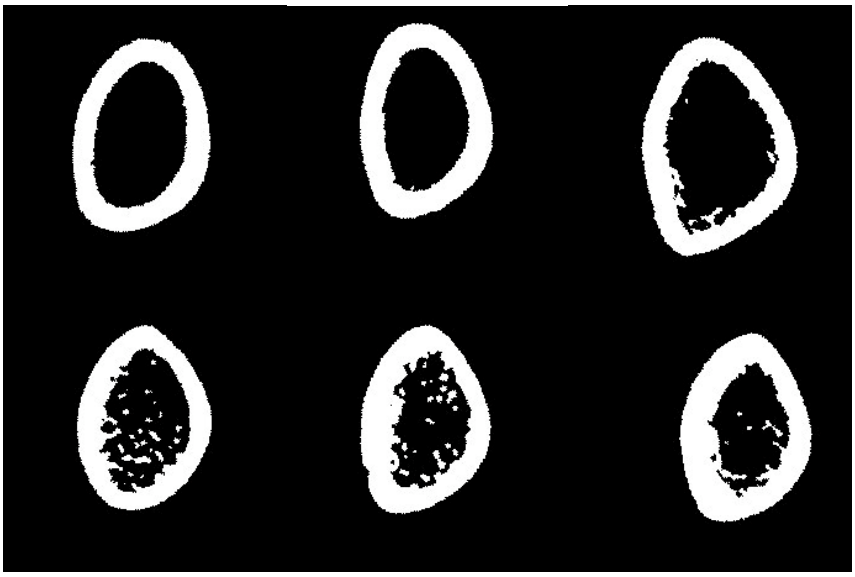


Figure 23: Images of 30% Bone Slices of Left and Right Femora from Groups A, B and C.
*Columns from left: Group A, Group B, Group C
**Rows from top: Left femur, Right femur



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Summary of Capstone Project

Radiotherapy, the medical use of ionizing radiation, is an important part of cancer therapy, used in addition to surgery for treatment of patients with soft tissue sarcomas, a cancer that arises from damaged cells of mesenchymal (germ layers) origin in connective tissue, and alternatively for treatment of patients with Ewing sarcoma of bone, a type of malignant round-cell tumor that arises in the bone. Treating pediatric extremity tumors with radiotherapy has been shown to have harmful effects on the epiphyseal plate (growth plate), resulting in permanent limb shortening and deformity when bone growth centers are exposed to radiation. Mechanical signals, specifically low-magnitude high-frequency vibrations (LMHFV), have been shown to be non-invasive and non-pharmacological growth factors in bone that have the potential to serve as a safe treatment for a number of clinical conditions, such as osteoporosis deterrence, fracture healing, muscle strengthening, orthodontic and craniofacial repair, tendon healing and connective tissue regeneration. Thus, this study was aimed at evaluating the possible beneficial effects of low-magnitude high-frequency mechanical vibration (LMHFV) stimuli applied to growing bone after irradiation and the possibility for restoration of function of the epiphyseal plate by LMHFV, a research topic that has never before been published in the literature.

Eighteen 3-week old young male albino rats were subjected to a standard radiation dose applied to right hind limbs, with the left leg serving as a non-irradiated control. Then, the animals were divided into three groups: *A)* rats

subjected to LMHFV only for 20 minutes once per day, 7 days/week, for 3 weeks, *B)* rats subjected to the same conditions of LMHFV plus an injection of spermine NONOate, a post-irradiation recovery agent that has shown weak positive results in this lab, and *C)* rats subjected to no treatment. After euthanizing the animals, skeletal growth of all eighteen animals was measured by taking x-rays and measuring bone lengths. Bone shape and form was measured by performing micro-CT scans of one set of femora from one animal in each group and comparing different sections and aspects of the three-dimensional images that were generated. Also, a colony-forming unit-fibroblasts (CFU-F) assay was performed on the femora of three animals from each group in order to measure the potential for stem cells flushed from femora bone marrow to mature into osteoblasts, the cells responsible for bone formation.

X-ray and CFU-F analyses show statistically significant differences between right and left limbs in all groups, showing that radiation inhibited skeletal growth and the formation of mature osteoblasts in all animals, as expected, since radiation has been shown to cause deformity in bone. No statistical significance was observed between vibration versus control groups, but trends suggest there could be some positive effect of vibration, although not statistically significant, showing that vibration did not recover the effects of radiation in groups subjected to vibration after radiation compared to the control group in a statistically significant manner, but trends do show weak positive effects of vibration. Micro-CT results show a clear difference between right and left limbs in all groups, also showing that radiation changed the shape and form of

limbs subjected to radiation versus control limbs. Regarding vibration versus control groups, micro-CT results are ambiguous, but do suggest that vibration may have altered local growth characteristics and stimulated local shape changes in the 20% region from the distal end of the femur, just above the growth plate, showing that vibration may have recovered some damage caused by radiation, but more animals are needed to substantiate this result (only one set of femora from one animal of each group was compared).

Despite the number of positive reports of LMHFV on bone, the present study did not reveal a clear, statistically significant effect on growth, structure or MSC colony formation. Thus, the effects of vibrational loading on irradiated growing bone are still unclear. However, findings in this paper suggest that LMHFV may have a weak positive effect, although this cannot be said with any statistical certainty. Thus, LMHFV applied to irradiated growing bone still has the possibility for restoration of function of the epiphyseal plate, but more studies on the effects of LMHFV on irradiated growing bone are needed in order to delineate the findings of this paper.