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Dynamic Hydraulic Flow Stimulation on Mitigation of Trabecular Bone Loss in a Rat Functional Disuse Model

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Abstract

Bone fluid flow (BFF) has been demonstrated as a critical regulator in mechanotransductive signaling and bone adaptation. Intramedullary pressure (ImP) and matrix strain have been identified as potential generator to regulate BFF. To elevate *in vivo* oscillatory BFF using ImP, a dynamic hydraulic stimulation (DHS) approach was developed. The objective of this study was to evaluate the effects of DHS on mitigation of bone loss and structural alteration in a rat hindlimb suspension (HLS) functional disuse model. Sixty-one 5-month old female Sprague-Dawley rats were divided into five groups: 1) baseline control, 2) age-matched control, 3) HLS, 4) HLS + static loading, and 5) HLS + DHS. Hydraulic flow stimulation was carried out daily on a “10 min on-5min off-10min on” loading regime, 5 days/week, for total of 4 weeks in the tibial region. The metaphyseal trabecular regions of the proximal tibiae were analyzed using μ CT and histomorphometry. Four weeks of HLS resulted in a significant loss of trabecular bone, leading to structural deterioration. HLS with static loading alone was not sufficient to attenuate the bone loss. Bone quantity and microarchitecture were significantly improved by applying DHS loading, resulting increase of 83% in bone volume fraction, 25% in trabecular number and mitigation of -26% in trabecular separation compared to HLS control. Histomorphometry analysis on trabecular mineralization coincided with the μ CT analysis, in which DHS loading yielded increases of 34% in histomorphometric BV/TV, 121% in MS/BS, 190% in BFR/BS and 146% in BFR/BV, compared to the HLS control. Overall, the data demonstrated that dynamic hydraulic flow loading has potentials to provide regulatory signals for mitigating bone loss induced by functional disuse. This approach may provide a new alternative mechanical intervention for future clinical treatment for osteoporosis.

Keywords

dynamic hydraulic stimulation; bone adaptation; mitigation of bone loss; disuse osteopenia; bone fluid flow; osteoporosis

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Disclosure

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Introduction

Complications associated osteopenia and osteoporosis, whether generated by aging or disuse, are commonly referred to low bone mass and microstructural deterioration of the skeleton, leading to fragile bones that are more susceptible for fractures. Common targets of disuse osteoporosis include patients subjected to prolonged immobility or bed-rest (e.g., due to spinal cord injury), astronauts who participate in long-duration spaceflight missions, and the aged population [1]. Effective and clinically applicable interventions are greatly needed to prevent such skeletal deterioration and to enhance bone structural quality.

Paralysis caused by spinal cord injury (SCI) outlines severe immobilizations and atrophic responses in musculoskeleton [2], and that SCI patients have significantly higher incidences of post-injury fractures [3, 4]. Studies of quantitative bone structural measurements have been performed in SCI patients. In a pQCT study of SCI patients, overall bone quantity decreased exponentially with time after injury. Between 2 months to 50 years of paralysis, SCI lead to 25–35% of decreases in mean cortical thickness at tibial and femoral diaphysis at steady state compared to the controls with mobile ability [5]. Because of the thinner cortical bone, the section moduli also decreased by 25% and 31%, respectively to these regions. Another study also showed that 1 year after SCI, decreases of 14–16% mean trabecular density and 7% average cortical density resulted in the tibia [6]. Studies have reported that SCI-induced osteopenia and/or osteoporosis can lead to up to 34% of fracture frequency [3, 7]. Similarly, analysis from space missions of 6 months have demonstrated an average loss of total bone mass of approximately 11% in the proximal femur region, in which trabecular bone mass and density declined by 14.4–16.5% [8]. Femoral and tibial cortical density decreased by about 2% after 6 month of spaceflight [9, 10]; while approximately 5% and 12% of trabecular density reductions were observed at the distal tibia and proximal femur, respectively. Furthermore, altered cortical and trabecular architecture have been observed in postmenopausal women with fractures; showing 12% and 19% decreases in cortical thickness and trabecular bone volume fraction [11].

Current treatments for osteoporosis include anti-resorptive and anabolic pharmacotherapies with noticeable benefits in clinic. Pharmacological interventions often require high costs and long-duration therapy, and easily give rise to adverse side effects (e.g. atypical fracture) [12]. Recent developments of non-pharmacological mechanical interventions, on the other hand, provide a non-invasive alternate approach. Mechanobiology and novel mechanical stimulations have been investigated for their abilities to regulate bone health. Bone adapts its overall shape and internal architecture in response to the applied mechanical stimuli [13]. Responsive skeletal adaptation to mechanical loading has been demonstrated with various modalities in the past research. *In vivo* studies have shown that load-driven strain from four-point bending [14, 15] and axial loading [16] can lead to bone formation. Low-magnitude, high-frequency vibrations have also been shown to be anabolic in both human [17] and animal [18] skeletons. Joint loading has recently been shown as a unique modality to stimulate trabecular and cortical bone formations [19–21]. Previous *in vivo* studies from our group demonstrated that dynamic electrical muscle stimulation successfully mitigated bone loss in a rat hindlimb suspension (HLS) model [22]. While increases of physical activities result in increased bone mass [23–25], removal of functional loads can result in bone mass reduction [26, 27]. Reduced trabecular number and thickness, bone mineral density, and increased bone resorption occurred in rodents on Cosmos spaceflights over 14–40 days periods [28–30]. Ground-based HLS rodent models were included in the Cosmos 2044 mission as controls and coincided the atrophic changes in bone with the ones on flight [31].

Due to the anabolic potential of mechanical loading as a natural determinant in maintaining bone strength [32], a biomechanical intervention based on the skeletal mechanotransduction

pathway is proposed as an alternative and potential approach for osteopenia and osteoporosis treatments. This requires an in-depth understanding of how mechanical signals are delivered to bone cells and how bone cells respond to such signals. A growing body of evidence has pointed bone fluid flow (BFF) as a communication media, bridging an external load and the bone cells, thereby regulating the bone remodeling process [33–37]. Thus, BFF has been a contributing mechanism through which mechanical stimulations act. Previous studies have shown that intramedullary bone fluid flow alone, in the absence of mechanical strain, can drive bone remodeling. Qin et al. applied a direct intramedullary pressure (ImP) to an isolated turkey ulna without deforming the bone tissue and found increased bone formation in response to the applied pressure [38]. Similarly, a recent study introduced a novel microfluidic system for generating dynamic ImP and interstitial fluid flow within the femurs of alert mice to induce osteogenic responses [39]. The observations from these *in vivo* studies clearly determined that BFF can be altered by ImP with minimal strain.

As a practical translational potential of ImP, a non-invasive method that directly couples an external stimulation and internal bone fluid flow is in great need. Here, we hypothesized that daily non-invasive dynamic hydraulic stimulation (DHS) induced bone fluid flow can initiate adaptive responses *in vivo* to elicit osteogenic activity during a 4-week study using a functional disuse model. To evaluate the potential of DHS mediated fluid flow on the skeletal system, the objective of the study was to demonstrate the effects of dynamic external pressure stimuli on trabecular bone structural properties in a rat hindlimb suspension model.

Materials and Methods

Experimental Design

All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Stony Brook University. Sixty-one 5-month old female Sprague-Dawley virgin rats (Charles River, MA) were used to investigate the effects of DHS on skeletal adaptation under disuse conditions. Animals were provided with standard rodent chow and water *ad libitum*, and housed in 18"×18"×24" (L×W×H) stainless steel HLS cages. The animal room was temperature-controlled and had a 12:12 hours light:dark cycle. Animals were randomly assigned to five groups: baseline control (n = 15), age-matched control (n = 12), hindlimb suspended (HLS, n = 10), HLS + static pressure (n = 10), HLS + DHS (n = 14). Functional disuse in the rat hindlimbs was introduced via the HLS procedure, similar to the setup from Lam's study [22]. Briefly, an animal's tail was lightly coated with tincture of benzoin after being cleaned with 70% alcohol. A piece of surgical tape was then attached to the dried and sticky tail, forming a loop close to the end of the tail. The surgical tape was secured with three strips of elastic adhesive bandage along the surgical tape that was placed on the tail. The loop formed from the surgical tape was attached to a tail harness apparatus and a swivel hook suspended from the top of the cage. The animal was suspended with an approximately 30° head-down tilt, having the hindlimbs about 2 cm above the cage bottom. Full access to the entire cage bottom was allowed for the animal's forelimbs. Animals' body weights and overall health were carefully monitored throughout the entire study.

Dynamic Hydraulic Stimulation Protocol

For the stimulated animals, daily DHS was applied in conjunction with HLS for the 4-week study. DHS was achieved through a customer design inflatable cuff placed around the right hindlimb of the tibia. The stimulation cuff was connected to an oscillatory actuator-driven syringe, a force-controlled syringe, and a pressure sensor (Figure 1). The inflation and deflation of the cuff was driven by the actuator-driven syringe controlled by a programmable

waveform/signal generator (HP33120A, Hewlett-Packard, Palo Alto, CA). The force-controlled syringe was included to maintain the static pressure, and the pressure sensor was used to monitor the hydraulic pressure throughout the procedure to maintain baseline static pressure. The magnitudes of the pressure stimulation were 30 mmHg static pressure + 30 mmHg (p-p) dynamic pressure, with a stimulation frequency of 2 Hz. The daily stimulation of “10 min on – 5 min off – 10 min on” was applied to each stimulated animal while under anesthesia (isoflurane inhalation) 5 days/week, for 4 weeks. The static pressure was released during the 5 min break. The rats were euthanized at study's end, and the right tibiae were obtained and preserved with 70% ethanol.

μCT Evaluation

The proximal metaphyseal portions of the tibiae were scanned for trabecular bone morphology using a high resolution μCT scanner (μCT-40, SCANCO Medical AG, Bassersdorf, Switzerland) with a spatial resolution of 30 μm. For image smoothing purposes and to define the desired analyzed objects, all images were evaluated using a Gaussian filter, with specific sigma, support and threshold values of 0.3, 1, and 320, respectively. A 1500 μm region of trabecular bone was analyzed in the proximal metaphysis, starting from 1800 μm distal to the growth plate. Values for bone volume fraction (BV/TV, given as %), connectivity density (Conn.D, 1/mm³), trabecular number (Tb.N, 1/mm), trabecular thickness (Tb.Th, mm) and trabecular separation (Tb.Sp, mm) were evaluated for each metaphyseal trabecular region.

Static and Dynamic Histomorphometry

Two doses of calcein (10mg/kg) were given to the animals through intraperitoneal injections on days 2 and 16 prior to euthanasia. After the μCT scans, the proximal portions of the tibiae were kept and dehydrated with isopropanol. The samples were then infiltrated and embedded with the mixture of methyl methacrylate, n-butyl phthalate, and benzoyl peroxide. The samples were sectioned to 8 μm slices longitudinally using a microtome (Leica 2165, Wetzlar, Germany). Osteomeasure software (OsteoMetrics Inc., Decatur, GA) was used to perform histomorphometric measurements by tracing calcein labels in the trabecular bone in the metaphyseal region (5 mm² × 3 mm²). Histomorphometric bone volume fraction (BV/TV – Histo, %), mineralizing surface/bone surface (MS/BS, %), mineral apposition rate (MAR, μm/day), and bone formation rate (BFR/BS, μm³/μm²/day; BFR/BV, μm³/μm³/day) were calculated by the software from the single- and double-label values.

Statistical Analyses

The statistical analysis of all results is reported as mean ± SD. Differences between groups were determined using GraphPad Prism 3.0 Software (GraphPad Software Inc, La Jolla, CA) for body weight measurements, bone structural analysis, and static and dynamic histomorphometric analysis. One-way ANOVA with Tukey's post-hoc test were performed on the μCT and histomorphometry data with normal equal variance to determine the differences among groups. The level of significance was established at $p < 0.05$.

Results

Body weight

The animals' body weights were carefully monitored for 5 times per week throughout the entire experimental period. The body weights were not significantly different between groups at the beginning of the study, with an average of 305g ± 16g. Age-matched control animals were able to maintain a steady body weight throughout the study, with only a +4.7% difference between the start and end dates. Animals subjected to 4-week functional disuse,

including HLS control and stimulated groups, lost a great amount of body mass. These weight reductions were similar in HLS, HLS + static, and HLS + DHS groups, with -5% for HLS ($p > 0.05$), -7% for HLS + Static ($p < 0.0001$), -5% for HLS+DHS ($p < 0.05$).

μCT – Trabecular bone

Results of μCT analysis with representative scanned images are shown in Figure 2 for the tibial proximal metaphyseal region. The lack of weight-bearing activity for 4 weeks significantly reduced trabecular bone quantity and quality, demonstrated by a 41% decrease in BV/TV ($p < 0.05$) (Figure 3), a 47% decrease in Conn.D ($p < 0.01$) (Figure 4), a 17% decrease in Tb.N ($p < 0.05$) (Figure 5), and a 27% increase in Tb.Sp ($p < 0.05$) (Figure 6) compared with the age-matched group. Animals with static pressure pre-load alone showed slight improvements in microarchitecture although it's not statistical significant. HLS + static animals showed an increase of 20% in BV/TV (Figure 3), an increase of 30% in Conn.D (Figure 4), an increase of 11% in Tb.N (Figure 5), and a decrease of 19% in Tb.Sp compared with age-matched group ($p > 0.05$) (Figure 6). Trabecular BV/TV in DHS treated animals was significantly greater than that of disused bone. Animals with DHS at 2 Hz showed an increase in BV/TV by 83% ($p < 0.001$). Measures of Conn.D, Tb.N, and Tb.Sp were also significantly affected by DHS at 2 Hz. There were up to 102% ($p < 0.001$) and 25% ($p < 0.01$) increases for Conn.D and Tb.N; and up to a 26% ($p < 0.01$) decrease for trabecular separation (Figure 3–6). When compared to HLS + static animals, HLS + 2 Hz animals showed 36% ($p > 0.05$), 54% ($p < 0.05$), and 17% ($p > 0.05$) increases for BV/TV, Conn.D, and Tb.N; and up to a 21% decrease for trabecular separation ($p > 0.05$) (Figure 3–6).

Static and Dynamic Histomorphometry

The static and dynamic histomorphometry data showed significant improvements in the histomorphometric BV/TV and other bone formation parameters in response to DHS (Table 1 and Figure 7). HLS significantly reduced BV/TV, MS/BS, MAR, BFR/BS and BFR/BV compared to age-matched controls by 29% ($p < 0.05$), 62% ($p < 0.001$), 28% ($p < 0.05$), 73% ($p < 0.001$), and 67% ($p < 0.001$), respectively. HLS + static pressure alone did not show significant difference compared to HLS, except for MS/BS. However, HLS + 2Hz DHS-treated rats showed significant increases in BV/TV and all other bone formation parameters, except for MAR. HLS + DHS had increases compared to the age-matched group by 34% in BV/TV ($p < 0.05$), 121% in MS/BS ($p < 0.001$), 34% in MAR ($p > 0.05$), 190% in BFR/BS ($p < 0.001$), and 146% in BFR/BV ($p < 0.001$).

Discussion

In the present study, a non-invasive dynamic hydraulic stimulation was developed to successfully mitigate bone loss and detrimental changes to trabecular architecture induced by a lack of daily weight-bearing activities, with only 4-week of loading. Specifically, 20-min bouts of daily DHS at low magnitude onto the hindlimb suspended rats increased trabecular bone volume fraction and microarchitecture, which contribute critical to a stronger skeleton. With the idea of direct coupling with oscillatory marrow fluid pressure, DHS serves as low-magnitude loads that are applied to the tibial diaphysis and potentially alters pressure in the intramedullary cavity, leading to influences in interstitial fluid flow and further bone adaptation. Moreover, DHS makes itself a valuable tool to investigate the anabolic and/or anti-resorptive potentials of interstitial fluid flow and its role in regulating skeletal mechanotransduction mechanisms. The promising data may ultimately offer some insights into future potential clinical applications.

Mechanical signaling has long been recognized for its importance in skeletal health, playing a critical roles in strong anabolic potential and naturally gauging bone strength [40]. A good understanding on the mechanism of how bone cells sense physical signals is needed to further develop the therapeutic potential of mechanical signals. Factors including strain and ImP-induced bone fluid flow are potential determinants of bone mass and morphology. While early investigations had indicated strain magnitude as a dominant factor for osteogenic responses, dynamic or cyclic stimuli were shown in the later studies as being more anabolic to bone than static stimuli [41]. Since bone will accommodate to prolonged routine loading, rest periods between bouts of mechanical loading are needed to reinstate bone's mechanosensitivity [40]. In our present study, a rest period of 5 min was inserted in between two bouts of 10-min DHS. Yet, static loading alone, even with the rest period and constant loading magnitude, was not effective in attenuating bone loss. From our data, we concluded that the effectiveness of DHS was greatly dependent on the dynamic components that were superimposed to the static pre-load. While static loading was unsuccessful in preventing osteopenia, 2 Hz DHS applied to the tibia was able to maintain trabecular bone mass. Strain information has also been hypothesized to accumulate and integrate over time [42], suggesting that low-magnitude mechanical strain induced by DHS, if any, may propagate in the skeleton and initiate osteogenic responses. Low-magnitude stimuli, such as vibration, have been shown in previous research to mediate bone formation and to enhance bone morphology and bone strength [18, 43]. Responsiveness to mechanical signals has been observed at the cellular level in bone, e.g. osteoblasts and osteocytes. Growth hormones and cytokines activated from subsequent mechanotransduction-induced signaling cascades can play a role in the balance of bone formation and resorption [44–47].

Another crucial factor, bone fluid flow induced by ImP, has also been proposed to account for adaptive bone response [48]. Murine femoral ImP measurements showed a 23% drop in ImP upon HLS and a 25% increase upon femoral vein ligation, strongly supporting the hypothesis that hindlimb disuse leads to decreased pressure gradients and subsequent reductions in interstitial fluid flow, which can be re-built by femoral vein ligation [49]. Previous *in vivo* study from our group demonstrated that muscle contraction using dynamic electrical stimulation was able to induce ImP, and this change was strongly correlated with the bone structural adaptive effects [22, 50]. Aimed at decoupling the matrix deformation factor from interstitial fluid flow, Qin et al. applied a direct ImP to the isolated turkey ulna without deforming the bone tissue and found increased bone formation in response to the applied pressure [38]. Similarly, a recent study introduced a novel microfluidic system for generating dynamic ImP and interstitial fluid flow within the femurs of alert mice to induce osteogenic responses [39]. The observations from these *in vivo* studies clearly determined that fluid flow can be altered by ImP alone. Moreover, modulation potential in bone remodeling through autocrine and paracrine signaling cascades may occur with changes in the fluid environment [49, 51, 52]. Interestingly, trabecular bone growth in the proximal tibia could result from the direct compression by DHS on the diaphysis. The linkage between the site of pressure application and the site of bone formation may be an ImP gradient that influences the magnitude of mechanotransductory signals on the trabecular bone that is exposed in the marrow cavity.

Current limitations in this field involve either the lack of ability to isolate the matrix strain and ImP, or using highly invasive methods to decouple these two factors. Directly related to translational potentials of ImP, a non-invasive method that couples external stimulation and internal bone fluid flow has been a challenge. As a direct circular compression with low magnitude pressure on surrounding soft tissues, DHS provides an excellent method to eliminate matrix deformation, i.e. axial compression or torsion, bending, or shear stress. As suggested in the theory of muscle pump, veins within skeletal muscle are compressed upon DHS and thus increasing the arteriovenous pressure gradient and promoting blood flow to

the capillary bed [53–55]. The pressure gradient in the muscle vasculature may be relayed to the nutrient vessels in bone, further increasing ImP and inducing fluid flow in bone [56]. Based on this theory, this pilot study of DHS first investigated 30mmHg as the dynamic loading magnitude. The reason behind it is this magnitude is large enough to temporarily block venous blood flow, while still allowing the arterial blood flow for pressure build-up.

Loading frequency is an important determinant of bone adaptation to mechanical loading. A positive correlation has been demonstrated repeatedly in various studies [57–59]. Mechanical stimulation with various loading frequencies forms a nonlinear relationship with femoral strain measured at the mid-diaphysis [42, 43, 60, 61]. However, effective loading frequency has also been shown to differ among various loading modalities [21, 57, 58]. Higher frequencies (> 30 Hz) in whole-body vibration are known to be more effective. On the contrary, lower frequencies (5 and 10 Hz) were reported to be more effective over higher frequencies (20 or 30 Hz) in an ulna axial loading study in mice [62]. In recent studies based on ImP/interstitial fluid flow loading theory, relatively lower frequencies 5 Hz and/or 10 Hz were effective in bone adaptation in the femur [39] and tibia [21], respectively. In our present study, functional disuse for 4 weeks significantly reduced the metaphyseal bone volume fraction, mainly via a decrease in trabecular number, thereby reducing its connectivity and augmenting the marrow space. DHS with 2 Hz proved capable of preserving bone mass, trabecular number, and connectivity. The promising histomorphometry data further verified and coincided with the μ CT analysis, in which DHS treatment had significant increases in histomorphometric BV/TV and other bone formation parameters compared to HLS. The precise mechanisms of how a potential cause of differential frequency responses in the skeletal tissue are not yet identified. Though, our finding implies that direct hydraulic coupling may influence adaptation in a more physiologic frequency range. In addition to strain, nonlinear ImP profile with loading frequencies has also been observed during dynamic mechanical stimulation [50].

Selecting an optimal loading regimen with the appropriate magnitude, frequency and duration is important to maximize the effectiveness of DHS. One limitation of this study is that we did not test a wide range of these parameters to determine their combinational effects in skeletal adaptive responses. Future studies will extend the investigation into various loading regimes. Further, while DHS has been shown to significantly improve the structural quality of the disuse bone, DHS can be likely to act through increased bone formation and/or decreased bone resorption. Our *ex vivo* methodology of the tibias precluded analysis that requires cellule markers. Therefore, future studies will also incorporate cellular assays, such as TRAP and ALP staining, to further determine this process.

In summary, dynamic hydraulic pressure stimulus, applied externally and non-invasively, provides a physiological fluid flow intervention to attenuate trabecular bone loss in a functional disuse rat HLS model. The results of this study suggested that low magnitude, 2–3 Hz short duration, external hydraulic fluid flow loading is able to provide sufficient mechanotransductive signals to attenuate disuse osteopenia, showing improvements on bone structural quality. The skeletal adaptive responses induced by fluid flow are highly depended on its dynamic components such as loading frequency and duration. The underlying mechanism may involve regulation of oscillatory marrow fluid pressure to initiate adaptation. Although we only focused on indices of bone structural morphology, it is likely that this structural change may be adapted through integrated improved bone formation and inhibition of bone resorption activities. Applied fluid pressure significantly mitigated bone loss with only 20min of daily loading at low magnitude. This will provide significant insights for future clinical applications.

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Highlights

- Dynamic hydraulic stimulation (DHS) was developed to mitigate rat disuse bone loss.
- Bone quantity and microarchitecture were significantly improved by DHS.
- Histomorphometry data on mineralization coincided with the structural analysis.
- DHS potentially regulates signals for mitigating functional disuse bone loss.
- DHS may provide a new alternative mechanical intervention for osteoporosis treatment.

Figure 1. Experimental setup

DHS is achieved through a costume design inflatable cuff. The stimulation cuff is connected to an oscillatory actuator-driven syringe, a force-controlled syringe, and a pressure sensor.

Figure 2.

Representative 3D μ CT images of proximal tibial trabecular bone. The proximal metaphysis of the tibiae were scanned for trabecular bone morphology using a high resolution μ CT scanner with a spatial resolution of $30\mu\text{m}$. A $1500\mu\text{m}$ region of trabeculae was analyzed in the proximal metaphysis, starting from $1800\mu\text{m}$ distal to the growth plate.

Figure 3.

Graphs show mean $\pm$ SD values for bone volume fraction (BV/TV, %). DHS at 2 Hz produced a significant change, compared with values obtained in 4-week HLS. * $p < 0.05$; *** $p < 0.001$.

Figure 4.

Graphs show mean $\pm$ SD values for connectivity density (Conn.D, 1/mm³). DHS at 2 Hz produced a significant change, compared with values obtained in 4-week HLS. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 5.

Graphs show mean $\pm$ SD values for trabecular number (Tb.N, 1/mm). DHS at 2 Hz produced a significant change, compared with values obtained in 4-week HLS. * $p < 0.05$; ** $p < 0.01$.

Figure 6.

Graphs show mean $\pm$ SD values for separation (Tb.Sp, mm). DHS at 2 Hz produced a significant change, compared with values obtained in 4-week HLS. * $p < 0.05$; ** $p < 0.01$.

Figure 7. Representative 2D images of calcein (green) labeled trabecular bone histomorphometry in the proximal metaphyseal region of the tibia. The calcein (green marker) labeled the mineralizing bone surfaces. Trabecular bone formation was clearly indicated by the double calcein labels in the HLS + DHS group.

Proximal tibia metaphysic histomorphometry. Trabecular bone histomorphological analyses on age-matched control, HLS, HLS + static, and HLS + DHS.

Table 1

	BV/TV (Histo, %)	MS/BS (%)	MAR (mm/day)	BFR/BS(mm3/mm2/day)	BFR/BV(mm3/mm2/day)
Age-matched	46.61 ±7.32	10.75±2.24	0.73±0.15	0.08±0.03	0.23±0.07
HLS	33.22±7.99*	4.12±0.84**	0.53±0.16*	0.02±0.01**	0.07±0.03**
HLS + Static	40.99±14.84	6.81 ±1.18***	0.59±0.19	0.04±0.01**	0.12±0.03**
HLS + DHS	44.65±8.99#	9.09±2.94##	0.71±0.18	0.06±0.02### ^a	0.18±0.05### ^a

Values are mean ± SD.

BV/TV, bone volume/tissue volume; MS/BS, mineralized surface/bone surface; MAR, mineral apposition rate; BFR/BS, bone formation rate/bone surface.

* $p < 0.05$ vs. age-matched;

$p < 0.05$ vs. HLS;

** $p < 0.001$ vs. age-matched;

$p < 0.001$ vs. HLS;

^a $p < 0.05$ vs. HLS + static.