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Alteration of Contraction-to-Rest Ratio to Optimize Trabecular Bone Adaptation Induced by Dynamic Muscle Stimulation

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Abstract

Disuse osteopenia has shown to decrease bone mineral density and compromise bone's integrity, i.e., in aging population and long term functional disuse. The degree of attenuation of trabecular bone loss and deterioration of its microarchitecture is closely dependent on the mechanical loading parameters within the regimen. Dynamic muscle stimulation as a preventive countermeasure for disuse osteopenia has been shown to be effective. The objective of this study is to determine whether the contraction-to-rest ratio is a crucial parameter to affect the skeletal adaptive responses under a functional disuse environment. Fifty-six skeletally matured Sprague-Dawley rats were divided into seven groups for the 4-week experiment: baseline control, age-matched control, hindlimb suspended (HLS), and HLS plus muscle stimulation with a contraction-to-rest ratio of 1/4, 2/8, 4/6, and 2/28 sec. Muscle stimulation was carried out for total of 10 minutes per day, 5 days per week, for 4 weeks. Trabecular bone in the distal femurs was analyzed with microcomputed tomography and histomorphometry. HLS alone for 4-week resulted in a 25 – 45% trabecular bone loss in the distal femur. Dynamic muscle stimulation, applied at 50 Hz frequency, with a 2/8 sec contraction-to-rest ratio demonstrated significant attenuation of trabecular bone loss against the 4-week disuse, with up to +74% in bone volume fraction, +164% in connectivity, +20% in trabecular number, and –18% in spacing ($p < 0.05$). Stimulation with 1/4 and 4/6 also showed similar effects but with a lesser percentage differences when comparing to the HLS animals. Similarly, histomorphometric analysis showed partial enhancement in mineralizing surface and mineral apposition rate. The results suggested the potentials of dynamic muscle stimulation in regulating skeletal adaptive responses and illustrated the effects of optimized contraction-to-rest in mitigation of bone loss, in which 2/8 sec has shown maximal adaptive response among all tested ratios.

Keywords

muscle contraction; bone adaptation; mitigation of bone loss; disuse osteopenia; bone fluid flow; rest period; osteoporosis

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Introduction

Musculoskeletal disorders, i.e., osteoporosis, spinal cord injury and fracture, can impose short- and long-term consequences to bone. Significant amount of bone mineral density (BMD) reduction at the lower extremities has been reported in numerous studies. [1,2] Following acute spinal cord injury (< 6 months), BMD was reduced by 17 to 25% at various sites. In addition to bone loss, such injuries can alter bone microarchitecture and increase lower extremity fracture incidence by 1 to 34%. [3–6] Therefore, successful interventions are necessary to prevent osteopenia and subsequent complications.

With the exposure to daily activities, bone experiences repetitive bouts of mechanical loading. Under disuse condition, due to injury or paralysis, external mechanical stimulus applied to bone has the potential to induce remodeling. [7,8] Many in vivo studies have determined parameters, such as frequency, strain magnitude, strain history, and fluid pressure, within stimulation regimens to be influential to bone adaptation. [9–12] The concept of inserting rest period has recently shown to enhance osteogenesis. Short rest duration, e.g., 10 seconds, inserted into a low frequency loading regime augmented anabolic response on the periosteal surfaces of isolated avian ulnae. [13] Even with a 10-fold decrease in cycle numbers, rest period implemented into a high frequency signal significantly increased the bone formation rate of immobilized tibia. [14] These stimuli applied direct loading to the skeleton, generating bone strain in the order of 1000 $\mu\epsilon$. It is thought that insertion of rest period improves osteogenic responses by avoiding mechanosensitivity saturation in bone tissue. [15]

We have previously demonstrated that dynamic electrical muscle stimulation (MS) has anti-catabolic effect on disused skeleton. [9,16] In particular, the level of osteopenic attenuation is depended on the stimulation frequency. Whether inserting rest period into MS regimen, with a specific frequency, can optimize bone's adaptive response remains uncertain. Among applied MS frequencies, 1Hz, 20Hz, 50Hz, and 100Hz, 50Hz MS has demonstrated maximal adaptive potential in the trabecular bone. Another factor to be considered is the fluid flow induced by MS. [17–19] The contraction of skeletal muscle generates an intramuscular pressure gradient and increases venous circulation. Once relax, intramuscular flows restore. The duration of the contraction and relaxation may partially regulate the mean blood flow rate in muscle and subsequently affects the fluid flow in the skeleton. [20] It has been shown that hindlimb suspension in rodents significantly reduced the intramedullary pressure (ImP) gradient and implied the decrease of interstitial fluid flow in bone. [21] On the contrary, dynamic MS, applied at mid-stimulation frequency, significantly increased the ImP. [10]

Given that dynamic MS can prevent osteopenia and the promising results from various rest insertion studies, altering the contraction-to-rest (C/R) ratio may enhance fluid perfusion in skeletal muscle and further drive fluid flow in bone. Here, we hypothesized that the C/R ratio is a critical parameter within the MS regimen. Its alteration can optimize the mitigation of trabecular bone loss and deterioration induced by the functional non-weight bearing activity.

Materials and Methods

Experimental Design

All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Stony Brook University. Fifty-six 6-month-old female Sprague-Dawley retired breeder rats (Taconic, NY) were used to investigate the effects of varying the C/R ratio of dynamic muscle stimulation (MS) on skeletal adaptation in a disuse environment. They were housed individually in 18"×18"×24" (L×W×H) stainless steel HLS

cages in a temperature-controlled room with a 12:12 hour light:dark cycle, and were provided with standard rodent chow and water ad libitum. Animals were transferred to these cages one week prior to the experiment start date in order to acclimate them to their environment. The animals were randomly assigned to seven groups with $n=8$ per group: (1) baseline control, (2) age-matched control, (3) HLS, (4) HLS+1/4 (1 sec contraction-to-4 sec rest) MS, (5) HLS+2/8 MS, (6) HLS+4/6 MS, and (7) HLS+2/28 MS. Functional disuse was induced by HLS. The description of the HLS setup was previously described. [9,16] The body weight of each animal was carefully monitored throughout the study.

Electrical MS Protocol

For the four stimulated groups, dynamic MS was applied in addition to HLS. Skeletal muscle contraction was induced with two needle-size electrodes (L-type gauge #3, Seirin, Weymouth, MA) while the animals remained anesthetized and suspended. One electrode was placed in the right lateral proximal quadriceps and the other was placed in the lateral distal quadriceps. The electrodes were then connected to a 100 MHz arbitrary waveform generator (Model 395, Wavetek) to transmit a 1 ms square pulse at a stimulation frequency of 50 Hz for 10 minutes per day, 5 days per week, for a total of 4 weeks. The electrical stimulation was given at 2V sinusoidal signal with measured current value of less than 40mA. The contraction was applied for either 1 second with 4 seconds rest (1/4), or 2/8, or 4/6, or 2/28 ratios, which will give different stimulation pulses and cycle numbers due to its tetanic MS characteristic. In addition, the age-matched and the HLS control groups were subjected to anesthesia for the same amount of time per day as the MS treatment groups to account for any potential affects from the isoflurane inhalation.

Microcomputed Tomography (μ CT) Analysis

The right femurs were harvested and preserved in 70% ethanol after the completion of the *in vivo* study. Using a high resolution μ CT scanner (μ CT-40, SCANCO Medical AG, Bassersdorf, Switzerland), the distal femurs were scanned with a spatial resolution of 15 μ m. For image smoothing purpose and to define the desired analyzed objects, all images were evaluated using Gaussian filter, with specific sigma, support, and threshold values of 0.5, 1, and 347, respectively. In order to access how different C/R ratios of the MS regimens affect the trabecular bone adaptation regionally, three consecutive 750 μ m regions of trabecular bone longitudinally along each whole femur (M1, M2 and M3) were analyzed in the distal metaphysis, immediately proximal to the growth plate. M1 is the section closest to the diaphysis, M2 is the middle section between M1 and M3, and M3 is the section closest to the growth plate. One 750 μ m region of trabecular bone was also analyzed in the distal epiphysis of each femur. Values for bone volume fraction (BV/TV, given as %), connectivity density (Conn.D, $1/\text{mm}^3$), structural model index (SMI), trabecular number (Tb.N, $1/\text{mm}$), thickness (Tb.Th, mm) and separation (Tb.Sp, mm) were evaluated for each region [22].

Static and Dynamic Histomorphometry

Two intraperitoneal injections of calcein (10mg/kg) were administered to each animal 2 days and 16 days prior to euthanasia. After scanning with μ CT, the distal portions of the femurs and the proximal tibia (10 mm) were cut and dehydrated with isopropanol. The samples were then infiltrated and embedded with mixture of methyl methacrylate, n-butyl phthalate, and benzoyl peroxide. Longitudinal slices were sectioned to 8 μ m using a Leica 2165 microtome (Leica, Wetzlar, Germany). Histomorphometric measurements were made by tracing calcein labels in the trabecular bone at the metaphyseal region (3 sections per bone, 3mm^2 per section), using the Osteomeasure software (OsteoMetrics Inc, Decatur, GA). Histomorphometric bone volume fraction (BV/TV – Histo, %), mineralizing surface

(MS/BS, %), mineral apposition rate (MAR, $\mu\text{m}/\text{day}$), and bone formation rate (BFR/BS, $\mu\text{m}^3/\mu\text{m}^2/\text{yr}$) were determined. [23]

Statistical Analysis

Results are reported as mean $\pm$ SD for μCT analyses and mean $\pm$ SE for histomorphometric analyses. For all measurements, significance differences between groups were determined using the SigmaStat 2.03 (Systat Software Inc, San Jose, CA). Analysis of variance (ANOVA) with Tukey's pairwise multiple comparison tests was performed on the μCT data with normal equal variance. For analysis at the distal femoral metaphysis region, two-way ANOVA was used, with the experimental groups and the various metaphyseal regions as the two factors. One-way ANOVA was applied for all other μCT and histomorphometric data. The level of significance was established at $p < 0.05$.

Results

Body Weights

The animals' body weights were carefully monitored via the course of the 4-week study. The body weights were not significantly different between groups at the beginning of the study, with an average of $302\text{g} \pm 30\text{g}$. Age-matched control animals maintained a steady body weight throughout the experiment, with a -0.46% difference between the start and end date. Animals subjected to the 4-week functional disuse experienced a significant body weight loss and MS did not have an effect on the overall body mass. The weight reductions over the 4 weeks were 7% for the HLS control ($p < 0.05$), 1/4 MS ($p < 0.01$), and 2/8 MS ($p < 0.001$), 4/6 MS ($p < 0.001$), and 2/28 MS ($p < 0.001$).

μCT Analysis

Representative μCT volume fraction changes showed that HLS resulted in 35% trabecular bone loss compared to age-matched controls, while 2/8 C/R mitigated 39% bone loss vs. HLS (Fig. 1).

Femur M1 Region

M1 is the distal metaphyseal region 1.5 mm above the growth plate. Four weeks functional disuse significantly demonstrated a 46% decrease in BV/TV, a 51% decrease in Conn.D, a 12% decrease in Tb.N, a 23% increases in SMI, and a 13% increase in Tb.Sp compared to age-matched controls ($p < 0.01$) (Figure 2). Similar results were observed when compared with baseline controls ($p < 0.01$). MS with 2/8 C/R ratio showed the greatest prevention against disuse, with a 74% induction in BV/TV ($p < 0.05$), a 164% induction in Conn.D ($p < 0.01$), a 20% induction in Tb.N ($p < 0.01$), and a reduction of 18% in Tb.Sp ($p < 0.01$). Statistically significance was also observed for the bone quality parameters between HLS and MS with 4/6 ratio, with +106% in Conn.D ($p < 0.01$), +17% in Tb.N ($p < 0.01$), and -14% in Tb.Sp ($p < 0.05$). Stimulation with 1/4 and 2/28 ratios displayed similar preventive trend for trabecular deterioration but the percentage changes compared to disuse were not as substantial as the effects seen with 2/8 C/R ratio.

μCT – Femur M2 region

M2 is the distal metaphyseal region of 750 μm above the growth plate. The removal of daily activities to the hindlimbs generated a 35% of significant trabecular bone loss and compromised the structural network by decreasing the Conn.D by 43% and Tb.N by 17%, increasing SMI by 22% and Tb.Sp by 21%, $p < 0.01$ for all (Figure 3). Like in other regions, Tb.Th was not altered by the 4-week disuse or MS. Similar to M1, 50 Hz MS with 2/8 ratio was the only stimulation that demonstrated positive significant effects in all measured

indices against functional disuse, with increased BV/TV (39%; $p < 0.05$), Conn.D (109%; $p < 0.01$), and Tb.N (23%; $p < 0.01$), and reduced Tb.Sp (20%; $p < 0.01$). The changes in trabecular bone parameters observed with 1/4 and 4/6 C/R ratios had a trend similar to those of the 2/8 ratio, but with smaller percentage differences, for up to +28% for BV/TV, +78% Conn.D ($p < 0.01$), +20% Tb.N ($p < 0.05$), and -17% Tb.Sp ($p < 0.05$). MS with 2/28 ratio produced only minimal, not significant, effects to the trabecular bone in this region. Furthermore, the inhibition responses evaluated in the M1 and M2 regions were compared and did not illustrate any significance differences for all the parameters.

μCT – Femur M3 Region

M3 is the distal metaphyseal portion directly above the growth plate. This region had the most abundant trabecular network and the loss of bone due to disuse was significantly less than the loss measured in the M1 region ($p < 0.05$). In contrast to the M1 and M2 regions, MS also induced the least amount of changes in the trabecular bone in this region. Animals subjected to disuse alone experienced a 25% bone loss, 41% decrease in Conn.D, 15% reduction in Tb.N and 18% more spacing within this region ($p < 0.01$) (Figure 4). Trabecular BV/TV was not significantly affected by the stimulation. However, 50 Hz MS with 1/4 and 2/8 C/R ratios maintained the trabecular structure, with as high as 64% and 19% enhancement in Conn.D and Tb.N, respectively, and 15% decline in the spacing ($p < 0.05$). Like the other metaphyseal regions, SMI and Tb.Th were not affected by the stimulation. The changes in BV/TV and Conn.D induced by MS in this metaphyseal region were significantly different from the effects observed in M1 ($p < 0.05$) but not in M2.

μCT – Femur E Region

The epiphyseal trabecular bone was not affected by the 4-week HLS or MS, with BV/TV of $0.35 \pm 0.06\%$ for HLS and $0.32 \pm 0.05\%$ for MS groups. The percentage changes were minor versus the metaphyseal regions, with -1.2% BV/TV, -11% Conn.D, -4% Tb.N, and +6% Tb.Sp. MS did not induce any positive effect, regardless of the C/R ratio. All stimulated values were not significant or comparable to age-matched and HLS animals, with approximately 9% lower in BV/TV, 7% greater in Tb.N, and a 7% reduction in Tb.Sp.

Static and Dynamic Histomorphometry

Trabecular BV/TV-Histo, measured by the 2-D histomorphometric method, was 33% and 48% lower in HLS group than in age-matched controls ($p < 0.001$) in the metaphysis regions of femur and tibia, respectively (Table 3). For the other bone formation indices, HLS animals also showed significant decline in MS/BS (68–74%, $p < 0.01$), MAR (68–71%, $p < 0.01$) and BFR/BS (83%, $p < 0.01$) in both areas. Similar to the μCT results, MS with 2/8 C/R ratio demonstrated positive protection on the histomorphometric indices during the 4-week disuse with an increase of 26% in BV/TV-Histo ($p < 0.05$), 142% in MS/BS ($p < 0.05$), 140% in MAR ($p < 0.05$) and 65% in BFR/BS. The tibia metaphyseal region also showed an increase of 77% in BV/TV-Histo ($p < 0.05$), 170% in MS/BS ($p < 0.05$), 43% in MAR and 61% in BFR/BS. In addition, MS with 1/4 and 4/6 ratios resulted in up to +130% for MS/BS, +88% for MAR, and 28% for BFR/BS in the femur, and up to +115% for MS/BS, 33% for MAR and 21% for BFR/BS in the tibia. The C/R ratio of 2/28 had the least effect in maintaining bone formation. Comparing to the age-matched controls, the histomorphometric values measured from the stimulated groups were still lower by 13–72% in MS/BS, 29–70% in MAR, and 70–87% in BFR/BS.

Discussion

The data from this study signified that dynamic muscle stimulation has the potential to inhibit trabecular bone loss and microarchitectural deterioration induced by 4 weeks of

functional disuse. Previously, we demonstrated the importance in selecting the stimulation frequency within the regimen to affect the skeletal adaptive responses. [9] Here, we further investigated another critical parameter, C/R ratio, in a frequency-specific MS regimen to potentially optimize the preventive measure against disuse. Our selection of the C/R ratios was mainly based on the number of individual 1 ms stimulation pulse applied to the muscle and the number of daily cycle number to bone.

As 50 Hz MS showed the greatest percentage of inhibition of bone loss and deterioration against disuse, we applied this mid-frequency for all of the stimulated groups in this study. One characteristic of applying MS greater than 10 Hz is the type of muscle contraction it produces – tetanic contraction. The stimulation cycle number generated by the tetanic contraction (MS at > 10 Hz) is sensed by the skeleton and is depended on the C/R ratio. For example, 50 Hz stimulation with 2/8 C/R ratio (2 sec contraction followed by 8 sec rest period) delivered 100 stimulation pulses and a tetanic contraction once every 10 sec. In a daily 10 minutes of stimulation, 50 Hz MS with 2/8 C/R ratio delivered 6000 stimulation pulses to the quadriceps and a cycle number of 0.1 Hz to the femur. Likewise, MS with 1/4 C/R ratio also delivered 6000 stimulation pulses but a cycle number of 0.2Hz. 4/6 C/R ratio doubled the stimulation pulses to 12,000 with a cycle number of 0.1 Hz. Lastly, 2/28 C/R ratio only delivered 2000 stimulation pulse and 20 cycles daily.

Although the cycle numbers varied between groups, several implications can be drawn from this study. First, doubling the cycle number from 0.1Hz to 0.2Hz applied to the femur daily during the entire study, by altering the C/R ratio from 2/8 to 1/4, did not enhance osteogenesis. Given the equation derived by Qin et al., stimulation with such small cycle number would need to compensate with higher strain level in order to maintain cortical bone mass. [24] Direct mechanical loading at 1 Hz generated greater than 1000 $\mu\epsilon$ and significantly increased cortical bone formation rate. [25] However, MS at 50 Hz generated extremely low level of bone strain, which is below 10 $\mu\epsilon$. [10] This discrepancy may be explained by the differences in stimulation and its frequency. It has been shown that the strain magnitude decreases in an inverse relation with the induced loading frequency. [26] Extremely low-magnitude strain (< 10 $\mu\epsilon$), perhaps arise from muscle activities, was measured thousands of times a day and may be a key determinant to maintain bone's formation and function. [27] In addition, induced low-magnitude mechanical signal has the ability to produce osteogenic responses on both cortical and trabecular regions if the stimulation is applied at a high frequency (> 30 Hz). [27,28] Consistent with the observations from this study, MS at 50 Hz with 2/8 C/R ratio for 10 minutes per day was sufficient to inhibit trabecular bone loss and maintain its structure against 4 weeks of hindlimb suspension, partially by retaining the amount of mineralizing surface and the mineral apposition rate. Even though 1/4 C/R ratio doubled the cycle number, the percentage of inhibition was not as great as the 2/8 C/R ratio, which implied that cycle number may not be an important factor in dynamic MS. Other mechanisms, such as fluid perfusion in relation to the rest period, may be involved.

Secondly, increasing the stimulation pulses delivered to the quadriceps from 120,000 to 240,000 by changing the C/R ratio from 2/8 to 4/6 also did not augment the inhibition responses in the disuse femur and tibia. It was stated that contraction period compressed the vasculature within the skeletal muscle, diminishing arterial blood inflow and that blood is rapidly expelled into the extramuscular venous circulation. [29,30] Muscle perfusion is recovered during the rest period. [30] Studies have confirmed that venous blood flow increased by 61 ml/min·100g during isometric tetanic contraction of canine sciatic nerve. [20] This augmentation in flow subsequently increased the oxygen uptake and the work performed by the muscle. [20] By examining the C/R ratios of 2/8 and 4/6, we would like to maximize the contraction period yet avoiding muscle fatigue that reduces the contractility.

Despite there is no statistically significant between the two groups, our analysis showed that the adaptive responses from MS with 4/6 C/R ratio were comparable to the 2/8 C/R ratio, but the percentage changes comparing to the HLS were still lower for all the parameters. Although no detrimental outcome was observed at the tissue level with the 4 sec of continuous tetanic contraction, vascular perfusion and the exchange of nutrient and waste in the vessels may be disrupted by the combination of longer contractile duration with the shorter rest period. [18,31] Taken both implications into account, a more in depth experimental design for future study should investigate the true consequences of the various contractile and non-contractile periods. Perhaps by extending the daily duration of the stimulation group, this way the same number of stimulation pulse and cycle number would be applied to the musculoskeletal tissues.

An alteration in the musculoskeletal fluid flow environment may also contribute to the osteogenic effects observed in this study. The close relationship between muscle and bone hypothesizes that a change in vascular perfusion within skeletal muscle can indeed cascade to a change in fluid pressure in bone. [10,21] The decline in femoral ImP due to hindlimb suspension and the increase in femoral ImP induced by MS firmly demonstrated the importance of fluid flow in mediating bone physiological processes. [10,21] Bone fluid flow can enhance mechanosensitivity at the cellular level has been illustrated via various studies. [32–35] Mechanical loading accentuates fluid shear stress on bone cells, thus releasing signaling molecules, e.g., nitric oxide, to further mediate bone remodeling. [32,33] In addition to circumvent muscle fatigue, the rest period inserted into the MS regimen may potentially heighten the mechanotransductive responses by allowing flow within the small fluid channel in bone to recover from the damping effects after each stimulation, increasing the convective transport of nutrient and signaling molecules that mediate bone adaptation. [36]

In conclusion, the devastation of bone loss associated with disuse osteopenia can lead to serious medical complications. The application of dynamic MS has been shown to be beneficial as a preventive intervention for such disease, and its effectiveness is highly depended on the stimulation parameter, i.e., frequency. This study attempted to optimize the skeletal adaptive responses by varying the C/R ratio and to further alleviate trabecular bone deterioration due to the lack of daily weight bearing activity. We showed that C/R ratio does play an important role in stimulating trabecular bone responses in both disused limbs, in which 2/8 sec has shown a sufficient and optimized C/R ratio among all tested C/R ratio, 1/4, 2/8, 4/6, and 2/28 sec. Although a more in-depth study is perhaps needed to determine the true effects of cycle number and the duration of rest insertion, the implications from this study presented the significance of the C/R ratio with this non-invasive stimulation and the preliminary layout of future investigations.

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Figure 1. Representative 3D μ CT images of trabecular bone volume fraction in the M2 region, age-match control, HLS disuse and HLS plus 50Hz, 2/8 C/R muscle stimulation. Significant bone loss was occurred in the HLS comparison to age-match control, while MS mitigated such bone loss with 10 min daily loading.

Figure 2.

μ CT analysis of the trabecular bone in the distal femoral M1 region (750 μ m, closest to femoral diaphysis). Graphs show mean + SD values for bone volume fraction (BV/TV, %), connectivity density (Conn.D, $1/\text{mm}^3$), trabecular number (Tb.N, $1/\text{mm}$), and separation (Tb.Sp, mm) at the M1 region. MS at 50 Hz with 2/8 C-R ratio produced significant changes in all indices, compared with values obtained from 4-week HLS. MS with 4/6 C-R ratio also demonstrated significant higher values for the bone quality parameters. [#] $p < 0.01$ vs. baseline; ⁺ $p < 0.01$ vs. age-matched; * $p < 0.05$ vs. HLS; ** $p < 0.01$ vs. HLS.

Figure 3.

μ CT measurement of the trabecular bone in the distal femoral M2 region (750 μ m, in between M1 and M3, 750 μ m above the growth plate). Graphs show mean + SD values for bone volume fraction (BV/TV, %), connectivity density (Conn.D, $1/\text{mm}^3$), trabecular number (Tb.N, $1/\text{mm}$), and separation (Tb.Sp, mm) at the M2 region. 50 Hz MS with 2/8 C-R ratio showed significant effects for all indices against 4-week HLS. MS with 1/4 and 4/6 C-R ratios also provided significant protection on the trabecular bone structure. # $p < 0.01$ vs. baseline; + $p < 0.01$ vs. age-matched; * $p < 0.05$ vs. HLS; ** $p < 0.01$ vs. HLS.

Figure 4.

μCT evaluation of the trabecular bone in the distal femoral M3 region (750 μm, immediately above the growth plate). Graphs show mean + SD values for bone volume fraction (BV/TV, %), connectivity density (Conn.D, 1/mm³), trabecular number (Tb.N, 1/mm), and separation (Tb.Sp, mm) at the M3 region. 50 Hz MS with 1/4 and 2/8 C-R ratios were able to maintain trabecular bone network during 4 weeks of the lack of weight bearing activity. [#] $p < 0.01$ vs. baseline; ⁺ $p < 0.01$ vs. age-matched; ^{*} $p < 0.05$ vs. HLS; ^{**} $p < 0.01$ vs. HLS.

Figure 5. Representative 2-D images of calcein labeled trabecular bone histomorphometry in the distal metaphyseal region of the femur. The calcein (green marker) labeled the mineralizing bone surfaces. Trabecular bone formation was clearly indicated by the double calcein labels in the 2/8 MS group.

Table 1

Distal femur metaphysis histomorphometry

	Control	HLS	1/4 MS	2/8 MS	4/6 MS	2/28 MS
BV/TV - Histo (%)	36.1 ± 2.6	24.3 ± 1.9**	27.1 ± 2.9	30.7 ± 1.9 [#]	29.0 ± 3.3 [#]	24.3 ± 3.1
MS/BS (%)	9.11 ± 1.3	2.37 ± 0.4*	5.45 ± 1.2	5.74 ± 1.2 [#]	4.70 ± 0.7	2.58 ± 0.5
MAR (µm/day)	1.18 ± 0.1	0.34 ± 0.1*	0.65 ± 0.2	0.83 ± 0.2 [#]	0.58 ± 0.2	0.49 ± 0.2
BFR/BS (µm ³ /µm ² /yr)	35.9 ± 6.2	6.03 ± 2.3*	7.75 ± 2.3	9.95 ± 2.7	5.88 ± 1.8	4.58 ± 2.1

Trabecular bone histomorphological analyses on control, HLS, and C-R ratios (1/4, 2/8, 4/6, and 2/28 min) MS. 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;

** $p < 0.01$ vs. age-matched;

[#] $p < 0.05$ vs. HLS.