Bone 48 (2011) 399–405

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Alteration of contraction-to-rest ratio to optimize trabecular bone adaptation induced by dynamic muscle stimulation

Hoyan Lam, Minyi Hu, Yi-Xian Qin *

Orthopeadic Bioengineering Research Laboratory, Department of Biomedical Engineering, Stony Brook University, Stony Brook, NY 11794-5281, USA

ARTICLE INFO

Article history:
Received 11 May 2010
Revised 1 September 2010
Accepted 13 September 2010
Available online 17 September 2010

Edited by David Burr

Keywords:
Muscle contraction
Bone adaptation
Mitigation of bone loss
Disuse osteopenia
Bone fluid flow
Rest period
Osteoporosis

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 s. Muscle stimulation was carried out for total of 10 min/day, 5 days/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 s 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 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 s has shown maximal adaptive response among all tested ratios.

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 s, 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 με. 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 dependent 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 of 1 Hz, 20 Hz, 50 Hz, and 100 Hz, 50 Hz 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.

* Corresponding author. Dept. of Biomedical Engineering, Stony Brook University, Bioengineering Bldg. Rm 215, Stony Brook, NY 11794-5281, USA. Fax: +1 631 632 8577.
E-mail address: Yi-Xian.Qin@sunysb.edu (Y.-X. Qin).

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doi:10.1016/j.bone.2010.09.018

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 in.×18 in.×24 in. (L×W×H) stainless steel HLS cages in a temperature-controlled room with a 12:12 h 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 s contraction-to-4 s 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 gage #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 min/day, 5 days/week, for a total of 4 weeks. The electrical stimulation was given at 2 V sinusoidal signal with measured current value of less than 40 mA. The contraction was applied for either 1 s with 4 s 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 (μCT) analysis

The right femurs were harvested and preserved in 70% ethanol after the completion of the *in vivo* study. Using a high resolution μCT scanner (μCT-40, SCANCO Medical AG, Bassersdorf, Switzerland), the distal femurs were scanned with a spatial resolution of 15 μm. 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 μm 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 μm 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/mm^3), structural model index (SMI), trabecular number (Tb.N, 1/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 (10 mg/kg) were administered to each animal 2 days and 16 days prior to euthanasia. After scanning with μCT, 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 μm 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, 3 mm^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, μm/day), and bone formation rate (BFR/BS, μm^3/μm^2/yr) were determined [23].

Statistical analysis

Results are reported as mean ± SD for μCT analyses and mean ± SE for histomorphometric analyses. For all measurements, significant 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 g ± 30 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).