

# Comparison of Morphological Changes of Muscle Fibers in Response to Dynamic Electrical Muscle Contraction and Dynamic Hydraulic Stimulation in a Rat Hindlimb Disuse Model

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## Summary

This study attempted to compare the muscle fiber morphological responses to dynamic electrical muscle stimulation (DEMS) and dynamic hydraulic stimulation (DHS) in rats under hindlimb suspension (HLS). DEMS at 1 Hz, 50 Hz and 100 Hz for 10 min/day, 5 days/week were introduced to the animals' right quadriceps. Static and 2 Hz DHS were introduced to the right tibiae of other animal groups on a "10 min on – 5 min off – 10 min on" loading regime for 5 days/week. In the end of the 4-week experiments, histological changes in the corresponding soleus, gastrocnemius and quadriceps of the stimulated sites were examined. Compared to age-matched, HLS led to muscle atrophy and strongly reduced muscle wet weights and averaged cross-sectional fiber areas. Among the tested DEMS frequencies, the averaged cross-sectional quadriceps fiber area in the 50 Hz group was 29 % larger than the 100 Hz group. In contrast, difference in the muscle fiber response to the static and 2 Hz DHS was not observed in either soleus or gastrocnemius. Muscle fiber morphological responses to the active DEMS was in a load frequency dependent manner under disuse condition. Relatively passive compressions, either *via* static or 2 Hz DHS, were unable to induce any difference in the muscle fiber responses under functional disuse.

## Key words

Disuse muscle atrophy • Hindlimb unloading • Mechanical stimulation • Muscle fiber regeneration

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## Introduction

Skeletal muscle and bone are closely interacted both anatomically and functionally (Cianferotti and Brandi 2014). Both of these tissues have been shown to be dramatically affected by the lack of gravity and immobilization (Bonewald *et al.* 2013). Biomechanical signals or the triggered systemic hormonal stimuli, elicited from conditions such as loading or disuse, are sensed and transduced by the endocrine properties of muscle and bone (Cianferotti and Brandi 2014). Despite the assumption that the major effect of a lack of gravity could be due to a lack of muscle loading on the bone, muscle and bone tissue may actually contribute to each other's restoration or repair (Bonewald *et al.* 2013). Therefore, a single duo-functional therapeutic that could maintain and heal not only bone but also muscle is most ideal and needed to be identified.

Decreases in muscle activity leading to muscle atrophy has been reported following space mission, spinal cord injury, bed rest and aging. The alteration in mechanical environment rapidly induces muscular adaptation in fiber size and metabolic properties (LeBlanc *et al.* 2000, Edgerton *et al.* 1995, Zhou *et al.* 1995, Gregory *et al.* 2003, Narici *et al.* 2003, Trappe *et al.* 2001). Short-term spaceflight caused significant losses in muscle volume by 5 to 17 %, fiber cross-sectional area by 11 to 24 %, and type I fiber by 6 to 8 % (LeBlanc *et al.* 2000, Edgerton *et al.* 1995). Likewise, 6-week post spinal

cord injury caused a 15 % lean muscle mass loss at the lower extremities and an averaged muscle cross-sectional areas reduction by 18 to 46 % (Castro *et al.* 1999, Spungen *et al.* 2003). These changes are often associated with increased muscle weakness and fracture risk (Zehnder *et al.* 2004).

Small animal models have been extensively studied to mimic the condition of disuse (Allen and Bloomfield 2003, Morey-Holton and Globus 2002, Hauschka *et al.* 1988, Morey-Holton and Globus 1998). Similar to human, rodent studies displayed a loss of skeletal muscle mass, greatest in the postural muscles, and decreased in muscle force (Boonyarom and Inui 2006, Fisher *et al.* 1998, Hurst and Fitts 2003, McClung *et al.* 2006). Chemical-induced paralyzed limb in rats showed the highest decline (~40 %) in muscle weight and fiber cross-sectional area in the soleus muscle (Dupont Salter *et al.* 2003). The degree of muscle atrophy was not as profound in the tibialis anterior and gastrocnemius, ~20 %. Rat hindlimb unloading also supported these findings, where there was significantly reduction in both type I and type II muscle fibers area for the soleus and plantaris muscles (Kyparos *et al.* 2005). In addition, hindlimb suspension decreased the percentage of type I muscle fibers by an approximately 40 %, while the number of type II fibers were augmented by ~20 % (Kyparos *et al.* 2005).

As an intervention, functional electrical stimulation produces isometric contractions in replacement for the lack of muscle activity (Baldi *et al.* 1998). The induced contractions have been shown to increase muscle volume, muscle cross-sectional area, and improve fatigue resistance in human (Gerrits *et al.* 2002, Skold *et al.* 2002). However, the effects of this external stimulus highly depended on the variability in the duration and frequency of the stimulation (Perez *et al.* 2002). Effect of dynamic electrical muscle stimulation (DEMS) on mitigation of disuse bone loss in rats has been shown in a frequency and contraction-to-rest ratio dependent manner (Lam and Qin 2008, Lam *et al.* 2011). On the other hand, the newly developed dynamic hydraulic stimulation (DHS) is suggested to act noninvasively as an external oscillatory muscle coupling. The dynamic components derived from the hydraulic loading have been shown to influence bone interstitial fluid pressure and mitigate disuse bone loss in a frequency and magnitude dependent manner (Hu *et al.* 2014, Hu *et al.* 2013a, Hu *et al.* 2012), possibly through the modulation on the bone marrow mesenchymal stem

cell population (Hu *et al.* 2013b) and osteogenic gene expressions (Hu and Qin 2014).

Electrical stimulation applied to animals has reported various effects on the skeletal muscle morphology and biochemistry. Numerous stimulation paradigms has been tested and generated mixed conclusion on the benefit of such stimulations. The discrepancies were mainly contributed by the applied stimulation frequency, the duration of the stimulation, and the type of muscle analyzed (Mabuchi *et al.* 1982, Kernell *et al.* 1987, Gorza *et al.* 1988, Jarvis *et al.* 1996). Stimulation at 10 Hz for 24 h per day increased fatigue resistance yet decreased the size of the tibialis anterior muscles (Salmons and Henriksson 1981). Lower frequency, such as 2 Hz, applied for 10 h per day demonstrated a marked reduction in paralyzed soleus atrophy (Dupont Salter *et al.* 2003). Most studies that showed positive effects of electrical muscle contraction at the tissue level have used extremely long stimulation protocols, from 2 to 24 h. In addition, the application of the stimulation frequency ranged from 1 to 100 Hz.

As skeletal muscle and bone are interrelated and inter-dependent, and that optimized stimulation signals are crucial for tissue regeneration, we were interested in investigating the frequency effect of DEMS and the effect of the dynamic components derived from DHS on the muscle fiber morphological responses under disuse condition. Separate animal studies were performed: (1) to verify the atrophic effect of hindlimb suspension on rats' soleus, gastrocnemius, and quadriceps muscles; (2) to evaluate the difference in muscle fiber morphological responses to DEMS at 1 Hz, 50 Hz and 100 Hz; and (3) to access the difference in muscle fiber morphological responses to static and 2 Hz DHS loading.

## Materials and Methods

### *Animal study 1 – Validation of HLS induced muscle atrophy*

The effect of lacking daily activity on muscle morphological change was investigated. Stony Brook University IACUC approved all the animal experimental procedures. Five-month-old female Sprague Dawley virgin rats (Charles River, MA) were randomly divided into three groups: (1) baseline, (2) age-matched and (3) HLS (n=5 per group). The animals in the baseline group were sacrificed at the beginning of the study. The HLS procedure was similar to the setup from our previous studies (Hu *et al.* 2012). Briefly, the tail of each animal

was cleaned with 70 % alcohol and lightly coated with tincture of benzoin (Bruce Medial Supply, MA), while the animal was under standard isoflurane anesthesia. The tail harness was made from a paperclip-looped plastic tab with a strip of skin tape. Once the tail was dried, the skin tape was adhered to the tail, covering  $\frac{3}{4}$  of its length. The tape was secured with two strips of filament tapes; one over the end of the skin tape around the base of the tail and the other about half-way up. The tail harness was then attached to a fish-line swivel apparatus that hangs from the top of the 18"x18"x24" (LxWxH) stainless steel cage. An approximately 30° head-down tilt was set to prevent contact of the animal's hindlimbs and the cage bottom. The animal's forelimbs were allowed full access to the entire cage bottom. Animals were provided with standard rodent chow and water *ad libitum*, and roomed under temperature-controlled and a 12:12 h light:dark cycle. Animals' body weights and overall health were carefully monitored throughout the 4-week HLS study. At the end of the study, the animals were euthanized by CO<sub>2</sub>; the soleus, gastrocnemius and quadriceps muscles were extracted. Measurements of the animals' end-point body weights normalized to the corresponded baseline, as well as the muscle wet weights of the right soleus, gastrocnemius and quadriceps were recorded. The samples were then snap-frozen using OCT compound (Tissue Tek, USA) and liquid N<sub>2</sub>-chilled 2-Methylbutane, and then stored at -80 °C.

#### *Animal study 2 – Effect of DEMS on muscle fiber morphology at various loading frequencies*

The effect of DEMS on muscle fiber morphology at various loading frequencies under disuse condition was examined *via* a 4-week HLS study. Animals were randomly assigned to groups of: 1) HLS+1 Hz DEMS (n=7), 2) HLS+50 Hz DEMS (n=4) and 3) HLS+100 Hz DEMS (n=4). Functional disuse was induced by HLS, using the same setup as described above. DEMS was applied in conjunction with HLS for 4 weeks. For the daily stimulation, DEMS was applied *via* two needle-size electrodes (L-type gauge #3, Seirin, Weymouth, MA) to the isoflurane anesthetized animals. One electrode was placed at the right lateral proximal quadriceps (rectus femoris), and the other was placed at the lateral distal quadriceps (rectus femoris). The electrodes were then connected to a function generator (Model 395, Wavetek, USA) to transmit a 1 ms square pulse with various stimulation frequencies (1 Hz, 50 Hz and 100 Hz). The stimulation duration was 10 min per

day for 5 days per week. To avoid muscle fatigue, a rest-insertion period (2 s contraction followed by 8 s rest) was added in the DEMS regimen. The animals were euthanized at the end of the 4-week study *via* CO<sub>2</sub>. Muscle samples (right soleus, rectus femoris of the right quadriceps and lateralis of the right gastrocnemius) were harvested immediately after sacrifice and then cryo-preserved.

#### *Animal study 3 – Effect of dynamic components of DHS on muscle fiber morphology*

A 4-week HLS study was conducted to evaluate the effect of the dynamic components of DHS on muscle fiber morphology. Five-month-old female Sprague Dawley virgin rats (Charles River, MA) were randomly divided into two groups: 1) HLS+static loading (n=5) and 2) HLS+DHS at 2 Hz (n=6). Daily 20 min loading (10 min on – 5 min off – 10 min on) for 5 days/week was applied in conjunction with HLS. To deliver DHS, a costume design inflatable cuff was placed over around the right hindlimb over the region of the tibia. An oscillatory actuator-driven syringe, a force-controlled syringe, and a pressure sensor were connected to the stimulation cuff to deliver dynamic compression with a constant static pressure by monitoring using the pressure sensor. A programmable waveform/signal generator (HP33120A, Hewlett-Packard, Palo Alto, CA) was used to control the actuator-driven syringe, which supported the inflation and deflation of the cuff. The DHS was applied to the stimulated animals at 30 mm Hg static pressure + 30 mm Hg (p-p) dynamic pressure with 2 Hz of loading frequency. Animals in the static group only received 30 mm Hg static pressure yet for the same amount of time. The rats were euthanized *via* CO<sub>2</sub> at the end of the study, and the right soleus and lateralis of the gastrocnemius were obtained and cryo-preserved.

#### *Histology and image analysis*

Cross-sections of the cryo-preserved muscle samples, containing both deep and superficial muscle layers, were made to 8 µm using a cryostat (Leica) at -20 °C. Three sections were then stained with Hemotoxilyn and Eoson (H&E, Polyscience, USA). Five random cross-sectional bright field images (20x) were captured from each muscle section with a Zeiss microscope (AxioVision 4.5, Germany). Averaged cross-sectional muscle fiber areas were determined by Image J software and normalized to the averaged HLS value.

### Statistical analysis

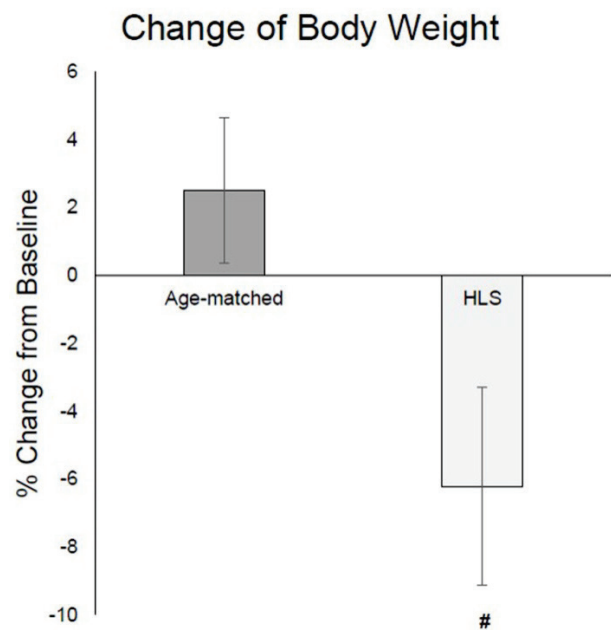
The percent body weight change was reported as mean  $\pm$  SE. The values of the muscle wet weight and evaluations of the averaged cross-sectional muscle fiber area were reported as mean  $\pm$  SD. For each analysis, significance differences between groups were determined using GraphPad Prism 3.0 Software (GraphPad Software Inc., La Jolla, CA). One-way ANOVA with Tukey's pairwise multiple comparison tests and unpaired Student's T Test was performed, respectively to the analysis on multiple groups or two groups. The level of significance was considered at  $p < 0.05$ .

## Results

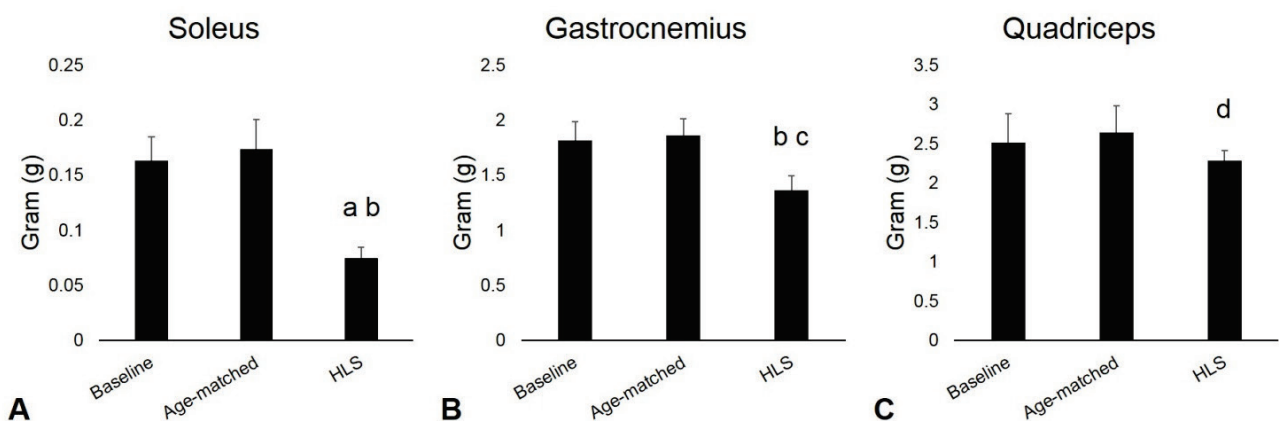
### HLS induced body weight change and muscle atrophy

To validate the impact of HLS on the animals' skeletal muscles, the animals' body weights, muscle wet weights and averaged cross-sectional muscle fibers were evaluated. Age-matched animals showed an increase in body weight by 2.5 % (Fig. 1). On the other hand, HLS reduced the body weight by 6.2 % ( $p < 0.05$  vs. age-matched). Additionally, HLS significantly decreased the right soleus muscle wet weight compared to the baseline (-54 %;  $p < 0.001$ ) and the age-matched (-57 %;  $p < 0.001$ ) groups, while the right soleus muscle wet weight of the age-matched group was similar to the baseline (Fig. 2). Similarly, the muscle wet weights of the right gastrocnemius were not significantly different between baseline and age-matched control. However, HLS significantly reduced the right gastrocnemius wet weight compared to the baseline (-25 %;  $p < 0.01$ ) and

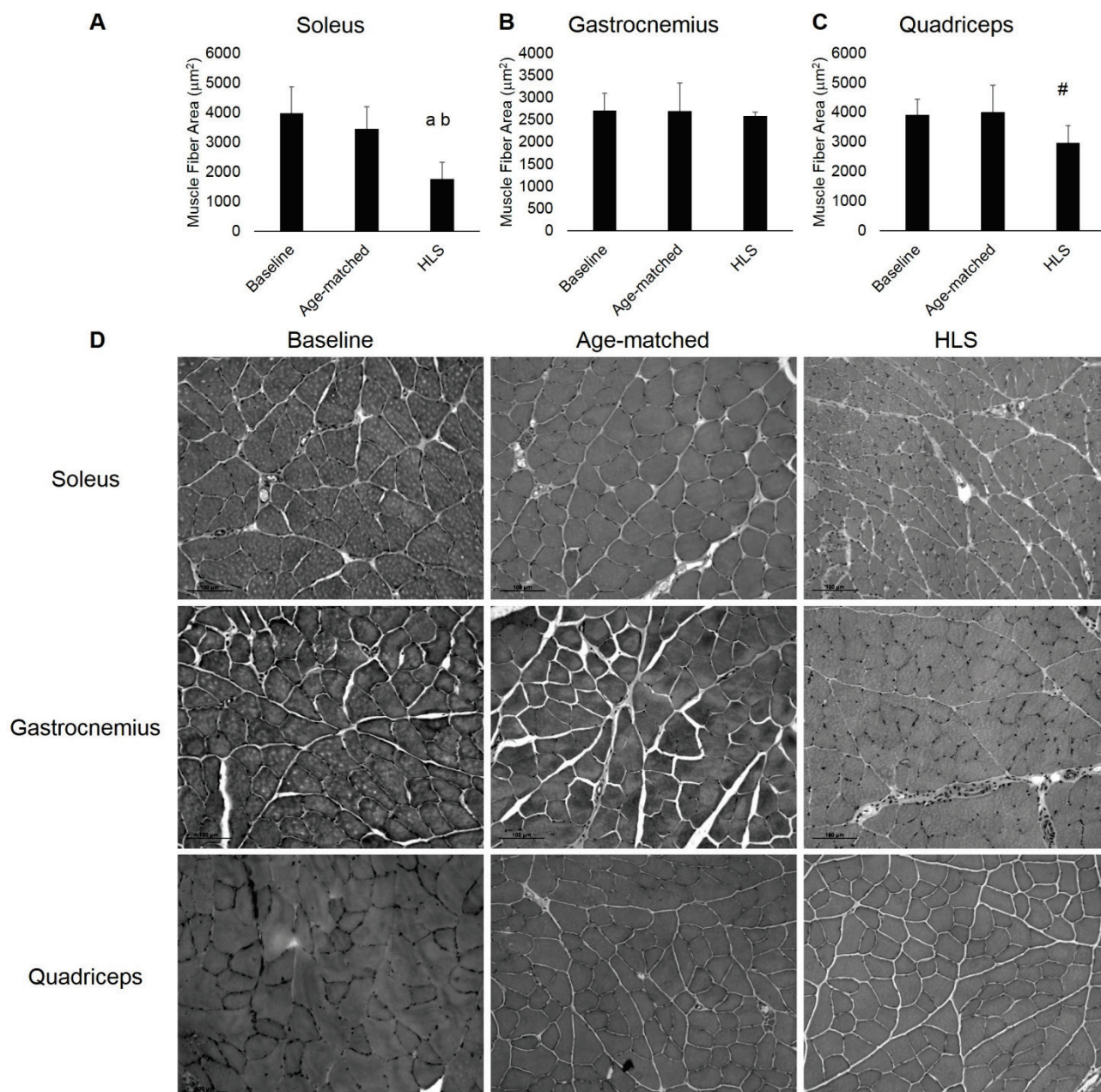
age-matched (-27 %;  $p < 0.001$ ) groups. HLS also significantly reduced the right quadriceps wet weight compared to the age-matched control (-14 %;  $p < 0.05$ ). For the averaged cross-sectional muscle fiber areas (Fig. 3), HLS negatively impacted the right soleus (-56 % at  $p < 0.001$  vs. baseline; -49 % at  $p < 0.001$  vs. age-matched) and right quadriceps (-26 % at  $p < 0.05$  vs. age-matched) but not the right gastrocnemius ( $p > 0.05$  vs. baseline or age-matched control).



**Fig. 1.** Graph shows mean  $\pm$  SE values for the percent body weight change from baseline. The age-matched animals showed an increase in body weight by 2.5 %. On the other hand, HLS affected the percent body weight change with a decrease of 6.2 % (#  $p < 0.05$  vs. age-matched control).



**Fig. 2.** Graph shows mean  $\pm$  SD values for the soleus (A), gastrocnemius (B) and quadriceps (C) muscle wet weights. The muscle wet weights of the age-matched group were similar to the baseline. However, HLS significantly decreased the soleus wet weight compared to the baseline (<sup>a</sup>  $p < 0.001$ ) and the age-matched (<sup>b</sup>  $p < 0.001$ ) groups, and decreased the gastrocnemius wet weight compared to the baseline (<sup>c</sup>  $p < 0.01$ ) and the age-matched (<sup>b</sup>  $p < 0.001$ ), as well as decreasing the quadriceps wet weight compared to age-matched (<sup>d</sup>  $p < 0.05$ ).



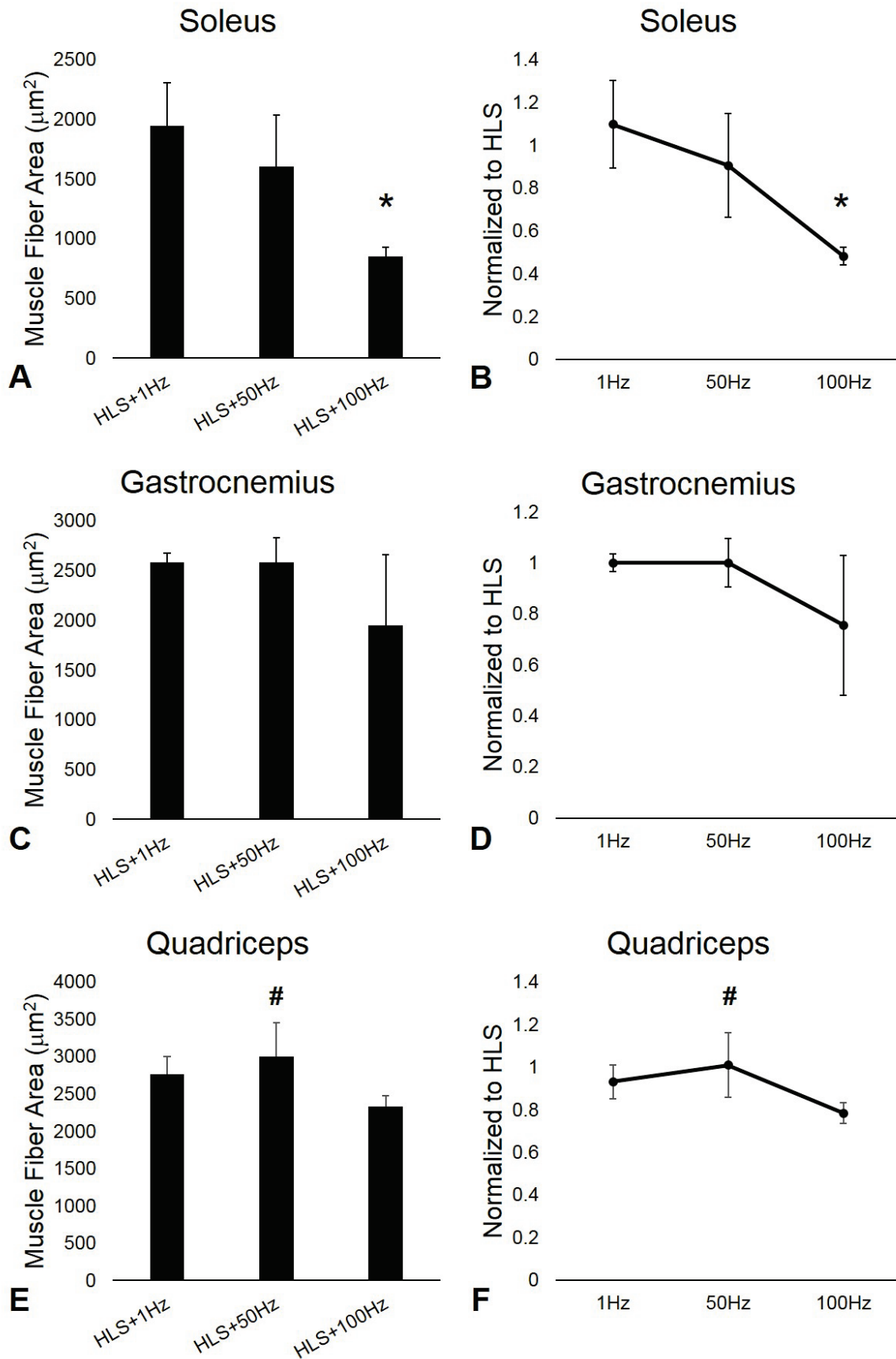
**Fig. 3.** Graph shows mean  $\pm$  SD values for the averaged cross-sectional soleus (A), gastrocnemius (B) and quadriceps (C) fiber area, along with representative histology images (D). HLS decreased the averaged cross-sectional muscle fiber area compared to baseline (for soleus) and age-matched control (for soleus and quadriceps). <sup>a</sup>  $p < 0.001$  vs. baseline; <sup>b</sup>  $p < 0.01$  vs. age-matched; <sup>#</sup>  $p < 0.05$  vs. age-matched.

*DEMS at various loading frequencies induced change of cross-sectional muscle fiber area*

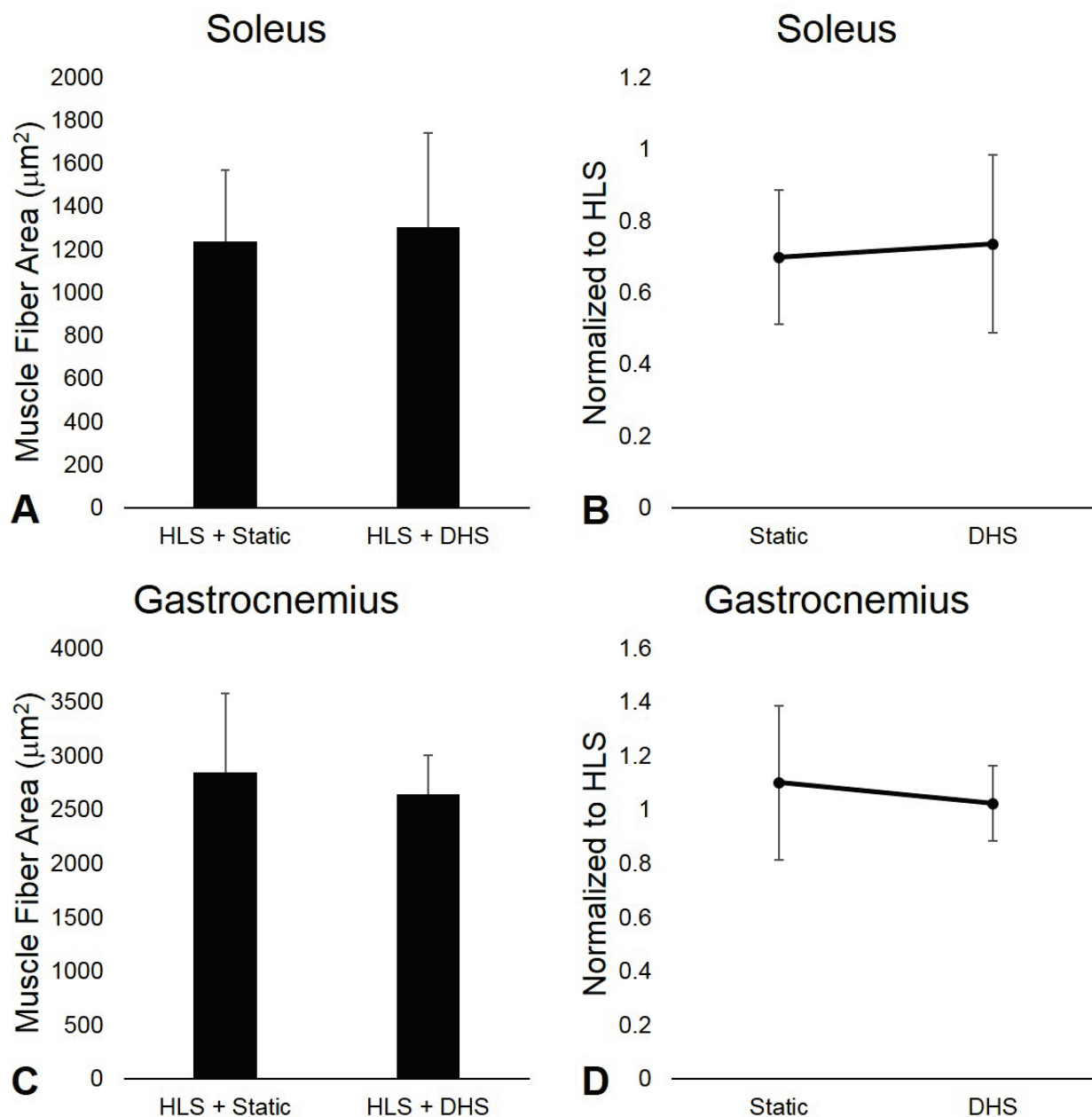
As shown in Figure 4, the averaged cross-sectional fiber area of the right soleus in the 50 Hz treatment group ( $1,605 \pm 430 \mu\text{m}^2$ ) and 100 Hz treatment group ( $855 \pm 75 \mu\text{m}^2$ ) were 18% ( $p > 0.05$ ) and 56% ( $p < 0.05$ ) lower than the 1 Hz treatment group ( $1,946 \pm 363 \mu\text{m}^2$ ), respectively. The right quadriceps in the 50 Hz treatment ( $2,998 \pm 453 \mu\text{m}^2$ ) group was 8%

larger than the 1 Hz treatment ( $2,765 \pm 236 \mu\text{m}^2$ ) group ( $p > 0.05$ ) and was 29% larger than the 100 Hz treatment ( $2,329 \pm 148 \mu\text{m}^2$ ) group ( $p < 0.05$ ). Although it was not statistically significant, the averaged cross-sectional gastrocnemius muscle fiber area showed a reduced value in the 100 Hz treatment ( $1,953 \pm 711 \mu\text{m}^2$ ) compared to the 50 Hz ( $2,585 \pm 247 \mu\text{m}^2$ ) and the 1 Hz ( $2,583 \pm 91 \mu\text{m}^2$ ) treatment groups ( $p > 0.05$ ). Normalization to the averaged HLS value showed similar trends.





**Fig. 4.** Graph shows mean  $\pm$  SD values for the averaged cross-sectional soleus, gastrocnemius and quadriceps fiber areas in response to DEMS at different loading frequencies (**A**, **C** and **E**), as well as the corresponding normalization to the average HLS value (**B**, **D** and **F**). \*  $p < 0.05$  vs. HLS+1 Hz; #  $p < 0.05$  vs. HLS+100 Hz.



**Fig. 5.** Graph shows mean  $\pm$  SD values for the averaged cross-sectional soleus and gastrocnemius fiber area in response to static pressure and DHS loading (**A** and **C**), as well as the corresponding normalization to the average HLS value (**B** and **D**). The averaged cross-sectional muscle fiber areas in the DHS were similar to the ones in the static group.

#### *Unchanged cross-sectional muscle fiber area in response to DHS*

As shown in Figure 5, the averaged cross-sectional fiber area of the right soleus in the DHS ( $1,303 \pm 439 \mu\text{m}^2$ ) group was similar to the one in the static ( $1,237 \pm 331 \mu\text{m}^2$ ) group ( $p > 0.05$ ). Likewise, the difference between the averaged cross-sectional fiber areas of the right gastrocnemius muscles between the DHS ( $2,643 \pm 363 \mu\text{m}^2$ ) group and the static ( $2,843 \pm 739 \mu\text{m}^2$ ) group was statistically un-significant ( $p > 0.05$ ). Normalization to the averaged HLS value

showed similar trends.

#### **Discussion**

This study investigated the frequency effect of DEMS and the effect of the dynamic components derived from DHS on the muscle fiber morphological responses under disuse condition. HLS negatively affected the muscle wet weights and averaged cross-sectional fiber areas, more so in the soleus than in the gastrocnemius and quadriceps. Under such disuse condition, DEMS at 50 Hz

had a significantly larger averaged cross-sectional fiber area compared to the 1 Hz or 100 Hz group in the quadriceps, and showed a similar trend in gastrocnemius. DEMS at 100 Hz showed a larger averaged cross-sectional fiber area compared to the 1 Hz and 50 Hz stimulations in soleus. The quadriceps and gastrocnemius muscle are examples of fast-twitch muscles, which is highly responsive to contraction with abundant of type II muscle fibers (Zhou *et al.* 1995, Perez *et al.* 2002, Punkt *et al.* 2004). In contrast, the soleus is considered as a posture muscle (slow-twitch) with abundant of type I fibers (Punkt *et al.* 2004, Perez *et al.* 2002, Zhou *et al.* 1995). It has been reported that slow-twitch fibers are more sensitive to HLS than fast muscle fibers, and that disuse induces slow-to-fast transformation (Marsh *et al.* 1992, Oishi *et al.* 1998).

The present data confirmed that the effectiveness of DEMS is highly dependent on the stimulation frequency. Mechanical stimulations have been researched to alleviate muscle atrophy. Long duration (10–24 h) electrical stimulations at low frequency significantly prevented weight loss in soleus and tibialis anterior (Dupont Salter *et al.* 2003, Salmons and Henriksson 1981). However, others found that stimulation with higher frequency can maintain muscle mechanical properties, i.e. twitch and tetanic force, as well as reducing muscle weight loss and fiber CSA (Eerbeek *et al.* 1984, Gorza *et al.* 1988). Daily 30 min of electrical stimulation at 45 Hz–60 Hz was previously studied with healthy individuals (Perez *et al.* 2002). Since the subjects did not experience any disuse-related muscle loss, the stimulation served as an enhancement and showed an increased in averaged CSA by 14 % and in the number of type II fibers. To our knowledge, this study was the first to examine the effect of such short-term duration (10 min) on disuse muscle. We have previously showed that DEMS, applied 10 min per day, can partially prevent trabecular bone loss in disused femur in a frequency dependent manner, where 50 Hz was found to be an optimized DEMS loading frequency for bone (Lam and Qin 2008). Correspondingly, the effect of DEMS on bone and muscle may ultimately give insights into the bone-muscle relationship.

The variation of stimulation frequencies within the DEMS regimen generated two types of isometric contraction at the quadriceps. DEMS at low frequency, such as 1 Hz, produced isometric twitch, in which there is a rapid increase in muscle tension and then gradually return to normal. This induced twitch contractions, which

failed to prevent muscle atrophy but was able to maintain maximum twitch tension (Yoshida *et al.* 2003). On the other hand, DEMS at mid to high frequency, such as 50 Hz or 100 Hz, generated isometric tetanus, where a peak muscle tension is achieved by a summation effect (Gorza *et al.* 1988). However, from the characteristics of tissue material point of view, e.g. viscoelastic property in the tissues, both muscle and bone could quickly damp the loads at high frequencies (Qin and Lam 2009). Therefore, DEMS at 100 Hz was not observed to be as effective as the stimulation at 50 Hz in this study. For our studies, we concentrated in determining the morphological consequence of DEMS on the muscles under disuse condition. Previous studies by Gorza *et al.* measured the maximum tetanic tension of an innervated soleus to be 1.9 N and a denervated soleus to be 0.046 N. The tension of the denervated muscle rose to 0.68 N with stimulation at 15 Hz and 1.1 N with stimulation at 100 Hz (Gorza *et al.* 1988). Similar results were found with HLS rats subjected to isometric exercise, the tetanic force of the soleus was increased by 30 % (Hurst and Fitts 2003). The degree of protection with the stimulation was less when measured in a fast-twitch muscle, such as extensor digitorum longus. Nevertheless, the tension–frequency relation could potentially be one of the key elements in selecting the optimal stimulation frequency within the DEMS paradigm.

The 2 Hz dynamic component of DHS did not make a difference in muscle morphology compared to the static loading, under disuse condition. A consideration in the design of DEMS and DHS regimen is the elevation of fluid flow induced by each stimulation. DEMS and DHS have been shown to be able to raise intramuscular pressure (Hu *et al.* 2014, Hu *et al.* 2013a, Qin and Lam 2009). It is thought that rhythmical contractions increase venous outflow by compressing the arteries and veins, the vessels are refilled during relaxation (Valic *et al.* 2005). This concept of muscle pump is well appreciated and may be a potentially mechanism in the effects of DEMS. Treadmill exercise and 1 second of tetanic stimulation at 30 Hz both decreased arterial blood flow and increased venous blood flow at the onset of contraction (Valic *et al.* 2002). Blood flow then returned to normal level during rest period. In addition, blood expelled from the venous system increased as function of increased stimulation frequency (Hogan *et al.* 2003). Extensive researches also suggested other mechanisms, i.e. vasodilation, to explain the rapid increase in blood flow during the first few second of contraction (Buckwalter *et al.* 1998, Welsh and Segal 1997).



Regardless of the possible mechanism, DEMS increased microvascular fluid perfusion is undeniable. Prolonged stimulation may enhance perfusion, augment oxygen and other metabolites delivery, and further protect the muscular tissues against the lack of functional activity. As a relatively passive stimulation, DHS's dynamic component at 2 Hz did not affect the muscle morphology under disuse, compared to the static application alone. Moreover, a limitation of this study was that the effect of DHS was only tested with a single frequency. While 2 Hz was found to be an optimized frequency for bone response (Hu *et al.* 2012, Hu *et al.* 2013a), it did not seem to induce muscle response. Further investigations should be done to optimize DHS's loading condition for muscle regeneration.

The quadriceps and gastrocnemius muscles are examples of fast-twitch muscles, which is highly responsive to contraction with abundant of type II muscle fibers. In contrast, the soleus is considered as a posture muscle (slow-twitch) with abundant of type I fibers (Zhou *et al.* 1995, Perez *et al.* 2002, Punkt *et al.* 2004). Studies on fast-twitch muscle groups, i.e., extensor digitorum longus (EDL), detected ~95 % of type II fibers *via* histochemical analysis and myosin heavy chain (MHC) II isoform *via* protein analysis (Jarvis *et al.* 1996, Punkt *et al.* 2004, Pette *et al.* 2002). Soleus expressed ~85 % of MHC II and 15 % of MHC I (Desaphy *et al.* 2005). Mammalian skeletal muscle fibers display great adaptive ability *via* adjustments of their molecular, functional, and metabolic properties in response to altered functional demands (e.g. mechanical signals). Fiber type transitions occur in a sequential order, which results from the adaptive changes in the expression of myofibrillar and other protein isoforms. Alteration factors of the functional demand affect muscle fibers on their functional transitions in the direction of slow or fast (Pette 2002). It has been reported that slow-twitch fibers are more sensitive to HLS than fast muscle fibers, and that disuse induces slow-to-fast transformation (Marsh *et al.* 1992, Oishi *et al.* 1998) which continues during disuse (Staron *et al.* 1998). Soleus muscle containing different populations of slow type I fibers have various sensitivity to altered physiological conditions (Caiozzo *et al.* 1997). Suspension hypokinesia exclusively influences type I fibers in postural muscles (e.g. SOL), and leads to arrest of muscle developmental conversion of type II to type I fibers (Asmussen and Soukup 1991). Marked increases of rat MHCIIId/x and MHCIIb isoforms as well as pronounced decreases in slow MHCI were induced by phasic high-frequency stimulation exceeded by far those

of denervation, leading to marked increases of these two isoforms, as well as to pronounced decreases in slow MHCI (Hamalainen and Pette 1996). A graded suppressive effect of thyroid hormone on the expression of MHC isoforms was in the order of MHCIIId less than MHCIIa less than MHCI, which was partially counteracted by elevated neuromuscular activity and showed a similar relationship for the slow MHCI and the slow MLC isoforms (Kirschbaum *et al.* 1990). One limitation of this current study is that we did not collect and specifically preserve the muscle samples for muscle fiber type analysis. As a preliminary study, we would like to perform initial assessments and comparison on the mitigation effects of DEMS and DHS on muscle atrophy under disuse condition. We will expand our research scope into muscle fiber type responses and systemic assessments (e.g. blood circulation) to further compare the effects and mechanisms of DEMS and DHS.

In conclusion, 10 min of daily DEMS to the quadriceps at 50 Hz showed an increase averaged cross-sectional quadriceps fiber area, compared to 1 Hz and 100 Hz. The variation of stimulation frequency has impacted on the morphological change of the muscle fibers under disuse condition. On the other hand, no difference in muscle morphology was found between the static and 2 Hz DHS stimulations. Although the mechanism between stimulation and tissue adaptation remain unclear, these studies demonstrated the importance in considering the type of stimulation that is being applied and the changes in fluid perfusion that is being generated by such stimulation. Future investigation may target the duration of the stimulations and supplement with the appropriate stimulation frequency within the DEMS and DHS regimens. It is aimed to optimize the DHS loading conditions (e.g. frequency and magnitude) effective on muscle and to determine the downstream mechanisms.

### Conflict of Interest

There is no conflict of interest.

### Acknowledgements

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