Published in final edited form as:

Exp Gerontol. 2019 January ; 115: 19–31. doi:10.1016/j.exger.2018.11.011.

Effects of Hindlimb Suspension and Reloading on Gastrocnemius and Soleus Muscle Mass and Function in Geriatric Mice

João Ricardhis S. Oliveira^{4,5}, Junaith S. Mohamed^{3,5}, Matthew Myers⁵, Matthew J. Brooks⁵, and Stephen E. Alway^{1,2,5}

¹Dept. of Physical Therapy, College of Health Professions, and the University of Tennessee Health Sciences Center, Memphis, TN 38163

²Department of Physiology, College of Medicine, University of Tennessee Health Sciences Center, Memphis, TN 38163

³Department of Clinical Laboratory Sciences, University of Tennessee Health Sciences Center, Memphis, TN 38163

⁴Interuniversity Exchange Undergraduate Program, CAPES Foundation, Universidade Federal de Pernambuco, Recife, PE 50670-901, Brazil

⁵Laboratory of Muscle Biology and Sarcopenia, Division of Exercise Physiology, Dept. of Human Performance & Applied Exercise Science, West Virginia University School of Medicine, Morgantown, WV 26506

Abstract

Reloading of atrophied muscles after hindlimb suspension (HLS) can induce muscle injury and prolong recovery after disuse in old rats, especially in fast contracting muscles. Less is known about the responses in mice and whether fast and slow muscles from geriatric mice will respond in a similar fashion to HLS unloading and recovery (HLS+R). Furthermore, while slow muscles undergo atrophy with disuse, they typically are more resistant to sarcopenia than fast contracting muscles. Geriatric (28 mo. of age) male C57BL/6 mice were randomly placed into 3 groups. These included HLS for 14 days (n=9), and HLS followed by 14 days of reloading recovery (HLS+R; n=9), or normal ambulatory cage controls (n=9). Control mice were not exposed to unloading. Electrically evoked maximal muscle function was assessed *in vivo* in anesthetized mice at baseline, after 14 days of HLS or HLS+R. As expected, HLS significantly reduced body weight, wet weight of gastrocnemius and soleus muscles and *in vivo* maximal force. There were no differences *in vivo* fatigability of the plantar flexor muscles and overall fiber size. There were only minor fiber type distribution and frequency distribution of fiber sizes that differ between HLS+R and control gastrocnemius and soleus muscles. Soleus muscle wet weight had recovered to control

*Correspondence and current address: S.E. Alway, Professor and Dean, College of Health Professions, University of Tennessee Health Sciences Center, Memphis, 930 Madison Avenue, Suite 622 TN 38163 salway@uthsc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

levels after reloading, but type I/IIA fibers in the soleus muscles were significantly smaller after HLS+R than control muscles. In contrast, gastrocnemius muscle wet weight did not recover to control levels after reloading. Plantar flexion muscle force (primarily influenced by the gastrocnemius muscles) did not recover in HLS+R conditions as compared to HLS conditions and both were lower than control force production. Signaling for apoptosis, autophagy and anabolic markers were not different between control and HLS+R gastrocnemius and soleus muscles in geriatric mice. These results suggest that molecular signaling does not explain attenuated ability to regain muscle wet weight, fiber size or muscle force production after HLS in geriatric mice. It is possible that fluid shifts, reduced blood flow, or shortened muscle fibers which failed to regain control lengths contributed to the attenuation of muscle wet weight after HLS and reloading and this affected force production. Further work is needed to determine if altered/loss of neural activity contributed to the inability of geriatric mice to regain gastrocnemius muscle weight and function after HLS and reloading.

Keywords

hindlimb suspension; muscle atrophy; apoptosis; autophagy

INTRODUCTION

Disuse results in muscle atrophy in response to unloading (i.e. microgravity, bed rest, or inactivity). Muscle wasting also occurs secondary to aging, inflammation, disease, poor nutrition, or fasting. Muscle disuse atrophy is characterized by decrements in muscle force, mass, and fiber cross-sectional area (CSA), and a phenotypic shift from an oxidative myosin heavy chain (MHC) isoform profile (i.e., Type I and Type IIA) to a profile consisting of more glycolytic MHC isoforms (Type IIB/X) (Alway, et al. 2015; Bennett, et al. 2013a; Egawa, et al. 2015; Fluck and Hoppeler 2003; Hanson, et al. 2010; Ohira, et al. 1992; Stelzer and Widrick 2003; Takahashi, et al. 2017). Muscle atrophy causes functional deficiency that leads to mobility impairment, decreases the quality of life in the elderly (Appell 1986; Appell, et al. 1988; Bodine 2013; Feng, et al. 2016; LeBlanc, et al. 1995; Rittweger, et al. 2005), impairs recovery and increases the risk for exercise induced injury (Kasper, et al. 1990; Reid and Moylan 2011; Widrick, et al. 2008).

The aging-associated loss of muscle mass and function, called sarcopenia, is exacerbated by increases in fat mass that causes sarcopenic obesity and mobility-related alterations (posture, gait, balance) in the aged (Alway, et al. 2017c; Alway, et al. 2011; Alway and Siu 2008; Del Campo, et al. 2018; Frontera, et al. 2000; Layne, et al. 2017; Roubenoff 2000; Thornell 2011). The societal impact of sarcopenia and sarcopenic obesity results in a poor life expectancy (Dalle, et al. 2017; Fielding, et al. 2017; Layne, et al. 2017; Wanigatunga, et al. 2017). Generally aging results in greater losses in muscles with a high type II fiber population, whereas disuse has a preferential loss of size in type I muscle fibers but both type I and type II fibers will undergo atrophy during disuse. While recovery of muscle mass after disuse-induced atrophy is feasible at young ages, it is generally attenuated in old age muscles from aged rodents (Alway, et al. 2015; Bennett, et al. 2013a; Brooks, et al. 2018; Takahashi, et al. 2017). However, the mechanisms that regulate muscle mass and function

during recovery after disuse-induced atrophy are poorly understood in fast and slow contracting muscles from geriatric mice.

Multiple pathways contribute to hindlimb suspension unloading (HLS)-induced atrophy and recovery, such as changes in apoptosis, autophagy and activation of muscle regeneration and anabolic/catabolic signaling pathways (Cannavino, et al. 2015; Darr and Schultz 1989; Egawa, et al. 2015; Joseph, et al. 2016; Marzetti, et al. 2013; Pellegrino, et al. 2011; Powers, et al. 2012; Prisby, et al. 2004; Takahashi, et al. 2017). Nevertheless, it is less clear how the signaling pathways that control unloading-induced muscle atrophy and loading-induced muscle mass are regulated at very old age and if changes in these pathways will account for any attenuation in recovery of muscle mass and/or function after recovery from disuse.

In this study, we evaluated muscle recovery in fast contracting gastrocnemius muscles and slow contracting soleus muscles of geriatric mice after hindlimb suspension (HLS) followed by recovery (HLS+R). We hypothesized that geriatric mice with severe sarcopenia would have severe muscle wasting and poor recovery of muscle mass and function upon reloading especially in fast contracting muscles.

METHODS AND EXPERIMENTAL DESIGN

Animals

Thirty-six male C57BL/6 mice (26-month-old) were obtained from the National Institute on Aging Colony (Harlan, Indianapolis, IN) and housed individually until they became geriatric (28 mo. of age). Young adult mice (16 weeks of age) C57BL/6 mice were obtained from Jackson Laboratories. The mice were maintained in standardized cages of the West Virginia University Health Science Center and the University of Tennessee Health Science Center animal facilities, which were both accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC). Young mice of same group. HLS, 14 days of HLS; HLS+R, 14 days of HLS followed by 14 days of reloading cage control ambulation. The three groups of young adult mice included: control (n=6) hindlimb suspension (HLS; n=6) and hindlimb suspension for 14 days followed by recovery for 14 days (HLS+R; n=6). The geriatric mice were controls (n=9), hindlimb suspension, (HLS, n=9), and HLS+R (n=18). As we were primarily interested in the responses of geriatric mice to unloading and reloading, the only variables assessed in the young adult mice were body weight and muscle weight. Our previous observations in aging and unloading in rodents indicated that muscle wet weight and function to hindlimb unloading has more inter-animal variability than cage control mice that do not undergo experimental intervention. Therefore, we examined more mice in the experimental HLS+R group than control mice to account for any increased in inter-animal variability and therefore to identify any statistical significance if it existed in response to loading and reloading.

Hindlimb Suspension (HLS) and Recovery after HLS (HLS+R).

HLS was conducted for 14 days as described previously (Alway, et al. 2014b; Alway, et al. 2015; Alway, et al. 2002; Alway, et al. 2013; Brooks, et al. 2018; Pistilli, et al. 2007; Takahashi, et al. 2017; Wang, et al. 2011). Briefly, the hindlimb muscles were unloaded by

placing orthopedic tape around the tail of the mice. The tape was attached to a metal swivel at the top of the cage, and the height of the swivel was adjusted to raise the hindlimbs from the floor of the cage and also allowing for 360° of rotation and movement of the animal. Food and water were provided *ad libitum* to the mice. To prevent excessive orthostatic intolerance and stress to the mice, the angle between the floor and the torso did not exceed 30°. The tip of their tails remained exposed to monitor tail blood flow. Food and water were provided *ad libitum*. The mice were euthanized after 14 days of HLS followed by 14-days of the recovery period. Cage control mice were treated similarly to HLS mice except the animals were not exposed to unloading by HLS.

Body weight

Body weight of HLS mice was assessed on Day 1, and Day 14. Body weight of reloaded mice was assessed on Day 1 (before HLS), day 14 (the last day of HLS) and day 28 (the last day of recovery).

Muscle function

To assess the effects of unloading and reloading, muscle function was measured longitudinally *in vivo*, in anesthetized geriatric mice at baseline, after 14 days of HLS and after 14 days of recovery following reloading after HLS. Electrically evoked contractions were assessed in anesthetized mice with a mixture of oxygen (97%) and isoflurane gas (3%) using a small animal anesthetic system (Isotec 5, Datex-Ohmeda, USA). The left hip was stabilized by placing a small animal rod on the lateral side of the knee. The left foot was secured to a footplate connected to a servomotor (300C-LR; Aurora Scientific Aurora, Canada). The ankle joint was aligned with the axis of rotation of the servomotor. Electrically evoked contractions of the plantar flexor muscles were made (10Hz, 25Hz, 50Hz, 75Hz, 100Hz, and 120Hz, 200 μ s pulses) of the tibial nerve, using a High-Power Bi-Phase Current Stimulator (701C; Aurora Scientific, Aurora, Canada). Stimulation of the tibial nerve occurred by inserting platinum electrodes (F-E7; Grass Instruments, Astro-Med, Inc., USA) through the skin so that they were positioned on either side of the nerve. Three minutes of rest occurred between each contraction to minimize the effect of fatigue on production of maximal muscle force. The Data Acquisition and Analysis System (605A; Aurora Scientific, Canada) was used to control the servomotor providing for the angular positions of the foot. Data files were analyzed by the Dynamic Muscle Analysis Software (615A, Aurora Scientific Inc., Canada). Plantar flexor muscle functional data were normalized to muscle weight. Repeated measures ANOVA was used to compare plantar flexor maximal force production in mice before, after HLS and after HLS+R conditions.

To assess muscle fatigability, the tibial nerve was stimulated at 40 Hz for 3 minutes utilizing a duty cycle of 330 ms of stimulation followed by 670 ms of rest (Brooks, et al. 2018; Chen and Alway 2001; Mohamed, et al. 2014). The Fatigue Index (FI) was calculated as the force from the last contraction expressed as a percentage of the force obtained on the first contraction. Differences in FI were compared by ANOVA analyses.

Tissue weight and preparation

At the end of the experimental period, and with the mice deeply anesthetized, the gastrocnemius and soleus muscles were removed from both limbs, blotted, and then weighed. The mice were euthanized by removing their hearts. The mid-belly of the soleus and gastrocnemius muscle was embedded in optimal cutting temperature compound (Tissue-Tek; Andwin Scientific, USA), snap frozen in liquid nitrogen cooled isopentane, and stored at -80°C for subsequent analyses.

Fiber morphology

Frozen cross-sections were cut from the mid-belly from soleus and gastrocnemius muscles on a freezing microtome (CM3050; Leica, Germany). The tissue sections were stained with hematoxylin and eosin. Average muscle fiber cross-sectional area (CSA) was determined by planimetry from 750–1200 fibers that were obtained from four non-overlapping regions of each tissue cross-section. Muscle fiber CSA was calculated using a public domain, Java-based image processing program (Image J; National Institutes of Health, USA) as published previously (Alway, et al. 2015; Alway, et al. 2017a; Alway, et al. 2013; Roman, et al. 1993). Differences between control and HLS+R fiber areas in geriatric mice were determined by One-Way ANOVA analyses.

Immunocytochemical staining for myosin heavy chain

Eight micron-thick frozen tissue cross-sections were placed on charged microscope slides (Superfrost Plus; ThermoFisher Scientific, USA), air-dried, and stored at -20°C until used for immunohistochemistry. The sections were washed in phosphate buffered saline (PBS) then incubated with 10% normal goat serum (S-1000; Vector Laboratories, USA) for 1hr to reduce non-specific protein binding. To detect fiber myosin heavy chain (MHC) isoforms, the tissue sections were incubated overnight in primary mouse antibodies for MHC type I (BA-D5; Developmental Studies Hybridoma Bank, USA), MHC type IIA (SC-71; Developmental Studies Hybridoma Bank, USA), and MHC type IIB (BF-F3; Developmental Studies Hybridoma Bank). After three washes in PBS, the sections were incubated with an appropriate secondary antibody labeled with Alexa Fluor- 488, Alexa Fluor-546 and Alexa Fluor-647 (Invitrogen-ThermoFisher) for 1h at room temperature followed by 3 washes in PBS. The tissue sections were counterstained with 4',6-Diamidino-2-phenylindole, dihydrochloride (DAPI) (P36941; Invitrogen-ThermoFisher, USA) to visualize all of the myonuclei. Tissue sections were imaged using a confocal microscope (LSM 710; Zeiss, Germany).

Western immunoblots

Approximately 75 mg of gastrocnemius muscle or ~6 mg of soleus muscle was homogenized in RIPA buffer (1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 10 mM Tris; pH 7.4) containing protease and phosphatase inhibitor (I3746; Sigma-Aldrich, St. Louis, USA). Protein concentration was measured using the BioRad DC protein assay (5000111; BioRad, Hercules, USA). Forty micrograms of protein was loaded into 4–12% gradient SDS-PAGE gel (NuPAGE-NP03222BOX; Invitrogen-ThermoFisher, USA) and separated using 120 V for 1 h followed by transfer to a nitrocellulose membrane for 1.5 h at 25 V.

Non-specific protein binding was blocked by incubating the membranes in 5% nonfat dry milk powder (9999, Cell Signaling Tech. USA) dissolved in TBS (SIG-32391; BioLegend, USA) containing 0.05% Tween 20 (TBST) (P1379; Sigma-Aldrich, USA) at room temperature. The membranes were incubated overnight in the appropriate primary antibody (1:1000). All antibodies (GAPDH, LC3A/B, ATG7, LC3, GCN5, SRC-3, Sirt1, Sirt2, Sirt3 and AIF) were purchased from Cell Signaling Technology, USA. After washing in TBST, the membranes were incubated in appropriate dilutions of secondary antibodies (1:5000 diluted in blocking buffer) conjugated with horseradish peroxidase (Sigma-Aldrich, USA) for 1h at room temperature. The signals were developed using an enhanced chemiluminescent substrate (32132; Pierce-ThermoFisher, USA) and the corresponding signals were assessed using a high-resolution gel document system (G:Box Bioimaging System; Syngene, USA). Band intensity was determined and normalized to GAPDH (Image J; NIH, USA).

Statistical analysis

The results are reported as means $\pm$ SE. Differences in means between groups for age (young and geriatric) and condition (control, HLS, HLS+R) was determined by a Two-Way Analysis of Variance (ANOVA). Group comparisons of fiber areas, fiber type and signaling protein levels were determined in the geriatric mice by a One-Way, and Hotelling's T-Square test (Prism, GraphPad Software, USA). Repeated measures ANOVA was used to compare time points across each group for maximal force production. Bonferroni post hoc analyses were performed between significant means. Chi Square analysis was used to evaluate cumulative frequency and fiber area-frequency distributions of binned fiber area (Alway, et al. 2014b; Alway, et al. 2015; Alway, et al. 1990; Alway, et al. 1989a; Alway, et al. 1989b; Hao, et al. 2011; Pistilli, et al. 2014). A P-value ≤ 0.05 was considered significant.

RESULTS

Body weight.

As shown in Figure 1, 14 days of HLS significantly reduced the body weight of mice, and body weight continued to decline throughout the experimental period and was not returned to the pre-experimental level during the recovery period in mice exposed to HLS and recovery. However, body weight in geriatric control mice also had lower body weight at day 28 as compared to day 1 of the study (Figure 1).

Muscle mass.

The Two-Way ANOVA confirmed the presence of sarcopenia as shown by a significantly lower muscle weight under each experimental condition in geriatric vs. young mice ($F=170.3$, $P<0.001$). As expected, the Two-Way ANOVA revealed a significant difference for treatments (Control, HLS and HLS+R) groups ($F=1765$ $P<0.001$). Specifically, HLS significantly reduced gastrocnemius muscle weight in both young adult and geriatric mice (Table 1). Gastrocnemius muscle weight did not return to control levels over the 14 days of reloading in the geriatric mice, whereas gastrocnemius muscle weight had recovered to control levels over the same time period in young adult mice (Table 1). Furthermore, there was a significant interaction across gastrocnemius variables for the experimental condition

($F=27.34$; $P<0.001$). The soleus muscle weight was significantly less in the HLS group than either the control group or the HLS+R group. Interestingly, control soleus muscle weight and HLS+R soleus muscle weight were not different, indicating that the soleus muscle weight had recovered muscle wet weight lost during HLS by 14 days after reloading (Table 1). There was no significant interaction across soleus muscle variables for the experimental condition.

Mean muscle fiber cross-sectional area obtained from hematoxylin and eosin stained tissue show that the soleus muscle fiber areas were significantly smaller after HLS+R ($1535 \pm 51 \mu\text{m}^2$) compared to the mean fiber area of control soleus muscle ($1745 \pm 43 \mu\text{m}^2$) (Figure 2A). There was a significantly higher frequency of smaller fibers that were less than $1800 \mu\text{m}^2$ in soleus muscle fibers of HLS+R vs. control mice (Figure 2B, 2C). However, there were no statistically significant differences in the mean muscle fiber cross-sectional area (Figure 2D), the cumulative frequency (Figure 2E) or the fiber area-frequency distributions of gastrocnemius muscles (Figure 2F).

Myosin Heavy Chain Distribution.

While hematoxylin and eosin staining provide a good approach for examining overall muscle morphology, it does not differentiate between muscle fibers with different myosin heavy chain composition. Therefore, we utilized immunocytochemistry to identify if one of the major fiber sub-types or hybrids of fibers, that co-expressed more than one myosin heavy chain (MHC) were differentially affected by unloading and reloading in geriatric mice. We wanted to make these comparisons because HLS has been reported to cause oxidative-to-glycolytic fiber type switching as well as targeted atrophy of oxidative fibers (Feng, et al. 2016). To determine if these effects occurred and remained in HLS+R muscles of geriatric mice, we evaluated the fiber type distribution and fiber area responses.

MHC staining showed that the population of II/IIA and IIX fibers was greater in soleus muscles from HLS+R mice as compared to the control soleus muscle (Figure 3A-C). There was a significantly greater type IIA/IIB fiber population in gastrocnemius muscles from HLS+R mice (Figure 3D-F).

Fiber cumulative and area-frequency distribution of fiber areas by fiber type.

The mean fiber area (Figure 4A), the cumulative frequency distribution (Figure 4B), and the fiber area-frequency distribution (Figure 4C) of type I fibers were similar in soleus muscles from control and HLS+R mice. In contrast, fibers that contained both type I and IIA myosin heavy chains were significantly smaller (Figure 4D) and this is also seen by a shift to the left (smaller fibers) in the cumulative frequency (Figure 4E) and fiber area-frequency distribution (Figure 4F) of HLS+R compared to control muscles. Similar to type I fibers, the mean type IIA fiber area (Figure 4G), cumulative frequency distribution (Figure 4H) and fiber area-frequency distribution (Figure 4I) were not different in HLS+R and control muscles.

In the gastrocnemius muscle, the mean fiber area (Figure 5A), the cumulative frequency distribution (Figure 5B) and the fiber area-frequency-distribution (Figure 5C) for type IIA fibers were similar in muscles from control and HLS+R mice. The mean area of fibers from

the gastrocnemius muscle that contained both type IIA and type IIB myosin heavy chains (IIA/IIB) were larger in gastrocnemius muscles from the HLS+R as compared to the control group (Figure 5D). There was also a significant shift to the right (larger fibers) of type IIA/IIB in HLS+R as compared to control muscles for fibers 800–1200 μm^2 (Figure 5E-F). However, fiber area (Figure 5G), cumulative frequency (Figure 5H), and the fiber area-frequency distributions for type IIB fibers (Figure 5I) were similar in HLS+R and control gastrocnemius muscles. Likewise, there was no difference in type IIX mean fiber area (Figure 5J), cumulative frequency (Figure 5K), or fiber area frequency distributions (Figure 5L) in control and HLS+R gastrocnemius muscles. Although representing only a small portion of the total population of fibers in the gastrocnemius muscle, type I/IIA fibers in the gastrocnemius muscle were significantly larger in the HLS+R as compared to the control muscles (Figure 5M) and this was reflected in a shift to the right of the cumulative frequency (Figure 5N) and the fiber area-frequency curves (Figure 5O). In contrast, while trending towards a mean smaller fiber cross-sectional area ($P=0.06$), type I fiber area was not statistically different in control and HLS+R gastrocnemius muscle samples (Figure 5P). Nevertheless, there is a significant shift to the left in the cumulative frequency distribution (Figure 5Q) and the fiber area-frequency distribution (Figure 5R) of type I HLS+R fibers of the gastrocnemius muscle indicating that type I fibers were smaller in the HLS+R mice as compared to muscles from control mice.

Maximal Muscle Force Production.

Maximal muscle plantar flexor force was measured at frequencies between 10–125 Hz (Figure 6). As muscle mass and bodyweight were also reduced, we examined relative muscle force (force/muscle weight) as an indicator of muscle function. Muscle force production was significantly lower in control muscles after 28 days, and there was the tendency to reduce force by 14 days of the study in control muscles (Figure 6A). In the HLS+R mice, force production at 100Hz and 125Hz was lower after HLS (Day 14) and also lower after HLS followed by 14 d of recovery in HLS+R (Day 28) as compared to pre-intervention at day 1 (Figure 6B). There was no statistical difference between maximal force production between control and HLS+R mice at submaximal and maximal frequencies of stimulation either before (Figure 6C), at day 14 (Figure 6D) or day 28 (Figure 6E). The maximal relative force was lower in control muscles at Day 14 and 28, compared to the beginning point of the study, which suggests that aging per se was responsible for the observed loss of force in the control muscles. Repeated measures ANOVA showed that maximal force decreased with unloading and reloading in experimental vs. control muscles, and muscles of the experimental animals did not recover to control levels after 14 days of reloading (Figure 6F).

Muscle Fatigue.

As expected, force declined throughout the fatigue protocol which consisted 116 repeated plantar flexor contractions that were stimulated at 40 Hz. The muscle fatigue curves did not vary over the course of the 28-day study in control mice (Figure 7A). Specifically, the fatigue responses were not statistically altered by HLS as compared to day 1, nor was there any change in fatigability after 14 days of reloading following 14 days of HLS (Figure 7B). The fatigue index was greater in HLS + R as compared to control muscles (Figure 7C).

Anabolic and Death Signaling.

Muscle fiber atrophy has been associated with increased apoptosis and decreased autophagy signaling during aging and after HLS (Alway, et al. 2014b; Alway, et al. 2013; Pistilli, et al. 2006; Siu and Alway 2005; Takahashi, et al. 2017). To determine if differences in protein levels of these pathways could explain the attenuated recovery of muscle mass or force production in muscles of geriatric mice, we used western blot analysis to quantify the relative abundance in a subset of signaling proteins in apoptotic, anabolic, and autophagy pathways. Although there were differences in protein abundance between soleus and gastrocnemius muscles, ANOVA analyses showed that there were no significant differences in proteins of autophagy or apoptotic pathways in control compared to HLS+R muscles (Figure 8).

DISCUSSION

Several studies have shown an attenuation of muscle recovery in fast and slow contracting muscles of aged rats after disuse-induced atrophy (Alway, et al. 2014a; Alway, et al. 2015; Alway, et al. 2013; Bennett, et al. 2013b; White, et al. 2015) but less was known about the recovery of muscle lost through disuse in aged mice. As mice are heavily used in aging research it was important to know if age slows or prevents muscle recovery in a universal fashion as it does in rats. This study evaluated if geriatric mice with severe sarcopenia would fail to recovery lost muscle mass and function upon muscle reloading after HLS-induced atrophy. In our study, sarcopenia was observed in both soleus and gastrocnemius muscles. We found that after 14 days of reloading after HLS, muscle mass, and muscle function had not returned to control levels in the gastrocnemius muscle of geriatric mice, but this could not be accounted for by altered signaling for autophagy or apoptosis. In contrast to the gastrocnemius muscle, the soleus muscle weight fully recovered muscle mass after the 14-day reloading period.

Muscle atrophy and recovery

Similar to studies in rats, this study in geriatric mice found that 14 days of HLS resulted in a significant loss of hindlimb gastrocnemius and soleus muscle mass. Unlike the young mice, gastrocnemius muscle wet in the geriatric mice of this study failed to fully recover muscle wet weight after reloading, whereas soleus muscle mass had recovered wet weight to control levels 14 days after reloading following HLS. Thus, geriatric mice appeared to have a preferential delay and/or loss of recovery in muscles with a high percentage of type II fibers. While there was a trend for the soleus to have some depression in recovery of muscle weight relative to control muscle weight ($P=0.06$) after 14 days of reloading, this did not reach statistical significance. However, unlike rats, type II fibers make up >40% of soleus muscle fibers, so recovery of soleus muscle mass indicates that both type I and type II fibers contributed to restoring soleus muscle mass after reloading. While muscle wet weight may reflect contractile tissue changes with unloading and unloading it is also affected by changes in fluid shifts (Farhat, et al. 2018) and this was not addressed in the current study.

Muscle fiber atrophy and recovery

While other studies in aged mice, showed improvement in cross-sectional area of both type I and type II fibers, and force after reloading (Feng, et al. 2016; Wang, et al. 2015), recovery from unloading in aged rats does not typically approach 80% of control fiber size. To determine if changes in muscle wet weight in the current study would be reflected by changes in fiber size, muscle fiber cross-sectional areas were examined. There is typically a significant loss of soleus mass with unloading in rats (Alway, et al. 2001; Chen and Alway 2000; Theilen, et al. 2018; Thomason and Booth 1990). Furthermore, type I fibers are considered to be sensitive to unloading (Thomason and Booth 1990) while more resistance to aging-associated atrophy (Baehr, et al. 2017; Daw, et al. 1988). In the current study, the soleus muscle from young adult and geriatric mice had a significant loss of muscle wet weight but muscle weight recovered to control levels after reloading. Interestingly, average fiber cross-sectional area was lower and this was accompanied by a shift to the left on the soleus cumulative fiber frequency distribution in HLS+R muscles. This apparent discrepancy between muscle wet weight and fiber size might be explained by the fact that during hindlimb unloading, the foot moves into plantarflexion, which shortens the muscle and enhances atrophy and loss of sarcomeres in the fibers of the hindlimb muscles. Muscle lengthening without a change in muscle mass results in a decrease in fiber cross-sectional area (Alway 1991; Alway, et al. 1990; Alway, et al. 1989b; Carson, et al. 1995) much like stretching an elastic band reduces the cross-sectional area of the band. Reloading would be expected to lengthen and stretch the reloaded muscles as compared to unloaded muscles. Therefore, the anabolic effects of muscle fiber lengthening (Alway 1991; Alway, et al. 1990; Alway, et al. 1989b; Carson, et al. 1995) would increase muscle wet weight by adding sarcomeres to the stretched muscles, even without a full recovery (or even a reduction) of muscle fiber cross-sectional area (Gomes, et al. 2004) so it is not surprising that there could be a shift to the left of the fiber area frequency even with a recovery of muscle wet weight. Unlike the rat soleus which is comprised of >80% type I fibers, the mouse soleus, is composed of both type I and type IIA myosin (Degens, et al. 1998; Feng, et al. 2016) which means that both type I and type IIA fibers in the fusiform soleus, would undergo stretch-induced growth after reloading. Less stretch would be expected in the pennate angled gastrocnemius muscle fibers which are also shorter and would have less individual stretch for a given overall length change in the gastrocnemius muscle than the same percent change in lengthening for the longer pennate fibers in the soleus muscle.

It was interesting to see that the cross-sectional area of soleus type I and type IIA fibers had recovered to control fiber sizes in the HLS+R groups, whereas type I fibers in the gastrocnemius muscle did not recover to control levels after reloading. One possibility is that impaired circulation may contribute to reduced muscle size and function in response to unloading and reloading (Ballak, et al. 2016; Omairi, et al. 2016; Snijders, et al. 2017). As blood borne compounds are important in muscle repair in aging (Brack, et al. 2007; Conboy, et al. 2005; Conboy and Rando 2012), reduced blood flow/capillarity would be expected to delay and/or prevent recovery of muscle mass and reduce muscle function to reloading after unloading. Indeed, satellite cell proliferation which is required during muscle repair and may be important in muscle recovery after disuse (Alway, et al. 2017b; Alway, et al. 2011; Alway, et al. 2014c; Alway, et al. 2013; Brooks, et al. 2018), is greater in fibers with a high

capillarity to fiber ratio (Joanisse, et al. 2018; Nederveen, et al. 2018). Thus, it is possible that refraction of capillarity and therefore blood flow during unloading (Desplanches, et al. 1990; Hirayama, et al. 2017) is reversed during reloading, especially in the soleus fibers, which have greater capillarity than the gastrocnemius muscle fibers (Myrhage 1978). The dissimilar responses in type I fibers in the gastrocnemius and soleus muscles might be the result of non-uniform changes in vascularity throughout skeletal muscle arteriolar networks between soleus and gastrocnemius fibers (Laughlin and Roseguini 2008) leading to different potentials for the type I fibers in gastrocnemius and soleus muscles to receive humoral anabolic compounds (e.g., myokines) during reloading.

Although overall soleus muscle analysis showed a smaller fiber CSA and a shift to the left in the frequency-area distributions (Figure 2) it is important to note that this shift appeared to be due to smaller type I/IIA fibers. This conclusion is based on a return to control muscle weight of HLS+R soleus muscles and also that soleus type I and type IIA fibers had recovered very well after reloading (Figure 4). In contrast, fiber size recovery was not universal in the soleus because the population of fibers that were comprised of both type I/IIA myosin heavy chains were significantly smaller and had a significant shift to the left in the HLS+R soleus muscles as compared to control muscles. Although this is a small population of fibers, there responses may be important because unloading induces a type I to a type II myosin heavy chain isoform shift (Ballak, et al. 2014; Brooks, et al. 2018). What was not expected was that the fibers which were presumably making this slow to fast transition (i.e. type I/IIA fibers) appeared to be unable to recover fiber size during 14 days of reloading in geriatric mice. Thus, while muscles that have a high composition of type I slow fibers are reported to be more sensitive to unloading than type II fibers (Alway, et al. 2003; Booth and Gollnick 1983; Degens, et al. 1998; Thomason and Booth 1990) the type I and IIA fibers in the soleus muscle recovered very well after reloading in geriatric mice. Nevertheless, while the type I/IIA fibers apparently did not fully recover with reloading, we cannot rule out the possibility that some of this population might have become denervated and therefore largely unable to recover from loading, but this speculation would require additional work to examine this possibility more closely.

Type IIA, IIB, IIX fibers recovered to control levels by reloading in the gastrocnemius muscles of mice. This was somewhat surprising because regrowth of type II fibers after atrophy in rats is limited in aging (Alway, et al. 2015; Degens 2010; White, et al. 2015) and we had expected this to be the same as in geriatric mice. However, in contrast to the soleus muscle, type I fiber cross-sectional area failed to recover in the gastrocnemius during reloading after HLS. As type I fibers make up almost 50% of the gastrocnemius, the failure of the gastrocnemius to fully regain muscle weight to control levels after 14 days of reloading may have been the result of a loss of type I fiber size recovery. As type I fibers have an abundance of mitochondria and unloading impairs mitochondrial function (Feng, et al. 2016), it is possible that impaired mitochondria structure and function of type I fibers in the gastrocnemius may be important for regulating several catabolic pathways (Alway, et al. 2017c; Siu and Alway 2006) and therefore catabolic signaling might prevent full recovery of type I fiber size in the gastrocnemius. However, if this is the case, it is not clear why mitochondrial signaling of type I fibers in the soleus would differ from the same fiber type in the gastrocnemius muscle. Additional experiments will be needed to determine if competing

signals for anabolism and catabolism may occur simultaneously in muscles of old mice during reloading after disuse atrophy.

A second possibility is that the atrophic type I fibers in the mouse gastrocnemius muscle were more vulnerable to reloading-induced damage to the basement membrane and this may have contributed to fragility and slowed repair in old gastrocnemius muscles (Kanazawa, et al. 2017). A third possibility is that reduced satellite cell number or function in muscles of old animals (Ballak, et al. 2015) might limit muscle growth or repair of injury due to reloading. This may be due in part to different biomechanical loading of type I fibers between the fusiform soleus muscle and the pennate fibered gastrocnemius muscles. Alternatively, it is possible that biomechanical loading differences account for the discrepancy in fiber size and muscle weight. For example, the shortened fibers of the pennate gastrocnemius muscle after HLS may not have fully lengthened with reloading (as would be the case in the fusiform soleus muscle). The shortened gastrocnemius fibers from unloading, should have the effect of increasing fiber size (like a shortened rubber band) and therefore we may have underestimated the effect of reloading on fiber size in the gastrocnemius muscle. Full recovery for some muscle fibers may require additional stimuli such as wheel running perhaps in part to increase muscle loading and lengthening or stimulate mitochondrial biogenesis in muscles of mice (Brooks, et al. 2018) to provide an adequate stimulation of satellite cell proliferation for adequate recovery. Nevertheless, we cannot rule out the possibility that fluid shifts occurring in unloading (Farhat, et al. 2018) had not normalized at the end of the reloading period and this may have contributed in part to muscle atrophy in the gastrocnemius muscle despite apparent recovery of fiber cross-sectional area.

Muscle function

Consistent with previous reports in rats (Alway, et al. 2014b; Alway, et al. 2015; Baehr, et al. 2016) longitudinal measures of plantarflexion force production in geriatric mice was clearly lower after HLS and reloading as compared with control muscles, indicating an inability to recover lost force during the reloading period. Although the loss of muscle force production may have been due to reduced contractile tissue in the HLS+R gastrocnemius muscles, as compared to control gastrocnemius muscles before HLS, we cannot rule out the possibility that the gastrocnemius muscles may have had a reduced force production, in part as a result of changes in muscle hydration and fluid shifts during the unloading and reloading periods, because function of fast contracting muscles is sensitive to changes in hydration, whereas type I fibers are not (Farhat, et al. 2018). Indeed, loss of bodyweight might be reflected in part by loss of fluid, and lean tissue.

Another important point to consider is that the model of disuse by HLS or aging per se may have caused not only muscle mass loss, but muscle damage, nerve damage and/or neuromuscular junction instability (Baehr, et al. 2016) that persists throughout 14 days of reloading. Although aging may accentuate this, the process of HLS creates a rapid loss of muscle strength that exceeds the loss of muscle mass. This drop in muscle quality is indicated by a decrease of maximal muscle force that is generated at 125 Hz, from ~5 g force/g to a drop of 3 g force/g over 28 days. This ~ 40% loss of maximal force production

over 4 weeks reflects a major loss of muscle quality and therefore could be consistent with damage (Frimel, et al. 2005). However, if muscle damage did occur by HLS, there was no attempt to activate satellite cells to repair the muscle during unloading, as we failed to find evidence for a significant increase in central nuclei from activated satellite cells. This could also mean that HLS did not induce muscle damage per se, but this does not rule out potential nerve damage or disruption to muscle innervation or damage to the neuromuscular junction in old mice (Baehr, et al. 2016). This is a potentially critically important point that should be considered when interpreting loss and recovery of force in response to HLS. Interestingly, the day-matched comparisons indicated that the loss of force per muscle mass was similarly affected between the animal groups. This also suggests that there is no evidence for differential fluid accumulation in controls and HLS mice and/or aggravated damage when the HLS is added to the electrical stimulation on day 1 to assess muscle contractile properties.

Despite lower plantarflexion force production and muscle wet weight in the gastrocnemius muscle, there were no differences in control and HLS+R muscle for SRC3, Sirt1, Sirt2, and Sirt3, which are involved in muscle cell proliferation and differentiation as part of anabolic pathways, and regulation of oxidative stress, and reducing atrophy in muscle fibers (Cheng, et al. 2016; Coste, et al. 2008; Hsu, et al. 2017; Li, et al. 2016; Qiu, et al. 2015; Sin, et al. 2015). Although aging associated changes in apoptosis and autophagy have been proposed to have roles in muscle atrophy and reduced muscle recovery after HLS in aged rats (Alway 2017; Alway, et al. 2015; Alway, et al. 2017a; Alway, et al. 2017c; Baehr, et al. 2016; Baehr, et al. 2017; Cannavino, et al. 2015; Takahashi, et al. 2017; White, et al. 2015), we did not observe improved signaling for autophagy or apoptosis of reloaded versus control muscles after HLS. For example, autophagy mRNA levels (White, et al. 2015) as well as protein levels for autophagy and apoptotic proteins ATG7, LC3 and AIF have been reported to be elevated relative to control levels in rat muscle after HLS (Takahashi, et al. 2017) but if this was the case in muscles from mice, then these proteins had returned to control levels after reloading because the autophagy markers were not different in control and HLS muscles after 14 days of reloading in the current study in muscles of old mice. These findings suggest that after 14 days of recovery following 14 days of HLS, there is no alteration of signaling for protein apoptosis/autophagy beyond basal levels. Generally, our data from geriatric mice agree with previous results from rats showing that signaling pathways or protein synthesis/degradation do not explain the blunted recovery of muscle following disuse in aging (Baehr, et al. 2016; Baehr, et al. 2017; White, et al. 2015).

In conclusion, aging per se decreases force capabilities of muscles in very old mice over the 28 days of the study in very old mice. These findings suggest that with sarcopenia, muscles in geriatric mice are already experiencing functional loss, so that disuse may have a lower overall absolute effect as compared to what would be expected from disuse associated loss in young adult mice. Recovery of soleus muscle mass in geriatric mice appears to be better than observed in old rats after unloading. However, consistent with other reports (Alway, et al. 2001; Suetta, et al. 2009), in this study, recovery of muscle mass by reloading is attenuated in the gastrocnemius of in geriatric mice. The reduced recovery in fast contracting gastrocnemius muscles in advanced ages, is compounded upon the effects of sarcopenia. However, with a lower basal muscle mass in aging, disuse may result in a lower absolute

loss of muscle mass in old mice as compared to young mice, so there may be a lesser need for recovery after disuse in advanced ages in mice. Nevertheless, additional studies are needed to identify if altered mitochondrial mechanics, fluid shifts, responses to biomechanical loading, neural damage and/or neuromuscular junction instability may account for reduced muscle function after reloading in geriatric mice.

ACKNOWLEDGMENTS

We would like to acknowledge the West Virginia University Microscope Imaging Facility, which is supported by the Mary Babb Randolph Cancer Center and NIH grant 5P20RR016440, P30RR032138/GM103488 and P20RR016477, and NIH Grant P20GM103434 to the West Virginia IDeA Network for Biomedical Research Excellence. Additional support was provided by the University of Tennessee Health Science Center Neuroscience Imaging Center and funding for this project was provided by the University of Tennessee Health Science Center.

Abbreviations

AIF	Apoptosis inducing factor
ATG	Autophagy-related gene
DAPI	4',6-Diamidino-2-phenylindole, dihydrochloride
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCN5	General control of amino acid synthesis protein 5
LC3	Microtubule-associated protein light chain 3
MHC	Myosin heavy chain
Sirt1	silent mating type information regulation 2 homolog 1
Sirt2	silent mating type information regulation 2 homolog 2
Sirt3	silent mating type information regulation 2 homolog 3
SRC-3	GCN5/steroid receptor co-activator-3

REFERENCES

- Alway SE, 1991 Perpetuation of muscle fibers after removal of stretch in the Japanese quail. *Am J Physiol Cell Physiol.* 260, C400–C408.
- Alway SE, 2017 Inflammation and Oxidative Stress Limit Adaptation to Stretch-Shortening Contractions in Aging. *Exerc Sport Sci Rev* 45, 194. [PubMed: 28704215]
- Alway SE, Bennett BT, Wilson JC, Edens NK, Pereira SL, 2014a Epigallocatechin-3-gallate improves plantaris muscle recovery after disuse in aged rats. *Exp Gerontol.* 50, 82–94. [PubMed: 24316035]
- Alway SE, Bennett BT, Wilson JC, Edens NK, Pereira SL, 2014b Epigallocatechin-3-gallate improves plantaris muscle recovery after disuse in aged rats. *Exp Gerontol* 50, 82–94. [PubMed: 24316035]
- Alway SE, Bennett BT, Wilson JC, Sperringer J, Mohamed JS, Edens NK, Pereira SL, 2015 Green tea extract attenuates muscle loss and improves muscle function during disuse, but fails to improve muscle recovery following unloading in aged rats. *J Appl Physiol* (1985). 118, 319–330. [PubMed: 25414242]
- Alway SE, Degens H, Krishnamurthy G, Chaudhrai A, 2003 Denervation stimulates apoptosis but not Id2 expression in hindlimb muscles of aged rats. *J Gerontol A Biol Sci Med Sci* 58, B687–B697.

- Alway SE, Degens H, Lowe DA, Krishnamurthy G, 2002 Increased myogenic repressor Id mRNA and protein levels in hindlimb muscles of aged rats. *Am J Physiol Regul Integr Comp Physiol.* 282, R411–R422. [PubMed: 11792650]
- Alway SE, Gonyea WJ, Davis ME, 1990 Muscle fiber formation and fiber hypertrophy during the onset of stretch-overload. *Am J Physiol Cell Physiol.* 259, C92–102.
- Alway SE, Grumbt WH, Gonyea WJ, Stray-Gundersen J, 1989a Contrasts in muscle and myofibers of elite male and female bodybuilders. *J Appl Physiol* (1985). 67, 24–31. [PubMed: 2759948]
- Alway SE, Lowe DA, Chen KD, 2001 The effects of age and hindlimb suspension on the levels of expression of the myogenic regulatory factors MyoD and myogenin in rat fast and slow skeletal muscles. *Exp Physiol.* 86, 509–517. [PubMed: 11445830]
- Alway SE, McCrory JL, Kearcher K, Vickers A, Frear B, Gilleland DL, Bonner DE, Thomas JM, Donley DA, Lively MW, Mohamed JS, 2017a Resveratrol enhances exercise-induced cellular and functional adaptations of skeletal muscle in older men and women. *J Gerontol A Biol.Sci Med Sci.* 72, 1595–1606. [PubMed: 28505227]
- Alway SE, McCrory JL, Kearcher K, Vickers A, Frear B, Gilleland DL, Bonner DE, Thomas JM, Donley DA, Lively MW, Mohamed JS, 2017b Resveratrol Enhances Exercise-Induced Cellular and Functional Adaptations of Skeletal Muscle in Older Men and Women. *J Gerontol A Biol Sci Med Sci.* 72, 1595–1606. [PubMed: 28505227]
- Alway SE, Mohamed JS, Myers MJ, 2017c Mitochondria Initiate and Regulate Sarcopenia. *Exerc Sport Sci Rev.* 45, 58–69. [PubMed: 28098577]
- Alway SE, Morissette MR, Siu PM, 2011 Aging and apoptosis in muscle. *Handbook of the Biology of Aging* 7th, 63–118.
- Alway SE, Myers MJ, Mohamed JS, 2014c Regulation of satellite cell function in sarcopenia. *Frontiers of Aging Neuroscience.* 6, 1–15.
- Alway SE, Pereira SL, Edens NK, Hao Y, Bennett BT, 2013 B-Hydroxy-B-methylbutyrate (HMB) enhances the proliferation of satellite cells in fast muscles of aged rats during recovery from disuse atrophy. *Exp Gerontol* 48, 973–984. [PubMed: 23832076]
- Alway SE, Siu PM, 2008 Nuclear Apoptosis Contributes to Sarcopenia. *Exerc Sport Sci Rev.* 36, 51–57. [PubMed: 18362685]
- Alway SE, Winchester PK, Davis ME, Gonyea WJ, 1989b Regionalized adaptations and muscle fiber proliferation in stretch- induced enlargement. *J Appl Physiol* (1985). 66, 771–781. [PubMed: 2523374]
- Appell HJ, 1986 Skeletal muscle atrophy during immobilization. *Int J Sports Med.* 7, 1–5.
- Appell HJ, Forsberg S, Hollmann W, 1988 Satellite cell activation in human skeletal muscle after training: evidence for muscle fiber neof ormation. *Int.J.Sports Med.* 9, 297–299. [PubMed: 3182162]
- Baehr LM, West DW, Marcotte G, Marshall AG, De Sousa LG, Baar K, Bodine SC, 2016 Age-related deficits in skeletal muscle recovery following disuse are associated with neuromuscular junction instability and ER stress, not impaired protein synthesis. *Aging (Albany NY).* 8, 127–146. [PubMed: 26826670]
- Baehr LM, West DWD, Marshall AG, Marcotte GR, Baar K, Bodine SC, 2017 Muscle-specific and age-related changes in protein synthesis and protein degradation in response to hindlimb unloading in rats. *J Appl Physiol* (1985). 122, 1336–1350. [PubMed: 28336537]
- Ballak SB, Buse-Pot T, Harding PJ, Yap MH, Deldicque L, de HA, Jaspers RT, Degens H, 2016 Blunted angiogenesis and hypertrophy are associated with increased fatigue resistance and unchanged aerobic capacity in old overloaded mouse muscle. *Age (Dordr.).* 38, 39. [PubMed: 26970774]
- Ballak SB, Degens H, de HA, Jaspers RT, 2014 Aging related changes in determinants of muscle force generating capacity: a comparison of muscle aging in men and male rodents. *Ageing Res.Rev.* 14, 43–55. [PubMed: 24495393]
- Ballak SB, Jaspers RT, Deldicque L, Chalil S, Peters EL, de HA, Degens H, 2015 Blunted hypertrophic response in old mouse muscle is associated with a lower satellite cell density and is not alleviated by resveratrol. *Exp.Gerontol.* 62, 23–31. [PubMed: 25562814]

- Bennett BT, Mohamed JS, Alway SE, 2013a Effects of resveratrol on the recovery of muscle mass following disuse in the plantaris muscle of aged rats. *PLoS One* 8, e83518. [PubMed: 24349525]
- Bennett BT, Mohamed JS, Alway SE, 2013b Effects of resveratrol on the recovery of muscle mass following disuse in the plantaris muscle of aged rats. *PLoS One*. 8, e83518. [PubMed: 24349525]
- Bodine SC, 2013 Disuse-induced muscle wasting. *Int J Biochem Cell Biol*. 45, 2200–2208. [PubMed: 23800384]
- Booth FW, Gollnick PD, 1983 Effects of disuse on the structure and function of skeletal muscle. *Med Sci Sports Exerc*. 15, 415–420. [PubMed: 6645872]
- Brack AS, Conboy MJ, Roy S, Lee M, Kuo CJ, Keller C, Rando TA, 2007 Increased Wnt signaling during aging alters muscle stem cell fate and increases fibrosis. *Science*. 317, 807–810. [PubMed: 17690295]
- Brooks MJ, Hajira A, Mohamed JS, Alway SE, 2018 Voluntary wheel running increases satellite cell abundance and improves recovery from disuse in gastrocnemius muscles from mice. *J Appl Physiol* (1985). 124, 1616–1628. [PubMed: 29470148]
- Cannavino J, Brocca L, Sandri M, Grassi B, Bottinelli R, Pellegrino MA, 2015 The role of alterations in mitochondrial dynamics and PGC-1 α over-expression in fast muscle atrophy following hindlimb unloading. *J Physiol*. 593, 1981–1995. [PubMed: 25565653]
- Carson JA, Yamaguchi M, Alway SE, 1995 Hypertrophy and proliferation of skeletal muscle fibers from aged quail. *J Appl Physiol* (1985). 78, 293–299. [PubMed: 7713826]
- Chen KD, Alway SE, 2000 A physiological level of clenbuterol does not prevent atrophy or loss of force in skeletal muscle of old rats. *J Appl Physiol* (1985). 89, 606–612. [PubMed: 10926644]
- Chen KD, Alway SE, 2001 Clenbuterol reduces soleus muscle fatigue during disuse in aged rats. *Muscle Nerve*. 24, 211–222. [PubMed: 11180204]
- Cheng A, Yang Y, Zhou Y, Maharana C, Lu D, Peng W, Liu Y, Wan R, Marosi K, Misiak M, Bohr VA, Mattson MP, 2016 Mitochondrial SIRT3 Mediates Adaptive Responses of Neurons to Exercise and Metabolic and Excitatory Challenges. *Cell Metab*. 23, 128–142. [PubMed: 26698917]
- Conboy IM, Conboy MJ, Wagers AJ, Girma ER, Weissman IL, Rando TA, 2005 Rejuvenation of aged progenitor cells by exposure to a young systemic environment. *Nature*. 433, 760–764. [PubMed: 15716955]
- Conboy IM, Rando TA, 2012 Heterochronic parabiosis for the study of the effects of aging on stem cells and their niches. *Cell Cycle*. 11, 2260–2267. [PubMed: 22617385]
- Coste A, Louet JF, Lagouge M, Lerin C, Antal MC, Meziane H, Schoonjans K, Puigserver P, O'Malley BW, Auwerx J, 2008 The genetic ablation of SRC-3 protects against obesity and improves insulin sensitivity by reducing the acetylation of PGC-1 α . *Proc Natl Acad Sci USA*. 105, 17187–17192. [PubMed: 18957541]
- Dalle S, Rossmeislova L, Koppo K, 2017 The Role of Inflammation in Age-Related Sarcopenia. *Front Physiol*. 8, 1045. [PubMed: 29311975]
- Darr KC, Schultz E, 1989 Hindlimb suspension suppresses muscle growth and satellite cell proliferation. *J Appl Physiol* (1985). 67, 1827–1834. [PubMed: 2600016]
- Daw CK, Starnes JW, White TP, 1988 Muscle atrophy and hypoplasia with aging: impact of training and food restriction. *J Appl Physiol* (1985). 64, 2428–2432. [PubMed: 3403425]
- Degens H, 2010 The role of systemic inflammation in age-related muscle weakness and wasting. *Scand J Med Sci Sports*. 20, 28–38. [PubMed: 19804579]
- Degens H, Yu F, Li X, Larsson L, 1998 Effects of age and gender on shortening velocity and myosin isoforms in single rat muscle fibres. *Acta Physiol Scand*. 163, 33–40. [PubMed: 9648621]
- Del Campo A, Contreras-Hernandez I, Castro-Sepulveda M, Campos CA, Figueroa R, Tevy MF, Eisner V, Casas M, Jaimovich E, 2018 Muscle function decline and mitochondria changes in middle age precede sarcopenia in mice. *Aging (Albany NY)*. 10, 34–55. [PubMed: 29302020]
- Desplanches D, Kayar SR, Sempore B, Flandrois R, Hoppeler H, 1990 Rat soleus muscle ultrastructure after hindlimb suspension [published erratum appears in *J Appl Physiol* 1991 Dec; 71(6):following Author Index]. *J Appl Physiol*. 69, 504–508. [PubMed: 2228859]
- Egawa T, Goto A, Ohno Y, Yokoyama S, Ikuta A, Suzuki M, Sugiura T, Ohira Y, Yoshioka T, Hayashi T, Goto K, 2015 Involvement of AMPK in regulating slow-twitch muscle atrophy during hindlimb unloading in mice. *Am J Physiol Endocrinol Metab*. 309, E651–E662. [PubMed: 26244519]

- Farhat F, Grosset JF, Canon F, 2018 Water deprivation decreases strength in fast twitch muscle in contrast to slow twitch muscle in rat. *Acta Physiol (Oxf)*, e13072.
- Feng HZ, Chen X, Malek MH, Jin JP, 2016 Slow recovery of the impaired fatigue resistance in postunloading mouse soleus muscle corresponding to decreased mitochondrial function and a compensatory increase in type I slow fibers. *Am J Physiol Cell Physiol*. 310, C27–C40. [PubMed: 26447205]
- Fielding RA, Guralnik JM, King AC, Pahor M, McDermott MM, Tudor-Locke C, Manini TM, Glynn NW, Marsh AP, Axtell RS, Hsu FC, Rejeski WJ, group L.s., 2017 Dose of physical activity, physical functioning and disability risk in mobility-limited older adults: Results from the LIFE study randomized trial. *PLoS One*. 12, e0182155. [PubMed: 28820909]
- Fluck M, Hoppeler H, 2003 Molecular basis of skeletal muscle plasticity--from gene to form and function. *Rev Physiol Biochem Pharmacol*. 146, 159–216. [PubMed: 12605307]
- Frimel TN, Walter GA, Gibbs JD, Gaidosh GS, Vandenborne K, 2005 Noninvasive monitoring of muscle damage during reloading following limb disuse. *Muscle Nerve*. 32, 605–612. [PubMed: 16003743]
- Frontera WR, Suh D, Krivickas LS, Hughes VA, Goldstein R, Roubenoff R, 2000 Skeletal muscle fiber quality in older men and women. *Am J Physiol Cell Physiol*. 279, C611–C618. [PubMed: 10942711]
- Gomes AR, Coutinho EL, Franca CN, Polonio J, Salvini TF, 2004 Effect of one stretch a week applied to the immobilized soleus muscle on rat muscle fiber morphology. *Braz J Med Biol Res*. 37, 1473–1480. [PubMed: 15448867]
- Hanson AM, Stodieck LS, Cannon CM, Simske SJ, Ferguson VL, 2010 Seven days of muscle reloading and voluntary wheel running following hindlimb suspension in mice restores running performance, muscle morphology and metrics of fatigue but not muscle strength. *J Muscle Res Cell Motil*. 31, 141–153. [PubMed: 20632203]
- Hao Y, Jackson JR, Wang Y, Edens N, Pereira SL, Alway SE, 2011 B -Hydroxy-B-methylbutyrate reduces myonuclear apoptosis during recovery from hind limb suspension-induced muscle fiber atrophy in aged rats. *Am J Physiol Regul Integr Comp Physiol*. 301, R701–R715. [PubMed: 21697520]
- Hirayama Y, Nakanishi R, Maeshige N, Fujino H, 2017 Preventive effects of nucleoprotein supplementation combined with intermittent loading on capillary regression induced by hindlimb unloading in rat soleus muscle. *Physiol Rep*. 5.
- Hsu YJ, Hsu SC, Hsu CP, Chen YH, Chang YL, Sadoshima J, Huang SM, Tsai CS, Lin CY, 2017 Sirtuin 1 protects the aging heart from contractile dysfunction mediated through the inhibition of endoplasmic reticulum stress-mediated apoptosis in cardiac-specific Sirtuin 1 knockout mouse model. *Int J Cardiol*. 228, 543–552. [PubMed: 27875732]
- Joanisse S, Snijders T, Nederveen JP, Parise G, 2018 The Impact of Aerobic Exercise on the Muscle Stem Cell Response. *Exerc Sport Sci Rev* 46, 180–187. [PubMed: 29664744]
- Joseph AM, Adhihetty PJ, Leeuwenburgh C, 2016 Beneficial effects of exercise on age-related mitochondrial dysfunction and oxidative stress in skeletal muscle. *J Physiol*. 594, 5105–5123. [PubMed: 26503074]
- Kanazawa Y, Ikegami K, Sujino M, Koinuma S, Nagano M, Oi Y, Onishi T, Sugiyo S, Takeda I, Kaji H, Shigeyoshi Y, 2017 Effects of aging on basement membrane of the soleus muscle during recovery following disuse atrophy in rats. *Exp Gerontol*. 98, 153–161. [PubMed: 28803135]
- Kasper CE, White TP, Maxwell LC, 1990 Running during recovery from hindlimb suspension induces transient muscle injury. *J Appl Physiol* (1985). 68, 533–539. [PubMed: 2318764]
- Laughlin MH, Roseguini B, 2008 Mechanisms for exercise training-induced increases in skeletal muscle blood flow capacity: differences with interval sprint training versus aerobic endurance training. *J Physiol Pharmacol*. 59 Suppl 7, 71–88. [PubMed: 19258658]
- Layne AS, Hsu FC, Blair SN, Chen SH, Dungan J, Fielding RA, Glynn NW, Hajduk AM, King AC, Manini TM, Marsh AP, Pahor M, Pellegrini CA, Buford TW, Investigators LS, 2017 Predictors of Change in Physical Function in Older Adults in Response to Long-Term, Structured Physical Activity: The LIFE Study. *Arch Phys Med Rehabil*. 98, 11–24 e13. [PubMed: 27568165]

- LeBlanc A, Rowe R, Schneider V, Evans H, Hedrick T, 1995 Regional muscle loss after short duration spaceflight. *Aviat Space Environ Med.* 66, 1151–1154. [PubMed: 8747608]
- Li FH, Yu HT, Xiao L, Liu YY, 2016 Response of BAX, Bcl-2 Proteins, and SIRT1/PGC-1 α mRNA Expression to 8-Week Treadmill Running in the Aging Rat Skeletal Muscle. *Adv.Exp.Med Biol.* 923, 283–289. [PubMed: 27526155]
- Marzetti E, Csiszar A, Dutta D, Balagopal G, Calvani R, Leeuwenburgh C, 2013 Role of mitochondrial dysfunction and altered autophagy in cardiovascular aging and disease: from mechanisms to therapeutics. *Am.J Physiol Heart Circ.Physiol.* 305, H459–H476. [PubMed: 23748424]
- Mohamed JS, Wilson JC, Myers MJ, Sisson KJ, Alway SE, 2014 Dysregulation of SIRT-1 in aging mice increases skeletal muscle fatigue by a PARP-1-dependent mechanism. *Aging (Albany NY).* 10, 820–834.
- Myrhaage R, 1978 Capillary supply of the muscle fibre population in hindlimb muscles of the cat. *Acta Physiol Scand.* 103, 19–30. [PubMed: 665257]
- Nederveen JP, Joannis S, Snijders T, Thomas ACQ, Kumbhare D, Parise G, 2018 The influence of capillarization on satellite cell pool expansion and activation following exercise-induced muscle damage in healthy young men. *J Physiol.* 596, 1063–1078. [PubMed: 29315567]
- Ohira Y, Jiang B, Roy RR, Oganov V, Ilyina-Kakueva E, Marini JF, Edgerton VR, 1992 Rat soleus muscle fiber responses to 14 days of spaceflight and hindlimb suspension. *J Appl Physiol (1985).* 73, 51S–57S. [PubMed: 1388148]
- Omairi S, Matsakas A, Degens H, Kretz O, Hansson KA, Solbra AV, Bruusgaard JC, Joch B, Sartori R, Giallourou N, Mitchell R, Collins-Hooper H, Foster K, Pasternack A, Ritvos O, Sandri M, Narkar V, Swann JR, Huber TB, Patel K, 2016 Enhanced exercise and regenerative capacity in a mouse model that violates size constraints of oxidative muscle fibres. *Elife.* 5.
- Pellegrino MA, Desaphy JF, Brocca L, Pierno S, Camerino DC, Bottinelli R, 2011 Redox homeostasis, oxidative stress and disuse muscle atrophy. *J Physiol.* 589, 2147–2160. [PubMed: 21320887]
- Pistilli EE, Alway SE, Hollander JM, Wimsatt JH, 2014 Aging alters contractile properties and fiber morphology in pigeon skeletal muscle. *J Comp Physiol B.* 184, 1031–1039. [PubMed: 25150060]
- Pistilli EE, Siu PM, Alway SE, 2006 Molecular regulation of apoptosis in fast plantaris muscles of aged rats. *J Gerontol A Biol Sci Med Sci* 61, 245–255. [PubMed: 16567372]
- Pistilli EE, Siu PM, Alway SE, 2007 Interleukin-15 responses to aging and unloading-induced skeletal muscle atrophy. *Am J Physiol Cell Physiol.* 292, C1298–C1304. [PubMed: 17135303]
- Powers SK, Smuder AJ, Judge AR, 2012 Oxidative stress and disuse muscle atrophy: cause or consequence? *Curr Opin Clin Nutr Metab Care.* 15, 240–245. [PubMed: 22466926]
- Prisby RD, Nelson AG, Latsch E, 2004 Eccentric exercise prior to hindlimb unloading attenuated reloading muscle damage in rats. *Aviat Space Environ Med.* 75, 941–946. [PubMed: 15558992]
- Qiu G, Li X, Che X, Wei C, He S, Lu J, Jia Z, Pang K, Fan L, 2015 SIRT1 is a regulator of autophagy: Implications in gastric cancer progression and treatment. *FEBS Lett.* 589, 2034–2042. [PubMed: 26049033]
- Reid MB, Moylan JS, 2011 Beyond atrophy: redox mechanisms of muscle dysfunction in chronic inflammatory disease. *J Physiol.* 589, 2171–2179. [PubMed: 21320886]
- Rittweger J, Frost HM, Schiessl H, Ohshima H, Alkner B, Tesch P, Felsenberg D, 2005 Muscle atrophy and bone loss after 90 days' bed rest and the effects of flywheel resistive exercise and pamidronate: results from the LTBR study. *Bone.* 36, 1019–1029. [PubMed: 15811637]
- Roman WJ, Fleckenstein J, Stray-Gundersen J, Alway SE, Peshock R, Gonyea WJ, 1993 Adaptations in the elbow flexors of elderly males after heavy- resistance training. *J Appl Physiol (1985).* 74, 750–754. [PubMed: 8458791]
- Roubenoff R, 2000 Sarcopenic obesity: does muscle loss cause fat gain? Lessons from rheumatoid arthritis and osteoarthritis. *Ann NY Acad Sci.* 904, 553–557. [PubMed: 10865804]
- Sin TK, Yu AP, Yung BY, Yip SP, Chan LW, Wong CS, Rudd JA, Siu PM, 2015 Effects of long-term resveratrol-induced SIRT1 activation on insulin and apoptotic signalling in aged skeletal muscle. *Acta Diabetol.* 52, 1063–1075. [PubMed: 25959421]
- Siu PM, Alway SE, 2005 Id2 and p53 participate in apoptosis during unloading-induced muscle atrophy. *Am J Physiol Cell Physiol.* 288, C1058–C1073. [PubMed: 15601750]

- Siu PM, Alway SE, 2006 Aging alters the reduction of pro-apoptotic signaling in response to loading-induced hypertrophy. *Exp Gerontol* 41, 175–188. [PubMed: 16377111]
- Snijders T, Nederveen JP, Joannis S, Leenders M, Verdijk LB, van Loon LJ, Parise G, 2017 Muscle fibre capillarization is a critical factor in muscle fibre hypertrophy during resistance exercise training in older men. *J Cachexia Sarcopenia Muscle*. 8, 267–276. [PubMed: 27897408]
- Stelzer JE, Widrick JJ, 2003 Effect of hindlimb suspension on the functional properties of slow and fast soleus fibers from three strains of mice. *J Appl Physiol* (1985). 95, 2425–2433. [PubMed: 12949008]
- Suetta C, Hvid LG, Justesen L, Christensen U, Neergaard K, Simonsen L, Ortenblad N, Magnusson SP, Kjaer M, Aagaard P, 2009 Effects of aging on human skeletal muscle after immobilization and retraining. *J Appl Physiol* (1985). 107, 1172–1180. [PubMed: 19661454]
- Takahashi H, Suzuki Y, Mohamed JS, Gotoh T, Pereira SL, Alway SE, 2017 Epigallocatechin-3-gallate increases autophagy signaling in resting and unloaded plantaris muscles but selectively suppresses autophagy protein abundance in reloaded muscles of aged rats. *Exp Gerontol*. 92, 56–66. [PubMed: 28286171]
- Theilen NT, Jeremic N, Weber GJ, Tyagi SC, 2018 Exercise Preconditioning Diminishes Skeletal Muscle Atrophy after Hindlimb Suspension in Mice. *J Appl Physiol* (1985).
- Thomason DB, Booth FW, 1990 Atrophy of the soleus muscle by hindlimb unweighting. *J Appl Physiol* (1985). 68, 1–12. [PubMed: 2179205]
- Thornell LE, 2011 Sarcopenic obesity: satellite cells in the aging muscle. *Curr Opin Clin Nutr Metab Care*. 14, 22–27. [PubMed: 21088571]
- Wang F, Zhang P, Liu H, Fan M, Chen X, 2015 Proteomic analysis of mouse soleus muscles affected by hindlimb unloading and reloading. *Muscle Nerve*. 52, 803–811. [PubMed: 25656502]
- Wang Y, Hao Y, Alway SE, 2011 Suppression of GSK-3 β activation by M-cadherin protects myoblasts against mitochondria-associated apoptosis during myogenic differentiation. *J Cell Sci*. 124, 3835–3847. [PubMed: 22114306]
- Wanigatunga AA, Tudor-Locke C, Axtell RS, Glynn NW, King AC, McDermott MM, Fielding RA, Lu X, Pahor M, Manini TM, 2017 Effects of a Long-Term Physical Activity Program on Activity Patterns in Older Adults. *Med Sci Sports Exerc*. 49, 2167–2175. [PubMed: 29045323]
- White JR, Confides AL, Moore-Reed S, Hoch JM, Dupont-Versteegden EE, 2015 Regrowth after skeletal muscle atrophy is impaired in aged rats, despite similar responses in signaling pathways. *Exp Gerontol*. 64, 17–32. [PubMed: 25681639]
- Widrick JJ, Maddalozzo GF, Hu H, Herron JC, Iwaniec UT, Turner RT, 2008 Detrimental effects of reloading recovery on force, shortening velocity, and power of soleus muscles from hindlimb-unloaded rats. *Am J Physiol Regul Integr Comp Physiol*. 295, R1585–R1592. [PubMed: 18753267]

Highlights

- The consequences of disuse followed by reloading were studied in geriatric mice.
- Hindlimb disuse reduced gastrocnemius and soleus muscle mass, maximal plantarflexion force production and soleus muscle type I/IIA fiber size, but reloading did not restore gastrocnemius muscle mass or force production, whereas soleus muscle weight recovered to control levels by reloading in geriatric mice
- Molecular signaling could not account for failure to regain gastrocnemius muscle mass or function because signaling for apoptosis and autophagy was similar in geriatric mice after disuse and reloading vs. control muscles.
- Disuse followed by reloading did not amplify fast or slow muscle dysfunction and loss associated with aging-induced sarcopenia.

Figure 1. Body Weight.

The body weight of control and experimental (HLS) mice were obtained before unloading (Day 1) after 14 days of unloading (Day 14), and after 14 days of recovery following reloading (Day 28). Control mice-maintained cage ambulation conditions over the 28 days of the study. Values are presented as mean $\pm$ SE. * P 0.05 vs. Control at the same experimental day. ** P 0.05 vs. Control after 28 days vs. day 1.

Figure 2. Mean fiber area, cumulative frequency distribution and fiber area-frequency histogram.

Mean muscle fiber area, cumulative frequency distribution and fiber area-frequency histograms were obtained from all of the fibers measured in hematoxylin and eosin stained tissues from soleus (A,B,C) and gastrocnemius muscles (D, E, F) from control mice (n=9), or HLS+R mice (n=18). There was a greater frequency of smaller fibers that were smaller than 1800 μm^2 in soleus muscle fibers of HLS+R vs. control mice. * $P < 0.05$, control vs. HLS +R mean fiber area.

Figure 3. Myosin heavy chain distribution.

Representative images and percent fiber distribution data for the soleus (A, B, C) and gastrocnemius (D, E, F) muscles cross-sections from 9 mice that were cage controls (A, D), or after 14 days of hindlimb suspension (HLS), followed by 14 days of normal cage ambulatory reloading activity in 18 geriatric mice (B, E). Immunohistochemically stained for myosin heavy chain (MHC): (i) DAPI (blue) to identify all myonuclei, (ii) MHC Type IIA (green), (iii) MHC Type IIB (red), (iv) MHC Type I (fuchsia), and (v) the merged image. MHC Type IIX fibers were unstained/black. Quantification for the percent fiber type distribution in the soleus (C) and the gastrocnemius (D) muscles are presented at Mean $\pm$ SEM. $P < 0.05$, HLS+R vs. control.

Figure 4. Soleus Muscle Mean Fiber Area, Cumulative Frequency Distribution and Fiber Area-Frequency Histogram.

Immunocytochemistry was used to identify fibers containing one or a combination of myosin heavy chains. Mean fiber area was determined in control muscles (n=9) and in muscles of mice (n=18) after HLS and 14 days of recovery (HLS+R) in type I (A), type I/IIA (D), type IIA (G). Data for type IIX fibers were very few (data not shown). The cumulative fiber area distribution frequency distribution is shown in type I (B), type I/IIA (E), and type IIA (H) fibers. The fiber area-frequency distribution is shown in type I (C), type I/IIA (F), and type IIA (H) fibers.

Figure 5. Gastrocnemius Muscle Mean Fiber Area and Cumulative Frequency Distribution.

Immunocytochemistry was used to identify fibers containing one or a combination of myosin heavy chains. Mean fiber area was determined in muscles from control geriatric mice (n=9) and in muscles of geriatric mice after HLS and 14 days of recovery (HLS+R) (n=18) for type IIA (A), type IIA/IIB (D), type IIB (G), type IIX (J), type I/IIA (M), and type I (P) fibers. The cumulative fiber area distribution was also determined in type IIA (B), type IIA/IIB (E), type IIB (H), type IIX (K), type I/IIA (N) and type I (Q) fibers. The fiber area-frequency distribution was also shown in type IIA (C), type IIA/IIB (F), type IIB (I), type IIX (L), type I/IIA (O) and type I (R) fibers.

Plantar Flexion Force

Figure 6. Muscle Force Production.

Maximal *in vivo* force was determined in the plantar flexor muscles and presented relative to muscle mass in control geriatric mice (n=9;**A**) and HLS+R (n=18;**B**) mice after Day 1 (**C**) Day 14 (**D**); and Day 28 (**E**) of the study. Longitudinal maximal absolute plantar flexor force was shown for the same control geriatric mice at day 1, 14 and 28 (n=9), and longitudinal maximal plantar flexor force records are given for the experimental geriatric mice (n=18) at Day 1, and after HLS, and HLS+R (**F**). *P<0.01 compared to day 1. **P<0.01 compared to day 1 of experimental animals and all days in control animals.

Figure 7. Muscle Fatigue.

The loss in muscle force was measured over 116 contractions at 40 Hz in: **(A)** the Control Group (n=9), and **(B)** the HLS+R mice (n=18). **(C)** The fatigue index (Initial force-final force in 116 contractions/initial force x 100) is shown in experimental mice before (Day 1), after 14 days of HLS (Day 14) and after 14 days of hindlimb suspension followed by 14 days of reloading (HLS+R). Control mice were examined at days 1, 14 and 28 of the study but did not undergo any intervention.

Figure 8. Western blotting of Muscle Samples.

Muscle samples were obtained from the soleus (**A**) and gastrocnemius (**B**) muscles on day 28 and evaluated for signaling proteins associated with apoptosis (AIF; Sirt1), autophagy (LC3, ATG7), anabolism and reduction of oxidative stress (Sirt1, Sirt2, Sirt3, SRC-3). Control muscles (n=9) were from mice that were cage controls with normal ambulation over 28 days. HLS+R muscles (n=18) were exposed to 14 days of HLS followed by 14 days of reloading. The signals were normalized to GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) and expressed as mean $\pm$ SEM. (**C**) Representative blots for soleus and gastrocnemius muscles from control and HLS+R mice. LC3, microtubule-associated protein-1 light chain 3; SRC3: steroid receptor coactivator-3; ATG7: autophagy-related protein-7; Sirt-1: Sirtuin 1; Sirt2: Sirtuin 2; Sirt-3: Sirtuin 3; AIF: apoptosis inducing factor.

Table 1

Muscle wet weight in control conditions and after 14 days of hindlimb suspension and 14 days of suspension followed by 14 days of reloading in young and geriatric mice

Muscle	Young mice			Geriatric mice		
	Control	HLS	HLS+R	Control	HLS	HLS+R
Gastrocnemius	152 ± 5 mg	137 [*] ± 2 mg	148 ± 2 mg	121 [†] ± 3.1 mg	109 ^{*†} ± 2.7 mg	112 ^{*†} ± 3 mg
Soleus	11.1 ± 0.4 mg	8.8 [*] ± 0.4 mg	10.4 ± 0.2 mg	8.6 [†] ± 0.4 mg	6.8 ^{*†} ± 0.4 mg	9.1 [†] ± 0.3 mg

Data were compared by a Two-Way ANOVA.

^{*} P<0.05 compared to Control Group.

^{**} Young vs. geriatric groups.

[†] P<0.05 compared to Young mice of same group. HLS, 14 days of HLS; HLS+R, 14 days of HLS followed by 14 days of reloading cage control ambulation. The three groups of young adult mice included: control (n=6) hindlimb suspension (HLS; n=6) and hindlimb suspension for 14 days followed by recovery for 14 days (HLS+R; n=6). The geriatric mice were controls (n=9), hindlimb suspension, (n=9), and HLS+R (n=18). There was a significant interaction across gastrocnemius variables for the experimental condition (F=27.34; P<0.001)