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Epigallocatechin-3-gallate improves plantaris muscle recovery after disuse in aged rats

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Abstract

Aging exacerbates muscle loss and slows the recovery of muscle mass and function after disuse. In this study we investigated the potential that epigallocatechin gallate (EGCg), an abundant catechin in green tea, would reduce signaling for apoptosis and promote skeletal muscle recovery in the fast plantaris muscle and the slow soleus muscle after hindlimb unloading (HLS) in senescent animals. Fischer 344 × Brown Norway inbred rats (age 34 mo.) received either EGCg (50 mg/kg body weight), or water daily by gavage. One group of animals received HLS for 14 days and a second group of rats received 14 days of HLS, then the HLS was removed and they recovered from this forced disuse for 2 weeks. Animals that received EGCg over the HLS followed by 14 days of recovery, had a 14% greater plantaris muscle weight ($p < 0.05$) as compared to the animals treated with the vehicle over this same period. Plantaris fiber area was greater after recovery in EGCg ($2715.2 \pm 113.8 \mu\text{m}^2$) vs. vehicle treated animals ($1953.0 \pm 41.9 \mu\text{m}^2$). In addition, activation of myogenic progenitor cells was improved with EGCg over vehicle treatment (7.5% vs. 6.2%) in the recovery animals. Compared to vehicle treatment, the apoptotic index was lower (0.24% vs. 0.52%), and the abundance of pro-apoptotic proteins Bax (–22%), and FADD (–77%) were lower in EGCg treated plantaris muscles after recovery. While EGCg did not prevent unloading-induced atrophy, it improved muscle recovery after the atrophic stimulus in fast plantaris muscles. However, this effect was muscle specific because EGCg had no major impact in reversing HLS-induced atrophy in the slow soleus muscle of old rats.

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Keywords

sarcopenia; catechin; apoptosis; muscle fiber area; muscle function; muscle atrophy

1. Introduction

The deleterious effects of prolonged muscle disuse, including a loss of muscle mass, strength, and function are exacerbated by aging (Hao *et al.*, 2011; Kortebein *et al.*, 2008). Muscle disuse in aging animals is accompanied by an increase in apoptotic signaling (Alway *et al.*, 2008; Hao *et al.*, 2011; Marzetti *et al.*, 2010; Siu *et al.*, 2005a). Resuming normal ambulation following disuse causes incomplete or failed improvement in regaining muscle function of aged animals, indicating that aging impairs muscle repair/recovery from an atrophic stimulus. This differs from young animals where recovery of muscle mass and function following disuse is usually complete within a few weeks of re-ambulation (Zarzhevsky *et al.*, 2001). Thus, strategies that reduce muscle loss during disuse and/or improve recovery of muscle force and mass following disuse are clinically relevant to aging populations, especially after periods of prolonged bed rest (i.e. hospitalization).

Epigallocatechin-3-gallate (EGCg) is the predominant catechin in green tea. EGCg has strong antioxidant and anti-inflammatory properties and it is believed to be responsible for most of the health benefits linked to green tea. Both disuse and reloading greatly increase the oxidative stress in the affected muscles (Andrianjafiniony *et al.*, 2010; Jackson *et al.*, 2010; Lawler *et al.*, 2012; Lawler *et al.*, 2003; Pellegrino *et al.*, 2011). Therefore, reducing the high basal levels of oxidative stress in aging could potentially reduce muscle loss during disuse conditions and/or improve muscle recovery during reloading (Jackson *et al.*, 2010). However, the potential for EGCg to improve muscle recovery in response to reloading following disuse in aging has not been previously examined. Recent data suggest that oxidative stress is reduced after eccentric exercise upon supplementation with green tea catechins (Haramizu *et al.*, 2011) and green tea catechins reduce the loss of soleus muscle force during a period of hindlimb suspension in mice (Ota *et al.*, 2011). In the current study we tested the hypothesis that EGCg would lower myonuclear apoptosis in the hindlimb muscles of aged rats in response to disuse and improve muscle recovery following reloading.

2. Methods

2.1 Animal care

Fischer 344 × Brown Norway (FBN) rats that were 34 months of age, were purchased from the National Institute on Aging (NIA) colony that was housed at Harlan (Indianapolis). The animals remained in the animal quarters for at least two weeks post shipment to allow them to accommodate to the new environment. The standards for animal care were consistent with the recommendations for the care of laboratory animals as advocated by the American Association for Accreditation of Laboratory Animal Care (AAALAC). All experimental procedures in this study carried approval from the Institutional Animal Care and Use Committee from the West Virginia University.

2.1 Hindlimb suspension (HLS)

The rats were randomly divided into experimental groups that included: hindlimb suspended (HLS) for 14 days (n=24), or 14 days of HLS followed by 14 days of recovery from HLS (n=24). Cage control animals were obtained for the HLS (n=12) or the recovery group (n=12). The HLS procedure was conducted for 14 days as described previously (Hao *et al.*, 2011; Pistilli *et al.*, 2007). Briefly, orthopedic tape was placed along the proximal part of the tail and then attached to a harness that was designed to provide 360° of movement around the cage. The exposed tail was monitored to ensure that it remained pink, indicating that the blood flow to the tail was not compromised. The suspension height was adjusted so that the torso angle of the rat did not exceed 30°, with respect to the cage floor. The forelimbs remained in contact with a grid floor, which allowed the animals to move, groom themselves, and obtain food and water freely. After 14 days of HLS, animals in the vehicle (n=12) and the EGCg groups (n=12) were anesthetized with 4% isoflurane. While under deep anesthesia, the animals were euthanized and the plantaris and soleus muscles were removed. In other animals, and the tail harness and tape were removed after the 14 days of HLS with the animals under anesthesia. Following recovery from anesthesia, the rats resumed cage ambulation for 14 days (recovery group). In the cage control group, the rats were allowed to move freely around their cages. The rats were housed individually. As it is likely that there is a more rapid collapse/deterioration of the skeletal muscle system at older ages, we coordinated the termination points of the experiments, so that animals in the HLS group and HLS+recovery group were sacrificed at the same biological age of 35.5 months. Thus, HLS was initiated with the animals at 35 months of age, whereas, animals in the HLS + recovery group had HLS at 34.5 months of age. This coordination of ages was important to more fully evaluate the regenerative state as a result of reloading after HLS.

2.2 Treatment with EGCg

Twenty-four animals in each experimental group received either 1 ml of EGCg (50mg/kg-Teavigo®, DSM) dissolved in distilled water or 1 ml of the vehicle (distilled water) by gavage feeding once a day. This dose was chosen from pilot studies in our lab that evaluated doses between 25 and 100 mg/kg EGCg, and 50 mg/kg resulted in the lowest dose that had the best effect on maintenance of muscle mass after hindlimb unloading and reloading. The animals in the final study were pretreated with EGCg or the vehicle for 1 week prior to HLS (48 animals; n=12/group), over the 14 days HLS period and/or over the 14 days recovery period (48 animals; n=12/group). All animals received AIN-93M diet and water ad libitum over the entire treatment period. Daily food intake was measured across groups.

2.4 Activation of myogenic stem cells during reloading

While still anesthetized during the removal of the HLS, a time-released 5-bromo-2-deoxyuridine (BrdU) pellet (0.02 mg/g body weight/day; Innovative Research, Sarasota, FL) was placed subcutaneously over the dorsum of the back of each animal (Peterson *et al.*, 2008) in the recovery group. BrdU is a thymidine analogue and therefore each myogenic stem cell that underwent proliferation during the reloading period would have incorporated the BrdU label into its DNA structure. This is a low level of BrdU that does not appear to

interfere with proliferation of myogenic cells in skeletal muscle of vertebrates (Alway *et al.*, 2003; Peterson *et al.*, 2008; Siu *et al.*, 2005b).

2.5 In vivo force measurements

Plantarflexor force measurements were made before HLS (day 0), after 14 days of HLS, and after reloading from HLS in the group of animals that were in the recovery group. All force measurements were assessed using a custom built rat dynamometer (Cutlip *et al.*, 1997), while the animals were anesthetized with a mixture of 95% oxygen and 4% isoflurane gas (Hao *et al.*, 2011; Ryan *et al.*, 2008) using methods modified from our previous work (Murlasits *et al.*, 2006). Briefly, the animals were positioned on a heated plate to maintain body temperature throughout the experiment. Measurement of force records was via a DC servomotor servomotor (1410 DC, Aerotech Inc., Pittsburgh, PA) that was controlled by a Unidex 100 motion controller (Aerotech Inc., Pittsburgh, PA). Using sterile techniques, fine needle electrodes were placed through the skin into the popliteal fossa. Indirect muscle stimulation of the plantarflexor muscle group was achieved by electrically activating the tibial nerve. Square wave electrical impulses were generated from a muscle stimulator (Model SD9, Grass Medical Instruments, Quincy, MA) and delivered at a pulse duration of 200 μ s using 4 Volts. The pulses were sent to the tibial nerve at 100Hz for a duration of 300 ms. Three maximal force records were averaged for each assessment point. The animals regained consciousness ~2-3 minutes after removing them from anesthesia and regained normal cage ambulation without any obvious effects from the electrical stimulation or anesthesia. It is important to note that the plantarflexor force records will reflect primarily the gastrocnemius, plantaris and soleus muscles. While the soleus has a much smaller contribution to total plantarflexor, it also atrophies more than the fast muscles during HLS (Chen *et al.*, 2000; Ota *et al.*, 2011; Siu *et al.*, 2006b).

2.6 Ex vivo muscle physiological analysis

Although the soleus muscle in old rats typically has greater atrophy after HLS than fast muscles hindlimb muscles, the soleus muscle also undergoes less aging-associated losses of mass and function than fast muscles of the hindlimb such as the plantaris muscle (Alway *et al.*, 2001; Chen *et al.*, 2000; Siu *et al.*, 2005a). As aging affects fast muscles to a greater extent than slow muscles, we were most interested in examining the effects of EGCg on fast muscles after disuse and reloading. We chose to examine the plantaris muscle because it undergoes significant atrophy with aging and also undergoes HLS-induced atrophy (Pistilli *et al.*, 2006).

Ex vivo isometric muscle tetanic force was obtained from the plantaris (PL) muscles of vehicle and EGCg-treated rats. The muscles were removed and placed in an oxygenated Ringer's solution (137 mM NaCl, 4.7 mM KCl, 3.4 mM CaCl₂, 1.2 mM MgSO₄ 1 NaH₂PO₄, and 112 D-glucose). The Ringer's solution was aerated with 95% O₂ and 5% CO₂ (pH 7.4) and the temperature of the bath was maintained at 20°C. Although this is a non-physiological temperature, it does provide a model in which deterioration of force is eliminated over the duration of the study, whereas warmer temperatures in the incubation bath caused deterioration in muscle force. The PL muscle was fastened between a plexiglass plate, and the lever arm of a 300C dynamometer (Aurora Scientific, Aurora Ontario,

Canada). Evoked force of the PL was obtained with a square-wave stimulus (12 V, 200 μ s pulse width, 100 Hz). The PL muscles were adjusted to the optimal muscle length (L_o) by a micromanipulator that controlled the base position of the electrode clamp. L_o was established as the muscle length that produced maximal isometric tension as previously described (Alway 1994; Alway 2002; Jackson *et al.*, 2011). In our hands, we found no deterioration of *ex vivo* maximal muscle force in the plantaris muscle when it is maintained in the bath for more than two hours. As the force measurements that were obtained in this study typically take ~30 minutes to complete including the time for resting between contractions, we assumed that perfusion of oxygen to the muscle was adequate, and even if perfusion was not optimal, it did not affect maximal force production in the plantaris muscle. This is not the case for the gastrocnemius which shows rapid deterioration of force under our *ex vivo* conditions. Thus, we selected the plantaris muscle as representative of contractile function in the plantarflexors rather than the gastrocnemius muscle.

2.7 Body weight and tissue preparation

Each animal was weighed at the beginning of the experiment, after 14 days of HLS and/or after 14 days of reloading. Hindlimb muscles were removed from both limbs with the animals deeply anesthetized. The muscles were blotted, and weighed. The soleus and plantaris muscles of the right limb and the soleus of the left limb were immediately frozen in liquid nitrogen and stored at -80°C for Western blotting. Following physiological measures the plantaris muscle from the left limb was bisected at the mid-belly, embedded in optimal cutting temperature (OCT) compound (Tissue-Tek; Andwin Scientific, Addison, IL), frozen in liquid nitrogen cooled isopentane, and stored at -80°C until immunocytochemical analyses were performed. Preliminary studies (data not shown) failed to find significant differences in the fiber morphology of muscles that were stimulated by our *ex vivo* protocol, as compared to the contralateral control plantaris muscle that was not stimulated.

2.8 Immunocytochemical staining of tissue

Eight micron-thick frozen sections were cut from the mid-belly from soleus and plantaris muscles, mounted on glass slides and stored at -80°C . Myonuclei with apoptotic DNA strand breaks were assessed by fluorescent labeling of terminal dUTP nick-end labeling (TUNEL) assay (11684795910; Roche Applied Science, Indianapolis, IN) with minor modifications from the methods previously reported (Siu *et al.*, 2005b). Briefly the tissue sections were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 and incubated at 4°C overnight, with a rat anti-laminin antibody (MAB 1914, Millipore, Billerica, MA) to visualize the basal lamina of each muscle fiber. The sections were then incubated with donkey anti-rat rhodamine conjugated antibody (712-025-150, Jackson ImmunoResearch Laboratories, West Grove, PA) and the TUNEL reaction mixture, in a humidified chamber at 37°C for 1 hour in the dark. Omission of the TdT enzyme in the TUNEL reaction mixture on the tissue sections was included as negative control. TUNEL- and DAPI-positive nuclei that were immediately adjacent, under, or on top of the basal lamina were quantified. Data were expressed as an apoptotic index, which was calculated by counting the number of TUNEL-positive nuclei divided by the total number of nuclei (i.e., DAPI-positive nuclei) multiplied by 100. The TUNEL index for each muscle was calculated

from the same four random, non-overlapping fields that were used for fiber CSA measurements.

2.9 Muscle morphology

Muscle fiber cross sectional area (CSA) was obtained (20×) from sections stained with the laminin antibody. Images from four to five, random non-overlapping regions were analyzed in each tissue cross section. The CSA of 550 to 800 fibers were measured from each muscle of each animal with ImageJ software.

2.10 Myogenic precursor cells

To identify muscle precursor cells that had undergone proliferation during the reloading period, tissue cross sections were incubated with anti-BrdU monoclonal antibody (BD Biosciences, San Jose, California, #560808). The incubations were conducted at 4°C overnight, to identify the myogenic stem cells in the tissue sections. On the following day, the tissue sections were incubated with donkey anti-rat rhodamine conjugated and donkey anti-mouse FITC conjugated fluorescence second antibody (#712-025-150 and #715-096-151, Jackson Immuno Research Laboratories, West Grove, PA) for 1 hour at room temperature in the dark. Omission of the primary antibodies on the tissue sections was included as negative controls for each tissue section. The sections were mounted with 4',6-diamidino-2-phenylindole (DAPI) mounting medium to visualize nuclei (Vectashield mounting medium; Vector Laboratories, Burlingame, CA). The sections were examined under a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss Microimaging Inc. Thornwood, NY).

The number nuclei that were positive for BrdU, which were also immediately adjacent, under, or on top of the basal lamina were counted. BrdU positive nuclei that were located in the extracellular matrix compartment were assumed to be non-myogenic cells and were not quantified. BrdU positive nuclei were expressed as a percentage of the total number of myonuclei as determined from the DAPI staining.

2.11 Western immunoblots

Approximately seventy-five milligrams of muscle was homogenized in RIPA buffer (1% Triton x-100, 150 mM NaCl, 5 mM EDTA, 10 mM Tris; pH 7.4), containing protease inhibitor cocktail (1:100; P8340), phosphatase inhibitor cocktail 1 (1:100; P2850) and phosphatase inhibitor cocktail 2 (1:50; P5726). These reagents were purchased from the Sigma-Aldrich Company (St. Louis, MO). The protein content of the homogenate was measured using the BioRad DC protein assay (500-0116; BioRad, Hercules, CA). Forty micrograms of protein were loaded into each well of a 4-12% gradient polyacrylamide gel (NP0335BOX; Invitrogen, Carlsbad, CA) and separated by routine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for 1 hour at 120V followed by transfer to a nitrocellulose membrane for 1.5 hours at 25V. Non-specific protein binding was blocked by incubating the membranes in 5% nonfat milk in Tris-buffered saline containing 0.05% Tween 20 (TBST) at room temperature. The membranes were incubated overnight (1:1000) in antibodies purchased from Cell Signaling Technology, Boston, MA including: Bcl-2 (#2876), Bax (#2772), Akt (#4685), phospho-Akt^{Ser473} (#9271), GSK3β, cleaved caspase-3

(#9664), cleaved caspase-9 (#9509) and AIF (#4642). The primary antibody for FADD (#AB3102) was purchased from Millipore. The membranes were incubated in appropriate dilutions of secondary antibodies (diluted in 5% non-fat milk) conjugated to horseradish peroxidase (Sigma-Aldrich, St. Louis, MO). The signals were developed using a chemiluminescent substrate (Lumigen TMA-6; Lumigen, Southfield, MI) and visualized by exposing the membranes to x-ray films (BioMax MS-1; Eastman Kodak). Digital images were captured with a Kodak 290 camera. The protein bands were quantified using 1D analysis (Eastman Kodak) software as optical density $\times$ band area, and the data were normalized to the GAPDH for each lane as the loading control, and the final data product was expressed in arbitrary units.

2.12 Statistical analysis

The results are reported as means $\pm$ SE. Differences in means between groups were determined by multiple analysis of variance (MANOVA), and Hotelling's T-Square test. Bonferroni post hoc analyses were performed between significant means. A p-value <0.05 was considered significant.

3. Results

3.1 Body weight

The animals were allowed to acclimate for seven days after arrival. There were no statistical differences among the body weights of any of the groups before HLS. However, EGCg significantly reduced the amount of bodyweight that was lost as a result of 14 days of HLS or 14 days of HLS + 14 days of recovery (Table 1). EGCg helped preserve body weight without increasing average daily food intake (Supplemental Fig. 1).

3.2 Muscle wet weight

Cage control animals (n=12/group) were euthanized after 14 days as controls for the HLS animals. A second cage control group (n=12/group) was euthanized after 28 days as controls for the animals in the recovery group. In addition to the cage control animals, muscle wet weight was determined in the plantaris and soleus (Figure 1) muscles of EGCg- and vehicle-treated animals after HLS (n=12/group) or recovery (n=12/group). HLS induced loss of plantaris muscle weight by 30.9% ($p<0.05$) in EGCg treated animals and 33.6% ($p<0.05$) in vehicle-treated animals as compared to cage control animals. Similarly, compared to the cage control soleus muscle, the HLS group had a 35% lower muscle weight in EGCg treated animals and 36.9% lower weight in vehicle treated animals. Thus, EGCg did not protect either muscle from HLS-induced atrophy.

The animals that received EGCg over the HLS followed by 14 days of recovery, had a 14% greater plantaris muscle weight ($p <0.05$) as compared to the animals treated with the vehicle over this same period. Unlike the plantaris muscle, EGCg did not confer any ability to improve recovery of the soleus muscle mass because soleus muscle weight was similar in both vehicle and EGCg treated animals after recovery (Figure 1).

3.3 Maximal isometric force

EGCg showed improvement in muscle recovery from HLS-induced atrophy occurred in the plantaris muscle but not the soleus muscle. Therefore we followed up this observation by determining if the improved recovery from atrophy contributed to improved function in the plantaris muscle. *In vivo* plantar flexor isometric force decreased by 31.2% and 26.1%, in vehicle and EGCg-treated animals, respectively. While there was no significant recovery of force in the vehicle treated animals, force in the EGCg-treated animals had recovered to 91.5% of the pre-HLS value ($p<0.01$, Figure 2A).

There was a large reduction in maximal plantaris muscle *ex vivo* force after HLS in both vehicle-treated (-25.1%) and EGCg treated animals (-10.6%) as compared to cage control animals (Figure 2B). *Ex vivo* force did not further improve in the plantaris of recovery animals in either the vehicle-treated or EGCg treated animals, however maximal evoked plantarflexor force remained greater in plantaris muscles of EGCg treated compared to vehicle treated animals. Isometric force was expressed as a ratio of plantaris muscle mass as an estimate of muscle “quality”. The data (Figure 2C) indicate that muscle force generating quality was not different among the experimental groups.

3.4 Changes of muscle fiber CSA

Mean fiber CSA was determined in the fast contracting plantaris and slow contracting soleus muscles. As there was no differences between fiber CSA in animals in the cage control groups, the cage control vehicle-treated and cage control EGCg treated animals were combined for analysis. Fiber CSA was similar in the respective muscles of vehicle or EGCg treated control animals. While plantaris fiber area was dramatically decreased in both treatment groups at the end of the HLS period, fiber atrophy was more pronounced in the vehicle-treated group, EGCg prevented some of the HLS-induced atrophy (Figure 3A). Consistent with the mean fiber area data, the cumulative fiber area frequency distribution showed a shift to the left following HLS (Figure 3B) and recovery (Figure 3C) indicating a greater frequency of small fibers under these conditions. The cumulative frequency data show that recovery of plantaris muscle fiber area was nearly complete in the EGCg group because the curve shifted back to the right and was almost superimposed on the cage control fiber area distribution (Figure 3C). Examples of fiber morphology with the basal lamina of each fiber identified by immunocytochemistry are shown in Figure 3D.

As expected, the soleus muscle fibers were also severely affected by HLS. Fiber cross sectional area in the soleus of vehicle and EGCg treated muscles also remained lower than cage control animals after recovery (Figure 4A). The cumulative frequency distribution of soleus fiber areas was shifted to left in both vehicle and EGCg treated animals after HLS (Figure 4B) and recovery (Figure 4C), indicating that most fibers had undergone atrophy under these conditions. However, in contrast to the plantaris, the shift to the left was similar in the vehicle treated and EGCg treated animals for both HLS and recovery and EGCg did not improve muscle fiber area as compared to the vehicle treatment during reloading. Examples of fiber morphology with the basal lamina of each fiber identified by immunocytochemistry are shown in Figure 4D.

3.5 EGCg increases myogenic progenitor cell activation during reloading

BrdU is a synthetic thymidine analog nucleoside that is incorporated into nuclei during DNA synthesis (Gallegly *et al.*, 2004; Peterson *et al.*, 2008). Unlike myonuclei, myogenic progenitor cells (including satellite cells) can incorporate BrdU during proliferation. BrdU positive nuclei that were located under the basal lamina were identified as myogenic progenitor cells that had proliferated, migrated, and/or became incorporated into a muscle fiber (Adams 2006). Only the BrdU-positive nuclei within the boundaries of the basal lamina were counted and examples of labeled nuclei are shown in Figure 5. The BrdU labeling index was significantly greater in reloaded muscles of both vehicle and EGCg treated plantaris and soleus muscles ($p < 0.05$) as compared to cage control muscles (Figure 5). While 14 days of reloading increased BrdU labeled nuclei in the plantaris from EGCg treated muscles (7.4%) compared to vehicle treated animals (6.3%), EGCg did not improve myogenic stem cell activation in the soleus muscle (Figure 5).

3.6 Akt signaling

To explore potential anabolic signaling mechanisms that may contribute to the effect of EGCg on improving muscle fiber area recovery after reloading, the abundance of total and phosphorylated Akt was estimated by Western blots as an indication of the growth potential signaling in skeletal muscle. Total Akt was 14% ($p < 0.05$) greater in the EGCg treated plantaris muscles after HLS vs. the vehicle treated muscle. Phosphorylated Akt protein abundance was 125% ($p < 0.05$) greater in EGCg than vehicle treated control plantaris muscles after HLS. Phosphorylated Akt was elevated in the recovery group of both EGCg and vehicle-treated animals as compared to cage control muscles, the two treatment groups had similar levels of phosphorylated Akt in the plantaris muscle of the recovery animals (Figure 6).

In the soleus muscle after HLS, phosphorylated Akt was 35% lower ($p < 0.05$) and 13% greater ($p < 0.05$) than the corresponding cage control levels in vehicle and EGCg treated animals, respectively. Phosphorylated Akt was therefore 103% greater ($p < 0.05$) in the EGCg treated animals after HLS as compared to vehicle treated animals (Figure 7). In contrast, after recovery, phosphorylated Akt and total Akt were similar in both vehicle- and EGCg-treated soleus muscles but the protein abundances of these proteins were ~30% lower ($p < 0.05$) than measured in the cage control animals.

3.7. GSK-3 β protein abundance

Studies in skeletal muscles have linked lower GSK-3 β to muscle wasting under various conditions (Verhees *et al.*, 2013; Verhees *et al.*, 2011). Furthermore, GSK-3 β activity has been shown to increase in aging and correlate to sarcopenia (Kinnard *et al.*, 2005). Therefore, GSK-3 β was measured by Western blot as an indicator of altering catabolic signaling potential during myogenic recovery after disuse. Although, GSK-3 β protein abundance in the plantaris muscle increased with HLS and remained high in the muscles of the recovery animals as compared to the cage control muscles, it was similar in vehicle and EGCg treated muscles (Figure 6). In the soleus, GSK-3 β was 58% lower ($p < 0.05$) in vehicle vs. EGCg treated soleus muscles after HLS and also significantly lower than control muscles. EGCg treated muscles had greater GSK-3 β than control muscles. GSK-3 β protein

abundance was not different in the soleus muscle in the control and treated animals in the recovery group (Figure 7).

3.8 Apoptosis and apoptotic signaling proteins

The activation of the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2 as well as essential effector caspases, including cleaved caspase-3 and cleaved caspase-9 were evaluated by Western blotting. Bcl2 was increased by HLS in both vehicle and EGCg treated plantaris muscles. The anti-apoptotic protein Bcl2 was 31% ($p<0.05$) lower in the vehicle vs. EGCg treated plantaris muscles after recovery (Figure 8). The pro-apoptotic Bax protein abundance was 28% ($p<0.05$) and 23% ($p<0.05$) greater in vehicle vs. EGCg treated plantaris muscles after HLS and recovery, respectively. Cleaved caspase 9 and cleaved caspase 3 abundance were 24.5% and 17.9% respectively, lower in plantaris muscles of EGCg treated plantaris muscles after HLS as compared to vehicle treated muscles, but they were both still higher than controls levels (Figure 8). However, cleaved caspase 9 remained elevated in the plantaris of reloaded muscles, but the protein abundance of cleaved caspase 9 was similar in vehicle and EGCg treated muscles (Figure 8). AIF protein abundance was ~40% ($p<0.05$) lower in EGCg treated plantaris muscles from animals in the recovery group, whereas FADD was 48% ($p<0.05$) and 72% ($p<0.05$) lower in EGCg treated plantaris muscles of both HLS and recovery groups, respectively, as compared to vehicle treated animals (Figure 8.)

The frequency of TUNEL positive nuclei (apoptotic index) in the plantaris muscles increased after HLS in both the EGCg and vehicle gavaged animals but there was no difference between the treatment groups. In contrast, the apoptotic index in the plantaris muscle of the EGCg treated group was 54% lower as compared to the vehicle treated group, after recovery (Figure 9).

In the soleus muscle, neither Bcl2 nor Bax were markedly altered by EGCg treatment as compared to vehicle treatment, after HLS or reloading conditions (Figure 10). HLS increased cleaved caspase 3 in both vehicle treated and EGCg treated animals. A similar response was seen after recovery, where, cleaved caspase 3 abundance was increased >85% ($p<0.05$) in soleus muscles of both vehicle and EGCg animals compared to controls. EGCg had an impact on FADD levels during HLS and recovery, as FADD protein abundance was 60% ($p<0.05$) less than levels in vehicle-treatment during HLS, and 40% ($p<0.05$) less than vehicle treated soleus during recovery. There was no significant treatment group differences in AIF levels for animals in either the HLS or the recovery groups, although AIF protein abundance was elevated ~25% ($p<0.05$) by HLS in both treatment groups as compared to the cage controls (Figure 10). All protein abundance levels were normalized to the GAPDH protein abundance for each lane.

TUNEL staining was identical in cage control animals that were treated with the vehicle or EGCg. The TUNEL labeling index in the soleus muscles increased by 2.5 fold after HLS and remained high during recovery, but there were no significant differences in TUNEL labeling between EGCg and vehicle treated animals (Figure 11).

4. Discussion

In this study we tested the hypothesis that EGCg would lower myonuclear apoptosis, and improve muscle recovery after limb reloading following disuse, in the hindlimb muscles of aged rats. Because the impact of disuse is so profound in aged muscles compared to young, we have focused on studying the effects of EGCg on hindlimb muscles of old animals only. Although EGCg did not appear to reduce the extent of muscle wasting during HLS, it did improve muscle recovery after HLS. The major novel findings of this study are that during the recovery period after HLS was removed: **(i)** EGCg treatment increased the recovery of muscle mass, muscle force production, fiber area, and myogenic stem cell activation while decreasing some apoptotic signaling in EGCg treated muscles from fast contracting plantaris muscles as compared to muscles from vehicle-treated senescent animals; **(ii)** EGCg treatment was unable to fully recover muscle mass or fiber area back to control levels 14 days after removing the unloading stimulus; and **(iii)** EGCg treatment did not improve the recovery of soleus muscle mass in old rats following removal of HLS.

4.1 EGCg improves muscle loss and force production during reloading of fast contracting muscles

Studies from our lab and others have demonstrated that HLS dramatically reduces skeletal muscle mass and force production in muscle from aged rats (Alway *et al.*, 2001; Pistilli *et al.*, 2007; Siu *et al.*, 2008; Siu *et al.*, 2005b; Siu *et al.*, 2006b; Wilson *et al.*, 2008). In the current study, *in vivo* plantarflexion force production as well as *ex vivo* force production, in the fast contracting plantaris muscle was greater in EGCg-treated old rats as compared to vehicle-treated aged animals over recovery. This improvement in absolute force production is explained, at least in part by the impact of EGCg on recovery of muscle mass and fiber area in the fast contracting plantaris muscles after reloading following HLS, because muscle “quality” as indicated by *ex vivo* force/muscle mass, was not altered by EGCg. During reloading, there was a marked shift to the right (larger fibers) in the cumulative frequency distribution of EGCg treated plantaris muscles, such that the plantaris fiber area of the EGCg treated muscles were quite similar to cage control muscles (Figure 3C). EGCg treatment did not improve soleus fiber area or the cumulative frequency distribution following reloading. These findings are consistent with that of Ota and colleagues (Ota *et al.*, 2011) who reported no improvement in soleus muscle mass in mice fed catechins during hindlimb unloading.

Activation of myogenic stem cells is critical for muscle fiber growth upon reloading after gravitational unloading (Kawano *et al.*, 2008). The EGCg treated plantaris muscles had an elevated proliferation of muscle stem cells as shown by greater BrdU labeling and this presumably contributed to better regeneration of damaged fibers. The presence of developmental myosin in both vehicle and EGCg treated plantaris muscles (Supplemental Figure 2) suggests that the activated myogenic progenitor cells were capable of early regeneration/differentiation in the reloaded muscles, but the extent of fibers expressing developmental myosin was not different in the plantaris of vehicle and EGCg treated animals. Nevertheless, because neither the size of the fibers expressing developmental myosin, nor the abundance of developmental/neonatal myosin was quantified nor fully differentiated and mature fibers would not be expected to express developmental myosin

isoforms, we do not know if the differentiation of activated myogenic precursor cells varied between the treatments in the recovery groups. Furthermore, it is possible but untested that EGCg reduced inflammation in the muscles of the recovery animals, because inflammation can affect satellite cell (myogenic precursor cell) activation and differentiation (Degens 2010).

While the activation of myogenic stem cells occurred in the reloaded soleus muscle, there was no improvement in the recovery of soleus muscle mass in the EGCg-treated as compared to the vehicle-treated animals. This might indicate that activation of myogenic cells per se is not sufficient to guarantee muscle growth in aged animals. On the other hand, because a reduction of antioxidant levels negatively affects the regeneration index of myoblasts and satellite cells (Fulle *et al.*, 2005), and high levels of oxidative stress cause apoptosis (Siu *et al.*, 2009), it is possible that high oxidative stress could have limited muscle satellite cell differentiation and survival (Urish *et al.*, 2009), thereby reducing muscle regeneration, even if proliferation of these muscle stem cells was adequate. This idea is supported by observations that oxidative stress is high during conditions of muscle unloading (Siu *et al.*, 2008) and muscle regeneration (Urish *et al.*, 2009), and reducing oxidative stress improves muscle repair (Gharaibeh *et al.*, 2012; Myburgh *et al.*, 2012). Although not tested in this study, it is possible that EGCg may have buffered excessive oxidative stress adequately at least for the plantaris after reloading (Lawler *et al.*, 2003; Ota *et al.*, 2011) and in doing so, could have improved muscle mass and force production. This is possible because catechins have previously been shown to reduce oxidative stress during eccentric loading and in unloading in mice (Haramizu *et al.*, 2011; Ota *et al.*, 2011), and excessive oxidative stress is known to reduce calcium release from the ryanodine receptor/channel in response to a given action potential (Bellinger *et al.*, 2008) thereby suppressing force.

It is further possible that because the soleus had the largest oxidative potential, and it is more severely affected by HLS, it would also have the greatest potential for mitochondrial-generated oxidative stress and high oxidative stress that is not adequately buffered might reduce satellite cell differentiation (Gharaibeh *et al.*, 2012) as compared to muscles like the plantaris that have a lower mitochondrial content. As there are data to suggest that at least in old Wistar rats, hypertrophy would be expected to precede nuclear acquisition (van der Meer *et al.*, 2011a), the importance of oxidative stress to negatively affect differentiation of activated satellite cells and muscle regeneration (El *et al.*, 2012) and thereby limit the ability to add new nuclei to the reloaded muscles as a mechanism to enhance hypertrophy (Wust *et al.*, 2007) and improve recovery from muscle wasting requires additional investigation.

Akt has an important role in regulating protein synthesis, cell proliferation and cell survival via the inhibition of glycogen synthase kinase-3 β (GSK-3 β) and the activation of mTOR (Haddad *et al.*, 2006; Wu *et al.*, 2011). Furthermore, activation of Akt has been shown to occur as part of the downstream anabolic signaling cascade in reloaded muscles after disuse-initiated atrophy (Sugiura *et al.*, 2005). In this study, we show that the EGCg-treated plantaris muscles had higher pAkt levels during HLS yet the muscles still underwent atrophy during this period. However, phosphorylated Akt (i.e., activated Akt) increased significantly during reloading in both vehicle and EGCg treatment plantaris muscles but there was no

enhanced effect of EGCg. Thus, the greater anabolic result that occurred in the plantaris with EGCg treatment appeared to be unrelated to enhancing Akt signaling, or if Akt signaling was involved, other negative factors were in place which offset this potential to improve the anabolic environment in the muscle.

Recent data indicate that GSK-3 β is required for muscle atrophy (Verhees *et al.*, 2011). Our data are consistent with this observation because there was no suppression of GSK-3 β in the plantaris of either EGCg- or vehicle-treated animals during reloading, despite increased phosphorylated Akt. The failure of EGCg to reduce GSK-3 β abundance during the recovery period might have contributed to the inability of EGCg treatment to fully restore plantaris fiber characteristics to control levels after 14 days of reloading. Our data differs from that of van der Velden and colleagues (van der Velden *et al.*, 2007), who reported that GSK-3 β decreased in the soleus during reloading in young mice after a period of unloading. It is possible that these discrepancies between these studies were due to differences that might exist between the animal species and the age of the animals that were used in these experiments.

4.2 EGCg reduces apoptotic signaling in fast contracting muscles during reloading

Recovery of unloaded muscle mass occurs within 14 days of reloading in young animals (Oishi *et al.*, 2008). However, our data are consistent with previous observations that show that aging limits the recovery of muscle mass and function following atrophy. Although EGCg improved muscle fiber area and muscle wet weight recovery in the plantaris our aged animals in the recovery group, this was an incomplete recovery of muscle mass even at the end of 14 days of reloading following disuse. One possibility to explain this could be due, at least in part, to a reduced number of myonuclei and decreased DNA accretion during reloading (Mozdziak *et al.*, 2001). This speculation assumes that myonuclei have a limited cytoplasmic domain and there is tight relationship between the domain size even within large variations in fiber size (smaller or larger fibers) (Hikida 2011). Thus, if nuclei are lost (e.g., by apoptosis), the amount of cytoplasm would be expected to be reduced to maintain the nuclei/cytoplasmic ratio. Nevertheless, it is not clear if such a direct relationship between nuclear number and fiber size exists under muscle wasting conditions, because although nuclei are lost during denervation-induced muscle atrophy, loss of fiber size appears to precede loss of nuclei (van der Meer *et al.*, 2011b).

While there is a significant amount of data from several labs showing that apoptosis is part of the atrophic process and that blocking or reducing apoptosis reduces muscle atrophy (O'Leary *et al.*, 2012; Quadrilatero *et al.*, 2011; Siu *et al.*, 2006a; Teng *et al.*, 2011; Zhu *et al.*, 2013), the idea that myonuclei are reduced at least in muscles of young animals has been challenged (Bruusgaard *et al.*, 2012). While we do not have direct evidence to show that there is a greater number of new nuclei that survive in reloading of EGCg treated muscles, our data (Supplement 3) are consistent with the hypothesis that nuclear loss occurs during HLS, whereas reloading increases myogenic cell proliferation and DNA accretion (Mozdziak *et al.*, 2001) to return nuclear to cytoplasmic ratios to control levels in reloading conditions. The increase in the proliferated myogenic precursor cell population is reasonable in our model of reloading after disuse, because an increase in satellite cells has been shown

to occur in fast muscle with only moderate loading exercise and without injury (Shefer *et al.*, 2013).

The lower apoptotic potential in the plantaris of EGCg treated animals during reloading (Figure 9), is explained at least in part by greater Bcl2, and lower Bax protein abundance. Lower AIF and lower FADD abundance (Figure 8), likely contributed to preserve the number of new nuclei (activated myogenic precursor cells) that survived during the reloading period. Presumably, maintenance of a greater pool of these myogenic precursor cells in our model (e.g., satellite cells) that can be activated during periods of muscle reloading after atrophy, should improve the ability for muscle to recover from muscle atrophy.

Our finding of a lack of improvement of EGCg on the soleus muscle during HLS or recovery differs from data from Ota et al. (Ota *et al.*, 2011) who reported that green tea catechins reduced the loss of soleus muscle force during a period of hindlimb suspension in mice. However, the fiber population of the soleus in the mouse is composed of ~30% fast myosin containing fibers (Miyazaki *et al.*, 2006), whereas the rat soleus has ~10% type II fibers (Deschenes *et al.*, 2001), so it is possible that the improvement seen in the soleus in mice, could have been partly the result of a preferential effect on fast muscle fibers in that muscle. Another possibility is that green tea catechins used in that study (Ota *et al.*, 2011) would have included more than just EGCg and some combination of EGCg and other green catechins could have a greater effect than EGCg alone.

4.3 Limitations

We did not compare our data from old rats, to young rats in this study. Although we do not view this as a study limitation because that was not the purpose of the study, we recognize that we have based our interpretations of the data in this study on the fact that we are affecting the treatment of muscles in old animals under the conditions of disuse and reloading, and this represents a potential limitation. Thus, while we cannot distinguish completely between the effects of EGCg on aging vs. the synergistic effects of aging and treatment, we have nevertheless shown that EGCg has significant mechanistic effects on plantaris muscles of old rats, particularly in regulating or augmenting myogenic progenitor cells differentiation and reducing the abundance of pro-apoptotic proteins in reloading conditions after disuse. We have assumed that adding new nuclei are an important component in determining the regeneration potential of muscle after disuse, however, it is not clear how tightly regulated the nuclear/cytoplasmic domain is to regain loss muscle back to basal levels (which might be different than purely inducing hypertrophy over basal levels). We did not determine if autophagy or proteolytic changes also occurred in response to EGCg in old rat muscles in response to reloading after disuse, but this would be interesting given the observation that there is a suppressed level of autophagic signaling in muscles of aged rodents (Wohlgemuth *et al.*, 2010) and in aged muscles under conditions of denervation-induced disuse (O'Leary *et al.*, 2013).

5. Conclusion

Although EGCg did not prevent skeletal muscle atrophy induced by HLS in aged FBN rats, EGCg did improve muscle recovery in part by reducing apoptotic signaling and improving satellite proliferation and presumably survival for differentiation during reloading after HLS in the fast plantaris muscle. Differences between vehicle and EGCg treatments were not due to differences in food consumption between the animals in each treatment group (Supplemental Figure 1). EGCg treatment appears to be most effective for muscles with a fast (type II) myosin composition (i.e., plantaris and gastrocnemius) and has minimal effects for the slow myosin containing soleus muscle.

These data represent potentially important observations with clinical implications for the population of elderly persons who suffer from acute disuse (e.g., hospitalization) and then go through some period of rehabilitation in an attempt to recover function. Subsequent studies should be conducted to test if like our observations in old rats, elderly humans have similar benefits from consuming EGCg during a period of rehabilitation following hospitalization or other disuse. Daily ranges of EGCg between 400 mg - 800 mg has been reported to be safe and mild enough to be consumed by humans with gastric ulcers (Clifford *et al.*, 2013), and ranges up to 3000 mg of green tea catechins have been used in human studies apparently without negative side effects (Kim *et al.*, 2011). Assuming an 80 kg human, 3000 mg/d would be equivalent to 37.5 mg/kg of EGCg but this is less than the 50 mg/kg used in our study in rats. It is possible that very high doses could have undesired effects, because mice that consumed a diet that was very high in EGCg (1% w/w), showed evidence of increased inflammation (Pae *et al.*, 2012; Pae *et al.*, 2013). Thus, while EGCg shows significant promise, the optimal doses of EGCg should be established in elderly humans to obtain the desired biological effects that improve muscle mass after periods of disuse, without incurring undesired side-effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Epigallocatechin-3-gallate (EGCg) was given to aged rats during unloading and reloading
- Plantaris muscle weight and fiber size were improved by EGCg during reloading
- EGCg reduced pro-apoptotic signaling during muscle reloading
- EGCg increases recovery of muscle following forced disuse in aging

Figure 1. Muscle wet weight

Muscle wet weight was obtained in the plantaris and the soleus muscles of the hindlimbs in cage control animals treated with the vehicle (n=12) or EGCg (n=12) or in animals that had received either EGCg after 14 days of hindlimb suspension (HLS, n=12/group) or after 14 days of hindlimb suspension followed by 14 days of reloading (Recovery, n=12/group). The animals received EGCg or the vehicle (water) daily by gavage, for a total of 21 days (HLS) or for 32 days (Recovery). * $p < 0.05$, Cage Control vs. treatment group; † $p < 0.05$, Vehicle vs. EGCg treatments. VC, vehicle cage control; EGCg-C, EGCg treated cage control; VHLS, vehicle-treated animals that received either 14 days of HLS only, or 14 days of HLS followed by 14 days of recovery; EGCg-HLS, EGCg treated animals that received either 14 days of HLS only, or 14 days of HLS followed by 14 days of recovery.

Figure 2. Muscle force

Maximal *in vivo* in the plantar flexor isometric force (**A**), *ex vivo* plantaris isometric force (**B**), and *ex vivo* force per plantaris muscle wet weight (**C**), was measured after 14 days of hindlimb suspension (HLS) and after 14 days of hindlimb suspension followed by 14 days of reloading (Recovery). The animals received either EGCg, or the vehicle (water) daily, for 7 days before and throughout the experimental period. * $p < 0.05$, Cage Control vs. treatment group; † $p < 0.05$, Vehicle vs. EGCg. VC, vehicle cage control; EGCg-C, EGCg treated cage control; VHLS, vehicle-treated animals that received either 14 days of HLS only, or 14 days of HLS followed by 14 days of recovery; EGCg-HLS, EGCg treated animals that received either 14 days of HLS only, or 14 days of HLS followed by 14 days of recovery.

Figure 3. Plantaris muscle fiber morphology

A. Mean fiber cross sectional area was obtained in plantaris muscle of cage control, after 14 days of hindlimb suspension (HLS), or after 14 days of hindlimb suspension followed by 14 days of reloading (Recovery). The animals received EGCg or the vehicle (water) daily by gavage, for a total of 21 days (HLS, Control) or for 32 days (Control and Recovery).

* $p < 0.05$, Cage Control vs. treatment group; † $p < 0.05$, Vehicle vs. EGCg.

Cumulative frequency distribution of plantaris muscle fibers after 14 days of HLS (**B**), or after 14 days of hindlimb suspension followed by 14 days of reloading (**C**). As the fiber area distribution of cage controls was the same, whether they were treated with the vehicle or EGCg, these two control groups were combined into a single control group for data analysis.

D. Top row. Representative plantaris cross sections after staining the basal lamina (red) that were obtained from that were cage control (Control), vehicle-gavaged HLS animals (Vehicle-HLS) or EGCg-gavaged HLS animals (EGCg-HLS) animals. **Bottom row.** Representative plantaris cross sections after staining the basal lamina (red) from cage control (Control), vehicle-gavaged recovery (Vehicle-Recovery) or EGCg-gavaged recovery (EGCg-Recovery) animals. The animals received EGCg or the vehicle (water) daily by gavage, for a total of 21 days (HLS) or for 32 days (Recovery). Nuclei were stained with DAPI.

Figure 4. Soleus muscle fiber morphology

A. Mean fiber cross sectional area was obtained in soleus muscles of cage control, after 14 days of hindlimb suspension (HLS), or after 14 days of hindlimb suspension followed by 14 days of reloading (Recovery). The animals received EGCg or the vehicle (water) daily by gavage, for a total of 21 days (HLS, Control) or for 32 days (Control and Recovery).

* $p < 0.05$, Vehicle vs. EGCg.

Cumulative frequency distribution of soleus muscle fibers after 14 days of HLS (**B**), or after 14 days of hindlimb suspension followed by 14 days of reloading (**C**). As the fiber area distribution of cage controls was the same, whether they were treated with the vehicle or EGCg, these two control groups were combined into a single control group for data analysis.

D. Top row. Representative soleus cross sections after staining the basal lamina (red) that were obtained from that were cage control (Control), vehicle-gavaged HLS animals (Vehicle-HLS) or EGCg-gavaged HLS animals (EGCg-HLS) animals. **Bottom row.** Representative soleus cross sections after staining the basal lamina (red) from cage control (Control), vehicle-gavaged recovery (Vehicle-Recovery) or EGCg-gavaged recovery (EGCg-Recovery) animals. The animals received EGCg or the vehicle (water) daily by gavage, for a total of 21 days (HLS) or for 32 days (Recovery). Nuclei were stained with DAPI.

Figure 5. BrdU labeling in reloaded muscles

Left panel. Representative tissue sections from the plantaris muscle (*top row*) and soleus muscle (*bottom row*). Fluorescent staining is shown for BrdU (green) to identify activated myogenic progenitor cells nuclei. DAPI identified all nuclei (blue). The basal lamina (red) was identified the membrane boundaries of the muscle fibers to confirm that the BrdU positive nuclei were myonuclei/myogenic progenitor cells.

Right panel. The BrdU labeling index was calculated from tissue cross-sections of the plantaris muscle (*top*) and the soleus muscle (*bottom*). The BrdU labeling index was determined from the number of BrdU positive nuclei per total nuclei in after 14 days of reloading. A time released BrdU pellet was placed in the animals after 14 days of HLS, and at the point of reloading the hindlimbs. * $p < 0.05$, Cage Control vs. treatment group; † $p < 0.05$, Vehicle vs. EGCg.

Figure 6. Western blots and analyses for the plantaris muscle

Akt, phosphorylated Akt, and GSK3- β protein abundance and were determined by Western blot analysis in the plantaris muscles of rats after 14 days of hindlimb suspension (HLS) or after 14 days of HLS followed by 14 days of reloading (Recovery). The animals received EGCg (E) or the vehicle (V) daily by gavage. The data were normalized to GAPDH and were expressed as mean $\pm$ SE. * $p < 0.05$, Cage Control vs. treatment group; † $p < 0.05$, Vehicle vs. EGCg.

Figure 7. Western blots and analyses for the soleus muscle

Akt, phosphorylated Akt, and GSK3- β protein abundance and were determined by Western blot analysis in the soleus muscles of rats after 14 days of hindlimb suspension (HLS) or after 14 days of HLS followed by 14 days of reloading (Recovery). The animals received EGCg (E) or the vehicle (V) daily by gavage. GAPDH was used as a loading control. The data were normalized to GAPDH and were expressed as mean $\pm$ SE. * $p < 0.05$, Cage Control vs. treatment group; † $p < 0.05$, Vehicle vs. EGCg.

Figure 8. Apoptotic signaling proteins in the plantaris muscle

Left panel. Apoptotic signaling protein abundance was determined by Western blot analysis in the plantaris muscles of rats under control, hindlimb suspension (HLS), or reloading (Recovery) conditions. The animals received EGCg (E) or the vehicle (V) daily by gavage. GAPDH was used as a loading control. *Right panel.* The band density and area from the respective apoptotic signaling proteins were quantified and the data were normalized to GAPDH and expressed as mean $\pm$ SE.

TUNEL Labeling - Plantaris

Figure 9. TUNEL labeling as an indication of apoptosis in the plantaris muscle

Left panel. Representative tissue sections from plantaris muscles, with fluorescent staining for TUNEL (green) to identify apoptotic nuclei in cage control animals and in rats after 14 days of hindlimb suspension (HLS) or following 14 days of reloading that occurred after the 14 days of hindlimb suspension (Recovery). DAPI identified all nuclei (blue). The arrows show TUNEL positive nuclei.

Right panel. The apoptotic index was calculated from tissue cross sections of the plantaris muscle by determining the ratio of TUNEL positive nuclei to total nuclei in plantaris muscles of control animals for cage control (Control), hindlimb suspension group (HLS), and after reloading (Recovery) groups. The animals received EGCg or the vehicle (water) daily by gavage. * $p < 0.05$, Cage Control vs. treatment group; † $p < 0.05$, Vehicle vs. EGCg.

Figure 10. Apoptotic signaling proteins in the soleus muscle

Left panel. Apoptotic signaling protein abundance was determined by Western blot analysis in the soleus muscles of rats under control, hindlimb suspension (HLS), or reloading (Recovery) conditions. The animals received EGCg (E) or the vehicle (V) daily by gavage. GAPDH was used as a loading control. *Right panel.* The band density and area from the respective apoptotic signaling proteins were quantified and the data were normalized to GAPDH and expressed as mean $\pm$ SE. * $p < 0.05$, Cage Control vs. treatment group; † $p < 0.05$, Vehicle vs. EGCg..

Figure 11. TUNEL labeling as an indication of apoptosis in the soleus muscle

Left panel. Representative tissue sections from soleus muscles, with fluorescent staining for TUNEL (green) to identify apoptotic nuclei in cage control animals and in rats after 14 days of hindlimb suspension (HLS) or following 14 days of reloading that occurred after the 14 days of hindlimb suspension (Recovery). DAPI identified all nuclei (blue). The arrows show TUNEL positive nuclei.

Right panel. The apoptotic index was calculated from tissue cross sections of the soleus muscle by determining the ratio of TUNEL positive nuclei to total nuclei in soleus muscles of control animals for cage control (Control), hindlimb suspension group (HLS), and after reloading (Recovery) groups. The animals received EGCg or the vehicle (water) daily by gavage. * $p < 0.05$, Cage Control vs. treatment group.

Table 1
Body weight

	<i>Before Treatment</i>		<i>14 days of HLS</i>		<i>Recovery</i>	
	Vehicle	EGCg	Vehicle	EGCg	Vehicle	EGCg
Cage control (grams)	549.3 ± 18.7	562.2 ± 18.7	539.3 ± 19.7	555.2 ± 19.6	539.3 ± 19.4	555.2 ± 19.4
HLS (grams)	562.8 ± 16.2	564.2 ± 15.2	492.4 ± 17.0	519.1 ± 16.1	----	----
Recovery (grams)	562.7 ± 17.3	552.1 ± 16.2	472.4 ± 18.2	534.1 ± 17.0*	483.1 ± 12.3	526.2 ± 11.5*

Vehicle; animals received 1 ml of water by gavage daily. EGCg; animals received 50 mg/kg of epigallocatechin-3-gallate (EGCg) by gavage in 1 ml of water daily. Control; cage control animals. HLS; animals that received 14 days of hindlimb suspension. Recovery; animals that received 14 days of hindlimb suspension, then the suspension was removed so that the animals had 14 days of cage ambulation.

* $p < 0.05$, EGCg vs. Vehicle treatment