Green tea extract attenuates muscle loss and improves muscle function during disuse, but fails to improve muscle recovery following unloading in aged rats

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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 118: 319–330, 2015. First published November 20, 2014; doi:10.1152/japplphysiol.00674.2014.—In this study we tested the hypothesis that green tea extract (GTE) would improve muscle recovery after reloading following disuse. Aged (32 mo) Fischer 344 Brown Norway rats were randomly assigned to receive either 14 days of hindlimb suspension (HLS) or 14 days of HLS followed by normal ambulatory function for 14 days (recovery). Additional animals served as cage controls. The rats were given GTE (50 mg/kg body wt) or water (vehicle) by gavage 7 days before and throughout the experimental periods. Compared with vehicle treatment, GTE significantly attenuated the loss of hindlimb plantaris muscle mass (−24.8% vs. −10.7%, P < 0.05) and tetcatic force (−43.7% vs. −25.9%, P < 0.05) during HLS. Although GTE failed to further improve recovery of muscle function or mass compared with vehicle treatment, animals given green tea via gavage maintained the lower losses of muscle mass that were found during HLS (−25.2% vs. −16.0%, P < 0.05) and force (−45.7 vs. −34.4%, P < 0.05) after the reloading periods. In addition, compared with vehicle treatment, GTE attenuated muscle fiber cross-sectional area loss in both plantaris (−39.9% vs. −23.9%, P < 0.05) and soleus (−37.2% vs. −17.6%) muscles after HLS. This green tea-induced difference was not transient but was maintained over the reloading period for plantaris (−45.6% vs. −21.5%, P < 0.05) and soleus muscle fiber cross-sectional area (−38.7% vs. −10.9%, P < 0.05). GTE increased satellite cell proliferation and differentiation in plantaris and soleus muscles during recovery from HLS compared with vehicle-treated muscles and decreased oxidative stress and abundance of the Bcl-2-associated X protein (Bax), yet this did not further improve muscle recovery in reloaded muscles. These data suggest that muscle recovery following disuse in aging is complex. Although satellite cell proliferation and differentiation are critical for muscle repair to occur, green tea-induced changes in satellite cell number is by itself insufficient to improve muscle recovery following a period of atrophy in old rats.

Sarcopenia is characterized as an age-associated loss of muscle mass and strength that contributes to an increased risk of mobility-disability. Muscle loss is further increased during periods of disuse (22), and recovery after disuse is blunted or prevented in aged animals (26, 74). Muscle disuse is accompanied by an increase in apoptotic signaling (8, 22, 35, 66, 69), which may mediate some of the responses to unloading or reloading in the muscle. Strategies that reduce muscle loss during disuse or improve recovery of muscle force and mass following disuse are clinically relevant to aging populations, especially after periods of prolonged bed rest such as occurs with hospitalization or rehabilitation after surgery.

Green tea extracted from the leaves of the Camellia sinensis plant contains high levels of catechins, a class of polyphenols that appears to have a significant level of biological activity. Green tea catechins that are of particular interest include epicatechin, gallocatechin, epigallocatechin, epicatechin-3-gallate, and epigallocatechin-3-gallate (EGCg). EGCg is by far the most abundant, and is often believed to be the among the most biologically active catechins in green tea, although this is largely based on its high antioxidant and anti-inflammatory properties (39).

Muscle satellite cells are critical for successful muscle regeneration and repair (14, 71). However, the potential for green tea to improve muscle recovery following forced disuse by reducing oxidative stress or apoptotic signaling, or improving activation of satellite cells, or a combination of these has not been previously examined in aging. Recent data suggest that oxidative stress after eccentric exercise is reduced upon green tea supplementation (24) and green tea catechins were shown to reduce the loss of soleus muscle force during a period of hindlimb suspension (HLS) in mice (46). Furthermore, in vitro studies have shown that purified EGCg can inhibit serum-starvation and staurosporine-induced apoptosis in human myoblasts (31), attenuate muscle protein breakdown, and increase muscle protein synthesis in C2C12 myotubes in response to serum starvation or incubation in tumor necrosis factor-α (TNF-α) (40).

Our recent data suggest that a purified (~95%) EGCg preparation reduces apoptotic signaling in fast skeletal muscle in aging in addition to having a positive effect on muscle mass and strength during the recovery phase (reloading) following unloading (5). In this study we tested the hypothesis that green tea extract (GTE) will reduce muscle wasting during hindlimb unloading and improve muscle recovery during reloading following disuse. The results show that green tea was able to reduce muscle wasting and loss of function during hindlimb unloading, but it did not provide any further improvement during the reloading period. Because GTE has only ~50% EGCg, it is possible that either low levels of EGCg are needed to slow muscle wasting or, perhaps, along with EGCg, one or...
more of the other catechins that are present in green tea (39) may also have an effect on regulating muscle wasting during disuse in old rodents.

METHODS

Animal care. Sixty male Fischer 344 Brown Norway rats (34 mo of age, senescent), were obtained from the National Institute on Aging colony at Harlan. Experiments were approved by the Animal Care and Use Committee of West Virginia University. Standards for animal care of laboratory animals as advocated by the American Association for Accreditation of Laboratory Animal Care were followed in this study.

HLS and reloading (recovery). Senescent rats were randomly divided into three groups: cage control, HLS for 14 days, and 14 days of recovery after HLS. HLS was conducted as described previously by our laboratory (5, 22, 49). In this approach, we applied tape to the proximal part of the tail. The tape was connected to a swivel harness that allowed the animal to move 360° around the cage. The animals had full access to water and food and were housed individually. The suspension height was set to a maximum torso angle of 30° with respect to the floor of the cage. The animals were euthanized and the hindlimb muscles were removed after either 14 days of HLS or after 14 days of normal cage ambulation exercise (recovery) following HLS. The cage control animals moved freely around their cages.

Activation of satellite cells during reloading. After 14 days of HLS, the animals in the vehicle and green tea groups were anesthetized with 4% isoflurane, and the tail harness and tape were removed. A time-released 5-bromo-2-deoxyuridine (Brdu) pellet (0.02 mg per gram of body weight per day; Innovative Research, Sarasota, FL) was placed subcutaneously over the dorsum of the back in each of the anesthetized animals (5, 47). Brdu is a synthetic thymidine analog nucleoside that is incorporated by satellite cells during their proliferation stage as they undergo DNA synthesis (20, 47). Because myonuclei do not proliferate, they cannot incorporate Brdu, and therefore only satellite cell nuclei that had divided during the reloading period will be labeled with Brdu, whereas myonuclei will not incorporate this label. In this way, both proliferated satellite cells and their progeny cells can be tracked during recovery from HLS.

Nutritional treatment with GTE. Ten animals in each experimental group received a single daily dose of either 1 ml of water (vehicle) or 1 ml of GTE (50 mg/kg body wt dissolved in water, Sunphonen 90D; Taiyo Kagaku, Japan). GTE is an extract that is >80% polyphenol-enriched and contains the following: (2)-epigallocatechin-3-O-gallate (18.0%), (2)-allocatechin-3-O-gallate (11.6%), (2)-epicatechin-3-O-gallate (4.6%), (2)-epigallocatechin (15.0%), (1)-gallocatechin (14.8%), (2)-epicatechin (7.0%), and (1)-catechin (3.5%) (29). Either the vehicle or GTE was given to animals by gavage feeding (5, 7). Animals in both control and treatment groups that received HLS or HLS and recovery were pretreated with GTE or vehicle for 7 days before HLS and given the compounds over the 14 days of HLS (a total of 21 days of treatment). Animals in the control and treatment recovery groups received the dietary intervention for an additional 14 days after removing HLS (a total of 35 days of treatment).

Ex vivo muscle physiological analysis. Ex vivo isometric muscle tetanic and peak twitch force (PT) were measured in the plantaris muscles of vehicle- and GTE-treated rats after HLS or recovery following HLS. 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 mM NaH₂PO₄, and 112 D-glucose pH 7.4) that was maintained at 20°C. The plantaris muscles were fastened to the lever arm of a 300C dynamometer (Aurora Scientific, Aurora, ON, Canada). PT and tetanic force were obtained by passing a square wave pulse (12 V, 200 μs pulse width, 100 Hz) between two platinum plates that were placed on each side of the muscle. Optimal muscle length was adjusted to obtain the maximal isometric tension (4). The contractile measurements were analyzed offline using commercial software (DMI; Aurora Scientific).

Body weight, food intake, and tissue preparation. The animals were weighed at the beginning of the experiment, after 14 days of HLS, and after 14 days of reloading for the recovery group. To determine the amount of food the rats consumed, the contents of the food container was weighed every 1 or 2 days throughout the 7 days that preceded the intervention, or over the 14 days in HLS or recovery periods. The amount of food consumed by each rat was estimated from the loss of the weight of food in the container, and this was averaged over the intervention period.

After completion of the respective intervention, the plantaris and soleus muscles were removed from both hindlimbs with the animals deeply anesthetized with 4% isoflurane. Excess moisture was removed by blotting, and the muscles were weighed. The muscles were embedded in optimal cutting temperature compound (Tissue-Tek; Andwin Scientific, Addison, IL), frozen in liquid nitrogen-cooled isopentane, and stored at −80°C until the muscles were used for analyses. The contralateral soleus and plantaris muscles were frozen in liquid nitrogen and stored at −80°C for Western blotting and immunocytochemistry.

Plantaris and soleus muscle morphology. The soleus muscle consists primarily of type I fibers, and the plantaris muscle consists of a high percentage of type II fibers. These two muscles provided a range of fiber types that allowed us to determine whether GTE had a specific muscle fiber-type effect on regenerating muscle following HLS. Frozen sections (8 μm thick) were cut from the midbelly of the soleus and plantaris muscles. Tissues were mounted on glass slides, fixed with fresh 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and incubated with a rat antilaminin antibody (Millipore, Billerica, MA) at 4°C overnight to identify the basal lamina of each muscle fiber. Digital images from the basal lamina-stained tissue sections were obtained from four to five random, nonoverlapping regions of each tissue section using a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss Microimaging, Thornwood, NY). Muscle fiber cross-sectional area (CSA) was obtained from ~800 fibers by tracing around the basal lamina of each muscle fiber using ImageJ software (National Institutes of Health, Bethesda, MD). Fiber area was determined by an investigator who was blinded to the sample groups.

Muscle, satellite cells. Satellite cells and daughter cells derived from the parent satellite cells were identified using slight modifications to methods that were previously reported from our laboratory (5, 7, 47). Frozen sections (8 μm thick) were cut from the midbelly of soleus and plantaris muscles, mounted on glass slides, and stored at −80°C. The tissue sections were fixed with fresh 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and incubated with a mouse antidystagphin (Stigma-Aldrich, St. Louis MO) to identify the sarcolemma around each muscle fiber. To identify muscle satellite cells that had divided during the reloading period, tissue cross-sections were incubated with anti-Brdu monoclonal antibody (BD Biosciences, San Jose CA) and colabeled with dystrophin. Omission of the primary antibodies on the tissue sections was included as a negative control for each tissue section. The sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) to visualize all of the nuclei in each tissue section. The sections were examined under a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss Microimaging).

Typically, five to seven tissue cross-sections that were taken a minimum of 100 μm apart were analyzed to determine the number of total nuclei and Brdu-positive nuclei. The sections were analyzed by an investigator who was blinded to the experimental group or condition. Approximately 500–600 fibers were measured in each muscle. Data were quantified from sarcolemmal- (green), DAPI- (blue), and Brdu-labeled (red) tissue sections. The total number of nuclei that were positive for Brdu, which were adjacent, under, or on top of the basal lamina, were counted. The Brdu labeling index was determined from the number of Brdu-positive nuclei per total myonuclei deter-
Table 1. Body weight for the HLS group

<table>
<thead>
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<th>Before Treatment</th>
<th>HLS</th>
<th>Percent Change Before vs. After HLS</th>
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<tbody>
<tr>
<td>Cage control for vehicle</td>
<td>595.4 ± 15.6</td>
<td>536.4 ± 20.8</td>
<td>-1.4 ± 0.3</td>
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<td>Vehicle</td>
<td>538.3 ± 24.6</td>
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<tr>
<td>Cage control for GTE</td>
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<tr>
<td>GTE</td>
<td>548.4 ± 16.8</td>
<td>463.2 ± 13.3</td>
<td>-15.5 ± 1.1</td>
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HLS, hindlimb suspension; GTE, green tea extract. *P < 0.05, treatment group vs. cage control of the same treatment type.

Results

**Body weight and food intake.** The animals were allowed to acclimate to the new cage for 7 days after arrival. Although there was some variability in animal body weights, there were no statistical differences among the weights of any of the groups before HLS. HLS significantly reduced the body weight in both the groups treated with vehicle (−9.5%) and GTE (−8.4%) (Table 1), but there was no significant difference in body weight loss between the treatment groups. Animals in the recovery group [both those treated with vehicle (−22.1%) and GTE (−21.3%)] continued to lose body weight compared with their weight immediately following HLS relative to their starting body weight (Table 2). The similarity of the changes in body weights between the treatment groups was reflected by a comparable amount of food consumed by the animals in each group over the experimental periods (Fig. 1).

Muscle wet weight. Muscle wet weight was determined in the plantaris and soleus (Fig. 2) muscles of animals treated with vehicle or GTE after HLS or recovery. The cage control animals were euthanized after 14 days and served as controls for the HLS animals. A second control group was euthanized after 28 days and served as controls for the animals in the recovery group. HLS induced a loss of both plantaris and soleus muscle wet weight. The loss in soleus muscle mass was similar in both groups treated with vehicle (−32.2%) and GTE (−32.6%) after HLS. Similarly, the loss of soleus muscle weight was similar in animals treated with vehicle (−22.4%) or GTE (−26.5%) after the recovery period. Although the plantaris muscle had lower muscle weight with HLS compared with muscles from cage control animals, GTE significantly attenuated this loss (−10.7%) relative to the vehicle-treated muscles (−24.8%). Even though the plantaris muscle of animals treated with GTE had not returned to control levels at the

Table 2. Body weight for the recovery group

<table>
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<th></th>
<th>Before Treatment</th>
<th>HLS</th>
<th>Percent Change Before vs. After HLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cage control for vehicle</td>
<td>558.5 ± 11.9</td>
<td>536.4 ± 24.7</td>
<td>-4.0 ± 0.3</td>
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<tr>
<td>Vehicle</td>
<td>590.4 ± 16.9</td>
<td>491.5 ± 11.3</td>
<td>-16.8 ± 1.4</td>
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<tr>
<td>Cage control for GTE</td>
<td>547.2 ± 26.7</td>
<td>522.1 ± 18.7</td>
<td>-4.6 ± 0.4</td>
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<tr>
<td>GTE</td>
<td>555.9 ± 20.9</td>
<td>467.6 ± 14.2</td>
<td>-15.9 ± 1.7</td>
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*P < 0.05, treatment group vs. cage control of the same treatment type. Vehicle treatment.

**RESULTS**

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end of the recovery period, the loss of mass was significantly less in the plantaris of animals treated with GTE (−16%) than in those treated with vehicle (−25.2%) compared with the respectively treated cage control muscles.

**Plantaris ex vivo isometric tetanic and twitch force.** Ex vivo peak isometric tetanic force in the plantaris muscle was significantly lower \((P < 0.05)\) after HLS in animals treated with vehicle (−43.7%) or GTE (−25.9%) compared with cage control animals (Fig. 3A). Reloading did not improve evoked tetanic force; however, tetanic force production was significantly greater in animals treated with GTE compared with those treated with vehicle after both the HLS and recovery periods.

Following HLS, there was a significant decrease \((P < 0.05)\) in PT in muscles from animals treated with vehicle (−51.2%) and GTE (−30.6%) compared with muscles from the cage control animals (Fig. 3B). GTE significantly attenuated this reduction in PT so that was significantly greater in GTE- vs. vehicle-treated muscles after HLS \((P < 0.05)\). There was no change in PT in either vehicle- or GTE-treated plantaris muscles during the recovery period compared with immediately after HLS \((P > 0.05)\); however, muscles of animals treated with GTE maintained greater PT compared with those treated with vehicle after the reloading period.

**Changes in muscle fiber CSA.** At baseline, fiber CSA was similar in the respective muscles of vehicle- and GTE-treated control animals. Although HLS significantly decreased fiber CSA in the plantaris and soleus muscles from both treatment groups compared with the cage control muscles \((P < 0.05)\), fiber atrophy was significantly more pronounced in the group treated with vehicle (plantaris −39.9%, soleus −37.2%) than the GTE group (plantaris −23.9%, soleus −17.6%) (Fig. 4, A and D). Both plantaris and soleus mean fiber area failed to recover significantly from HLS levels and neither returned to cage control levels during the period of reloading following HLS; however, muscle fibers in both the plantaris and soleus muscles treated with GTE continued to be larger than those of muscles treated with vehicle after the reloading period.
The fiber-area frequency data after HLS (Fig. 4, B and E) and χ² analyses of cumulative frequency data after reloading (Fig. 4, C and F) show that plantaris and soleus muscles of animals that received vehicle had a greater shift to the left (smaller fibers) compared with animals that received GTE.

**GTE increases myogenic proliferation and differentiation of satellite cells during reloading.** We evaluated the extent of satellite cell proliferation to determine whether the attenuation in plantaris muscle loss by GTE during unloading and reloading might have been due in part to the initiation of a repair program that included elevation of satellite cell number for an increased potential for transcriptional regulation for repair of injured muscle and/or recovery of muscle mass. BrdU-positive nuclei that were located adjacent to but on the inside of the basal lamina or between the sarcolemma and basal lamina (proliferated satellite cells) (Fig. 5) were identified as myogenic stem cells that had proliferated but had not migrated into a muscle fiber (1). The BrdU labeling index (% of total myonuclei that are BrdU-positive) was significantly greater in reloaded plantaris and soleus muscles of both vehicle- and GTE-treated animals ($P < 0.05$) compared with those of cage control animals (Fig. 6). Fourteen days of reloading significantly increased the number of BrdU-labeled nuclei ($P < 0.05$) in the plantaris muscle from GTE-treated (18.9 ± 1.3%) compared with vehicle-treated (9.1 ± 2.0%) animals. Similarly, reloading elevated the BrdU index in the soleus muscles of animals treated with vehicle or GTE in a similar pattern to that of the plantaris. GTE increased satellite cell proliferation in the soleus muscle after reloading (30.8 ± 3.1%), and this was significantly greater ($P < 0.05$) than the vehicle-induced increase in the BrdU index (13.0 ± 2.6%) (Fig. 6). Cage control animals had a basal BrdU labeling index of 1.8 ± 0.01% in the soleus muscle.

BrdU-positive nuclei that had migrated from outside the sarcolemma to inside the muscle sarcolemma (i.e., inside the muscle fiber) were used as an indication of differentiated progeny daughter cells (myoblasts) from satellite cell origins. An example of BrdU-labeled nuclei residing inside the muscle fiber (differentiated progeny myoblast cells) is shown in Fig. 7.
A, B, D, and E. Quantification of the percentage of BrdU-positive nuclei that were located inside the muscle sarcolemma vs. the total number of BrdU-positive nuclei (i.e., the number of BrdU-positive nuclei between the basal lamina and inside the sarcolemma) was measured to indicate the percent of satellite cells that had proliferated and whose progeny cells from the satellite cells then became differentiated. The data (Fig. 7, C and F) show that a significantly greater ($P < 0.05$) percentage of total BrdU-positive nuclei had also differentiated in both the soleus and plantaris muscles of animals treated with GTE during the recovery period compared with vehicle-treated animals.

Oxidative stress. As expected, both HLS and recovery induced a high level of oxidative stress in the hindlimb muscles as indicated by increased protein carbonylation (Fig. 8A) and 8-iso-PGF2α (Fig. 8B) compared with muscles from cage control animals. The level of protein carbonyl abundance increased by $\sim 100\%$ and $\sim 200\%$ in vehicle-treated plantaris and soleus muscles, respectively, after HLS (Fig. 8A). Protein carbonyl levels were also 50% and 45% greater in plantaris and soleus muscles, respectively, after the reloading period compared with muscles from cage control animals. GTE caused a significant decrease in protein carbonyl levels in both plantaris and soleus muscles of animals subjected to HLS or reloading.
compared with muscles from cage control animals. Although GTE continued to carry a benefit of reducing carbonyl levels after reloading in plantaris and soleus muscles, the level of suppression appeared to be less in the recovery group than in the HLS group (Fig. 8A).

Similarly, 8-iso-PGF2α was markedly increased after HLS (plantaris ~80%, soleus ~270%) and reloading (plantaris ~59%, soleus ~117%). GTE suppressed lipid peroxidation as shown by significantly lower levels of 8-iso-PGF2α in both plantaris and soleus muscles after HLS or reloading (Fig. 7B). Nevertheless, GTE was unable to fully block oxidative stress because 8-iso-PGF2α remained higher in GTE-treated muscles after HLS or recovery compared with cage control muscles.

SOD activity increased in plantaris muscles after both HLS and recovery but this was not improved by GTE. SOD activity was not changed in the soleus muscle of old rats after either HLS or recovery in either vehicle- or GTE-treated animals (Fig. 8C). This suggests that GTE treatment did not enhance the antioxidant defense in muscles of old rats during high levels of oxidative stress.

**Apoptotic signaling proteins in reloaded muscles.** Changes in proteins in the apoptotic signaling pathway have been shown to occur in conditions of muscle wasting (45, 64, 68) and reloading following disuse (9, 22, 45). Furthermore, increases in apoptotic signaling have been shown to correlate with reduced muscle function (36) and satellite cell dysfunction (19) in muscles of aged humans. Oxidative stress is one way to increase the signaling for proteins in the apoptosis pathway in skeletal muscle (9, 13, 30). Because the current study and others (32, 67) showed that oxidative stress was increased with this model, we sought to determine whether apoptotic signaling was altered by GTE treatment during the reloading period. We therefore evaluated the activation of proapoptotic proteins AIF and Bax and the antiapoptotic protein Bcl-xL as representatives of mitochondrial-associated apoptotic signaling. Western blot analysis showed that proapoptotic protein signaling for AIF and Bax, was significantly depressed by GTE after reloading in both plantaris and soleus muscles (Fig. 9). Relative to cage control animals, AIF protein abundance was increased (P < 0.05) in reloaded muscles but there was no statistical difference between the relative increase in AIF in plantaris muscles of animals treated with vehicle (49.6%) or GTE (29.7%). Similarly, in the soleus, the increase in AIF was similar in animals treated with vehicle (46.2%) and GTE relative to the cage control animals. In contrast to AIF, another proapoptotic protein, Bax, was significantly depressed by GTE after reloading in both plantaris and soleus muscles (Fig. 9). Western blot data showed that Bax protein abundance was significantly increased (P < 0.05) in reloaded plantaris mus-

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Fig. 7. Immunocytochemical localization of satellite cells. Representative tissue cross-sections that show immunocytochemical localization of satellite cells that had proliferated and taken up the BrdU label (red superimposed on blue DAPI-stained nuclei). A and B: plantaris muscle fibers. D and E: soleus muscle fibers. A and D: DAPI-stained (blue) nuclei to show all of the myonuclei. B and E: superimposed staining for DAPI (blue nuclei), BrdU (red), and the sarcolemma (green) of the fiber. An example of a proliferated satellite cell (red) that had moved inside the muscle sarcolemma (which was stained green) is shown by yellow arrows (A and B). The white open arrows (D and E) show a BrdU-positive nucleus that remained outside of the sarcolemma. The percent of BrdU-positive nuclei that were differentiated as indicated by those that were internal to the fiber and not associated with the periphery of the fiber were calculated for the plantaris (C) and soleus (F) muscles and expressed an index of differentiated satellite cells progeny cells. *P < 0.05 cage control vs. treatment group; †P < 0.05 vehicle vs. GTE.
animals. The antiapoptotic Bcl-xL protein abundance decreased significantly with reloading ($P < 0.05$) in the reloaded soleus muscles but it did not differ between animals receiving vehicle (~39.1%) or GTE (~51.3%) relative to cage control animals. However, the abundance of Bcl-xL was not changed ($P > 0.05$) in reloaded plantaris muscles after either treatment compared with cage control animals (Fig. 9). Thus in general, GTE suppressed proapoptotic signaling during reloading but it did not provide any further change throughout the reloading period.

**DISCUSSION**

The major novel finding of this study is that treatment with GTE reduced the extent of muscle wasting, improved force, attenuated fiber atrophy, increased myogenic stem cell proliferation and differentiation, and decreased apoptotic signaling in the fast plantaris muscle compared with muscles from vehicle-treated senescent animals after disuse followed by reloading. GTE also had positive benefits on attenuating soleus fiber CSA atrophy, which was not observed when aged rates were supplemented solely with EGCG (3).

**GTE attenuates the decrease in muscle mass and force production during HLS.** Data from our laboratory and others have shown that HLS dramatically reduces skeletal muscle mass and force production from aged rats (6, 49, 67–69, 72). We found decrements in the plantaris mass of ~25% in old vehicle-treated animals after HLS, which is generally consistent with most (6, 7, 11, 28) but not all studies (48), and the ranges in response for the plantaris may be due to slightly different ages or lots and types of animals that have been studied. GTE attenuated the loss of muscle mass (Fig. 2) and fiber area (Fig. 4), which reduced the decrement in ex vivo tetanic force and twitch force after HLS or reloading compared with that of vehicle-treated aged animals (Fig. 3). The better preservation of muscle mass in the plantaris with GTE was not the result of a greater food consumption compared with the vehicle-treated animals after HLS, which is generally consistent with most (6, 7, 11, 28) but not all studies (48), and the ranges in response for the plantaris may be due to slightly different ages or lots and types of animals that have been studied. GTE attenuated the loss of muscle mass (Fig. 2) and fiber area (Fig. 4), which reduced the decrement in ex vivo tetanic force and twitch force after HLS or reloading compared with that of vehicle-treated aged animals (Fig. 3). The better preservation of muscle mass in the plantaris with GTE was not the result of a greater food consumption compared with the vehicle-treated animals (Fig. 1). This may be a fiber type-specific effect because GTE treatment did not appear to significantly reduce soleus muscle mass loss with HLS, nor did it improve muscle recovery after HLS compared with the vehicle-treated animals. Unlike treatment with pure EGCG, which failed to preserve slow fiber area (5) after HLS, GTE partially preserved muscle fiber CSA in the slow soleus muscle after both HLS and recovery from HLS (Fig. 4C). This suggests that slow muscle fibers may respond better to non-EGCg catechins, but testing this possibility was beyond the scope of this study.

Green tea has been shown to increase intracellular calcium (60) and improve sarcoplasmic reticulum calcium store levels (75) in the myocardium. Therefore, we cannot rule out the possibility that improvements in force production that we observed in skeletal muscle from GTE-treated animals after HLS or recovery could also have been due in part to improved calcium kinetics in response to an evoked stimulus, but this was not assessed in the current study.

**Muscle function and GTE attenuation of oxidative stress.** Although low amounts of reactive oxygen species improve muscle force production, higher levels of oxidant stress reduces force production in both a time- and dose-dependent manner (42, 50–54). It is well known that both loading and aging increase oxidative stress in muscle (27, 58). In this study,
we show that GTE suppressed the negative effects of oxidative stress as shown by lower protein carbonyls and 8-iso-PGF2α levels (Fig. 7), indicating less oxidative damage to protein and lipids in skeletal muscle of old rats after HLS or recovery than vehicle-treated muscles. This is consistent with observations that catechins have previously been shown to reduce oxidative stress during eccentric loading and unloading in mice (23, 24, 46). Because GTE failed to improve the antioxidant SOD activity level in plantaris or soleus muscles, the lower levels of oxidative damage rather than elevated antioxidant levels in GTE-treated muscles may in part explain the improved ability to generate force in response to HLS. Although we did not measure calcium signaling in the current study, excessive oxidative stress is known to reduce calcium release from the ryanodine receptor/channel in skeletal muscle in response to a given action potential (10), so it is possible that oxidative stress may have had multiple effects in reducing force during unloading and reloading, which were buffered in part by GTE to improve muscle function.

**GTE reduces apoptotic signaling in response to reloading after disuse.** Apoptosis is elevated in response to muscle wasting conditions including muscle disuse and early recovery (3, 8, 17, 18, 45). The rationale behind a role for apoptosis is that a reduced number of nuclei and decreased DNA accretion during reloading (43), which could prevent or at least contribute to the lack of muscle recovery during reloading with aging. In the current study, we found that the proapoptotic proteins AIF and Bax were elevated in response to reloading after HLS in muscles from vehicle-treated animals. This observation is similar to those from other studies (18, 22, 27, 33, 66). GTE suppressed Bax protein abundance in reloaded plantaris and soleus muscles. Bcl-xL, a mitochondrial-associated antiapoptotic protein, decreased with reloading in the soleus although it was unchanged in plantaris after HLS or reloading, and GTE did not change this response in either muscle after HLS or reloading. Nevertheless, the lower Bax abundance (or the greater Bcl-xL/Bax ratio) in both muscles from GTE animals would be expected to promote a lower mitochondrial-induced apoptotic environment than that in vehicle-treated muscles. Although we previously found that deletion of Bax attenuated denervation-induced muscle wasting (65), it was interesting to note that in the current study, although GTE suppressed proapoptotic Bax protein abundance in reloaded plantaris and soleus muscles, it was unable to improve muscle recovery in

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**Fig. 9. Apoptotic signaling.** Abundance of apoptotic signaling proteins was determined by Western blot analysis in plantaris muscles (A, C) and soleus muscles (B, D) of rats that had received 14 days of reloading after HLS-induced muscle disuse. Animals received GTE or vehicle daily by gavage. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. The density and area from the respective apoptotic signaling protein bands were quantified and the signals were normalized to GAPDH. Data are means ± SE for plantaris (C) and soleus (D) muscles. *P < 0.05 cage control vs. treatment group; †P < 0.05 vehicle vs. GTE.
the reloaded muscles compared with vehicle-treated animals. Whereas increases in individual or multiple signaling proteins in apoptotic pathways likely increase the susceptibility for apoptosis, such changes in signaling cannot be interpreted to mean that removal of nuclei (nuclear apoptosis) will absolutely occur. Moreover, the importance or the full role of apoptosis in contributing to a loss of nuclei in postmitotic tissue is not clear, and apoptosis has not been reported in all models in which muscle loss occurs in young animals (12, 21), although this may differ in aging.

**GTE improves satellite cell proliferation and differentiation after muscle reloading.** Although there is support for the idea that loss of satellite cell function inhibits or prevents growth in adult and aged muscles (2, 41, 55–57), other data from transgenic mouse models challenge the paradigm that satellite cells are necessary for adult muscle growth (37). Nevertheless, there is large support for the notion that satellite cell activation and proliferation are indispensable for muscle regeneration (34, 44, 59). Muscle unloading followed by reloading induces significant levels of muscle damage (16, 38, 70, 73). Thus we would anticipate elevated satellite cell activity to occur as part of an attempted program to repair muscle damage following unloading. However, there is an aging-associated reduction in the number of muscle satellite cells and perhaps a reduction in systemic factors that activate satellite cells (15, 63), and these may be important contributors to impaired adaptation of skeletal muscle to loading or reloading after disuse in aging muscles (20, 61, 62). In the current study, we found that GTE improved both satellite cell proliferation (as indicated by BrdU labeling) and differentiation of their progeny daughter cells in muscle, as shown by BrdU-positive nuclei that were inside the sarcolemma of a muscle fiber (Figs. 5–7).

Reducing apoptosis has been shown to promote satellite cell proliferation and suppress myoblast differentiation, thereby reducing muscle regeneration by a BAI1-dependent mechanism (25). Although it is possible that a GTE-mediated reduction in apoptosis signaling in our current study had a role in enhancing satellite cell proliferation, GTE did not appear to prevent differentiation of satellite cells because more BrdU-positive nuclei were located inside the muscle sarcolemma of muscles of GTE-treated animals than in vehicle-treated animals. Nevertheless, the increased satellite cell proliferation and differentiation that was mediated by GTE was insufficient to improve muscle recovery following a period of atrophy in old rats. This highlights the fact that although satellite cells are important in muscle repair, simply having more satellite cells or their daughter cells does not guarantee improved muscle recovery, at least in aging.

In conclusion, GTE was effective in reducing the extent of muscle fiber area and force losses in response to muscle unloading, and this was largely maintained during the reloading period compared with vehicle-treated animals. GTE appears to be most effective for muscles that have a high percentage of fast (type II) myosin (5) such as the plantaris. Although some of the results of the current study were similar to our previous study of EGCg (5), there were some important differences that pointed to the potential that the benefits of GTE on muscle wasting may not be exclusively due to the effects of EGCg alone. Alternatively, perhaps along with EGCg, one or more of the other catechins that are present in green tea (39) may also have an effect on regulating muscle wasting during disuse in old rodents. This is potentially possible because smaller catechins that are contained in GTE are thought to be more bioavailable than larger catechins such as EGCg (39). Nevertheless, identifying which of the other catechins (if any) in GTE have a role in reducing muscle wasting was beyond the scope of the current study. These preclinical data show evidence for technical success and provide a rationale for conducting a clinical study that evaluates the effect of GTE in an elderly population experiencing bed rest or hospitalization.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


