Impact of Repetition Number on Muscle Performance and Histological Response

BRENT A. BAKER, ROBERT R. MERCER, KEN B. GERONILLA, MICHAEL L. KASHON, GERALD R. MILLER, and ROBERT G. CUTLIP

National Institute for Occupational Safety and Health, Health Effects Laboratory Division, Morgantown, WV

ABSTRACT

BAKER, B. A., R. R. MERCER, K. B. GERONILLA, M. L. KASHON, G. R. MILLER, and R. G. CUTLIP. Impact of Repetition Number on Muscle Performance and Histological Response. *Med. Sci. Sports Exerc.*, Vol. 39, No. 8, pp. 1275–1281, 2007. Skeletal muscle injury is major concern in sport- and occupation-related fields. **Purpose:** We investigated the effects of increasing stretch-shortening contraction (SSC) repetition number *in vivo* and the resulting changes in functional performance and quantitative morphometry in rat skeletal muscle. **Methods:** Functional testing was performed on the ankle dorsiflexor muscles of Sprague–Dawley rats, which were randomly exposed to 30 SSC, 70 SSC, 150 SSC, or 15 isometric contractions of equal duration. Changes in functional performance and muscle morphometry were assessed at 48 h after exposure. Stereology was used to quantify the volume density of degenerative myofibers and normal myofibers in the tibialis anterior muscle from each group, as well as measures of inflammation and swelling and changes in the interstitial space. **Results:** At 48 h there was a significant decline in isometric force for the 70- and 150-SSC groups ($P < 0.05$ and $P < 0.05$, respectively). Stereological measures indicated significant decreases in the percentage of volume density of normal myofibers in the 70- and 150-SSC groups ($P < 0.05$). Measures for percentage of volume density of degenerative myofibers and inflammation were increased ($P < 0.0001$ and $P < 0.05$, respectively) in the 70- and 150-SSC groups. Moreover, a significant increase in the percentage of volume density of degenerative myofibers in the 150-SSC group compared with the 70-SSC group was observed ($P < 0.05$). **Conclusion:** These data strongly suggest that exposure to increasing SSC repetitions results in increased functional decrements and morphometric indices of myofiber degeneration and inflammation, and that there is an apparent threshold (repetition number) at which this occurs. **Key Words:** MUSCLE INJURY, STEREOLOGY, DORSIFLEXOR MUSCLES, *IN VIVO*

Soft-tissue injury (including skeletal muscle) is pronounced in the workplace, accounting for approximately 38% of all medically diagnosed injuries (29). This leads to a significant increase in lost workdays and increased disability (28). The use of muscle contractions to study skeletal muscle injury mechanics is beneficial in understanding the etiology of work-related musculoskeletal disorders. Findings from volitional animal models of repetitive motion (4,5), human models of exercise overload (8–10,20,24), and electrically stimulated rat dynamometer models (2,3,13,23) demonstrate that the cellular pathways of activation and the accompanying inflammation and histopathology are congruent.

Stretch-shortening contractions (SSC; reciprocal eccentric/concentric contractions) are physiologic in nature and have been studied in the context of human locomotion and athletic performance (1). Exposure to SSC has been shown to produce muscle injury from the eccentric component of the cycle (11–13,16). Thus, the amount of mechanical loading does have a graded effect, both on changes in muscle performance and on the extent of myofiber injury (13). In fact, varying the amount of repetitions may shape the physiological response in the continuum from adaptation to maladaptation and injury.

Studies of acute muscle injury have focused on the force response of muscle to a single exposure using a broad range of muscle contractions from 1 to 1800 repetitions (6,7,18,19). The results from these studies indicate that muscle injuries may be caused solely by eccentric muscle actions. It is possible that mechanical damage may occur while muscles are absorbing energy. High mechanical forces produced during eccentric muscle actions have been causal in the underlying etiology of muscle strain injuries (26,27). However, the influence of multiple repetitions on surpassing the target muscle's safety threshold, or tolerance level, and initiating an injury response is not fully understood.

Address for correspondence: Robert G. Cutlip, Ph.D., 1095 Willowdale Road MS L-2027, Morgantown, WV 26505; E-mail: rgc8@cdc.gov.
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There is clear evidence that the number of forced lengthening contractions has an effect on the amount of resultant muscle injury and force deficit (15). Hesselink and colleagues (15) have demonstrated that in rat muscle, approximately 240 stretches may be the threshold for inducing the maximum loss in isometric force, with an insignificant additional loss in isometric force encountered after 300 repetitions. Still, these results only allow us to conclude what may occur at the ceiling of contraction-induced muscle injury, and a very crucial component of contraction-induced muscle injury may be overlooked: the safety threshold. Single-stretch models that have stretched muscle within the physiological range have not resulted in muscle damage or a pronounced force deficit (7,17). In other studies, it has required more than one stretch within the physiological range to produce muscle injury (13,14,26,30,31).

A variety of methods have been used to examine the physiological and cellular responses to contraction-induced muscle injury. Importantly, these methods have not established strict classification criteria for quantifying myofiber degeneration. Moreover, these methods have not addressed the interstitial space's response and modifications to the induced injury. By establishing rigorous exclusion criteria for categorizing cellular normality, degeneration, and inflammation, we have been able to rapidly and objectively determine the histological status of the muscle and interstitial space temporally following various SSC-injury exposures (2,3). However, the effect of repetition number *in vivo* using SSC and the resulting quantitative degree of myofiber inflammation and degeneration have not been studied to date.

In the current study, our purpose was to investigate the effects that increasing repetition number exhibited on rat skeletal muscle exposed *in vivo* to electrically evoked (supramaximal) SSC of the left limb that activated the dorsiflexor muscle group, including the tibialis anterior muscle. The specific aims of the current study were twofold: (i) to investigate the effects that increasing repetition number has on skeletal muscle functional performance 48 h after an acute SSC exposure, and (ii) to investigate the effects that increasing repetition number has on skeletal muscle morphology 48 h after an acute SSC exposure. Specifically, we hypothesized that animals exposed to increasing numbers of SSC repetitions would have increased functional performance decrements 48 h after acute SSC exposure. Additionally, we hypothesized that animals exposed to increasing numbers of SSC repetitions would have increased stereological indices of myofiber injury, inflammation, and increased disruption to the interstitial space 48 h after acute SSC exposure.

METHODS

Animals. Male Sprague–Dawley rats (422 ± 19 g, 12 wk of age) (N = 24) were used in the present study. Rats were housed in an AAALAC-accredited animal quarters where the temperature and light/dark cycle (dark cycle from 7:00 a.m. to 7:00 p.m.) were controlled and food and water were provided *ad libitum*. All rats were exposed to a standardized experimental protocol approved by the NIOSH animal care and use committee that complied with the *Guide for the Care and Use of Laboratory Animals*. Animals were randomly assigned to either an isometric control group (CON, N = 6), a 30-SSC group (N = 6), a 70-SSC group (N = 6), or a 150-SSC group (N = 6). A subset of animals, which constituted the CON group at 48 h and a 150-SSC group after 48 h of recovery, have been reported in a previous study (2).

Experimental setup. Animals were tested on a custom-built rodent dynamometer as previously described. Rat dorsiflexor muscles were exposed to an SSC protocol as previously described by Baker et al. (2). Briefly, rats were anesthetized with isoflurane gas in an "induction" tank (Surgivet Anesco Inc.); they were placed supine on a heated x–y positioning table of the rodent dynamometer, with an anesthetic mask over the nose and mouth of the animal. The knee was secured with a knee holder, and the left foot (exposed limb) was secured in the load cell fixture with the ankle axis (assumed to be between the medial and lateral malleoli aligned with the axis of rotation of the load cell fixture). Each animal was monitored during the procedure to maintain proper anesthetic depth and body temperature.

Functional testing. Platinum stimulating electrodes (catalog F-E2, Grass Medical Instruments, Quincy, MA) were placed subcutaneously to span the common peroneal nerve. Activation of the electrical stimulator resulted in muscle contraction of the dorsiflexor muscle group. Stimulator settings were optimized to maximize dorsiflexor contractile performance as previously described (13). Muscle stimulation for all protocols was conducted at 120-Hz stimulation frequency, 0.2-ms pulse duration, and 4-V magnitude. The joint angle of the rat ankle was defined as the angle between the tibia and the plantar surface of the foot. The angular position of the load cell corresponded with the foot position. Vertical forces applied to an aluminum sleeve fitted over the dorsum of the foot were translated to a load cell transducer (Sensotec, Inc.) in the load cell fixture. The force produced by the dorsiflexor muscles was measured at the interface of the aluminum sleeve and the dorsum of the foot. An isometric contraction was performed at a 1.57-rad ankle angle using 300-ms simulation duration. An isometric contraction was performed 2 min before (pre) and 2 min after (post) either 3 sets of 10 SSC (30 SSC), 7 sets of 10 SSC (70 SSC), 15 sets of 10 SSC (150 SSC), or 15 isometric contractions (CON group), and 48 h after SSC exposure (immediately before the animal was euthanized). All animals were returned to their home cages in the animal quarters after their respective treatment protocols, and they remained there with access to food and water *ad libitum* until time of

sacrifice. Animals were monitored after their treatment exposure and did not show any signs of distress.

Injury protocol. The contraction-induced injury groups were exposed to 30, 70, or 150 SSC. The SSC were electrically evoked (supramaximal) via stimulation of the common peroneal nerve of the left limb, which activated the dorsiflexor muscle group, including the tibialis anterior muscle for 100 ms, and then moving the load cell fixture from a 1.13- to 2.00-rad angular position at a velocity of 8.72 rad·s^{-1}, in a reciprocal fashion, for 10 oscillations. After 10 oscillations, the load cell fixture was stopped at an angular position of 1.13 rad, and the dorsiflexor group was deactivated 300 ms later. The total stimulation time per set was 2.8 s. The repetitions were conducted at 1-min intervals.

Isometric control protocol. The CON group was exposed to 15 isometric contractions at 1-min intervals. During each contraction, dorsiflexor muscles were stimulated for 2.8 s at 1.57 rad, using the same stimulation parameters and duration as in the SSC group.

Histology. After completion of the second posttest, rats from both the CON group and SSC group were weighed, anesthetized with sodium pentobarbital (ip, 10 mg·100 g^{-1} body weight), and exsanguinated at 48 h after SSC exposure. The left tibialis anterior muscle (LTA) and right tibialis anterior muscle (RTA) were dissected, cleaned, and weighed. The midbelly region was cut from the muscle and mounted on cork, immersed in optimal cutting temperature medium (OCT), frozen in isopentane cooled with liquid nitrogen, and stored at −80°C. The midbelly region was selected to obtain the maximum tissue sample. Transverse sections were cut at 12 μm, mounted on precoated microscope slides, air dried, and stained using a routine procedure with Harris H&E. Permount was used to attach coverslips to microscope slides. Stereological analyses were evaluated on a Leica DMLB microscope.

Myofiber definitions. Stereology was used to quantify the degree of myofiber degeneration and the accompanying changes in the interstitial space in the TA muscle from each group. Myofibers have been defined previously (2); normal myofibers demonstrate (i) complete contact with adjacent myofibers, (ii) a smooth outer membrane, *and* (iii) no presence of internal inflammatory cells. Degenerative myofibers display (i) a loss of contact with adjacent myofibers, (ii) presence of internal inflammatory cells, *and* (iii) an outer membrane interdigitated with inflammatory cells.

Stereology. Quantitative morphometric methods were used to measure the volume fraction and surface densities of normal myofibers, degenerative myofibers, and the interstitial space as previously described (25). The interstitium was divided into the endomysium and the perimysium space, which included capillaries. Stereology also was used to quantify the degree of inflammation, which was quantified as either noncellular interstitium (NCI) indicative of edema, or cellular interstitium (CI); CI consisted of all possible infiltrating cells such as, but not limited to, inflammatory, endothelial, and fibroblasts. A stage micrometer was used to identify the midpoint of the sample section. Point and intercept counts using a 121-point/11-line overlay graticule (12.5-mm square with 100 divisions) at 40× magnification were taken at five equally spaced sites across the section. This process was repeated, 2 mm on either side of the midpoint of the section, for a total of 1210 points and 110 intercept lines per section (Fig. 1). Volume density or the percent tissue volume was computed from the percentage of points over the tissue section to points over normal myofibers, degenerative myofibers, CI, and NCI. Intercepts over the line overlay were counted for the perimeter of normal myofibers, degenerative myofibers, and interstitium to myofiber transitions. Points and intercepts over blood vessels greater than 25 μm in diameter were excluded.

Statistical analysis. Statistical analyses were conducted using SAS version 8 (SAS Institute, Cary NC). Mixed-model analyses of variance with repeated measures were used for the analysis of isometric force measures. *Post hoc* analysis where main effects or interactions were significant was subsequently performed using Fisher's LSD. Stereological measurements of the volume of cellular and noncellular components were analyzed using two-way (treatment × limb) ANOVA, with the animal as the random factor accounting for measures in both limbs. *Post hoc* comparisons were made using Fisher's LSD tests. Data for the measurement of the percentage of volume density of degenerative myofibers was regarded as ordinal (because of the consensus of zero scores for the control and, therefore, the lack of variance that would be present as opposed to the exposed animals), so a nonparametric Kruskal–Wallis test was applied, and when differences were statistically significant, a Mann–Whitney *U*-test was performed. One section per animal with six animals per group was evaluated, and the results are expressed as means ± SEM.

RESULTS

Functional measurements. No difference was observed in the maximum preexposure isometric force between any of the SSC groups or the isometric control group (P = 0.4402, Fig. 2). However, 48 h after exposure, the isometric control group exhibited a 5.6% increase in force, whereas animals exposed to the SSC exhibited a loss

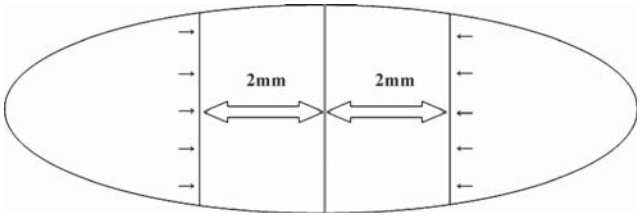

FIGURE 1—Representative drawing of a tibialis anterior muscle cross-section prepared on a slide. The *vertical midline* represents the middle of the section, and the *vertical black lines* represent the sampling fields (separated from the midline by 2 mm on either side). Each *horizontal arrow* represents the equidistant site that is sampled using a 121-point/11-line overlay graticule.

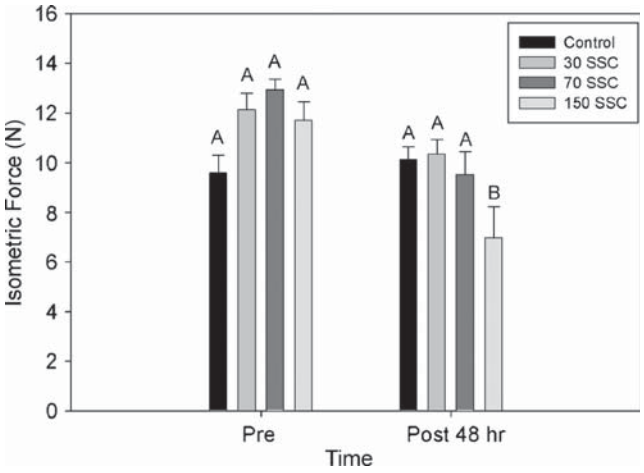

FIGURE 2—Isometric force change of muscles 48 h after SSC of varying repetition numbers. Different letters denote significance (*A, B*) and are reported at the $P < 0.05$ level. Group ($N = 6$) data shown are mean values ± SEM.

of force of 14.9, 26.4, and 40.5% respectively, for the 30-, 70-, and 150-SSC groups. Thus, at 48 h after exposure, the isometric force generated by the 150-SSC group was statistically lower than its preexposure isometric force test, the CON group ($P < 0.05$), the 30-SSC group ($P < 0.05$), and the 70-SSC group ($P < 0.05$, Fig. 2). However, all other comparisons were not statistically different.

Quantitative Morphology

Stereological analyses of normal myofibers. There was no difference in the percentage of volume density of normal myofibers in the contralateral control, isometric control, or 30-SSC groups. However, there was a decrease in percent volume density of normal myofibers in the 70- and 150-SSC groups ($P < 0.05$, Fig. 3). Additionally, there was a significant decrease in the percent

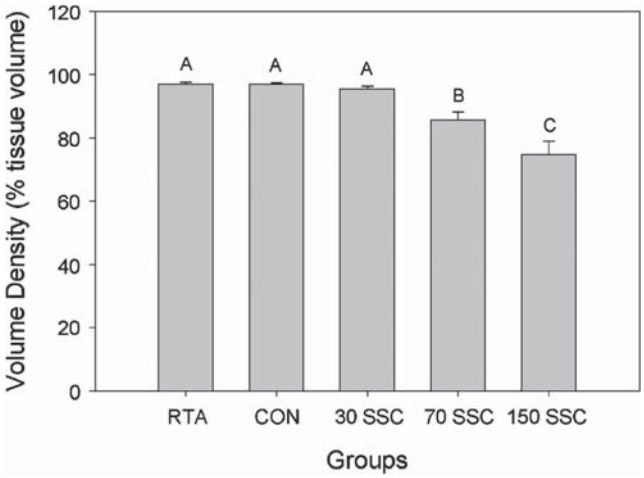

FIGURE 3—Percent volume density of normal myofibers of muscles exposed to SSC of varying repetition numbers. Different letters denote significance (*A, B, C*) and are reported at the $P < 0.05$ level. Group ($N = 6$) data shown are mean values ± SEM.

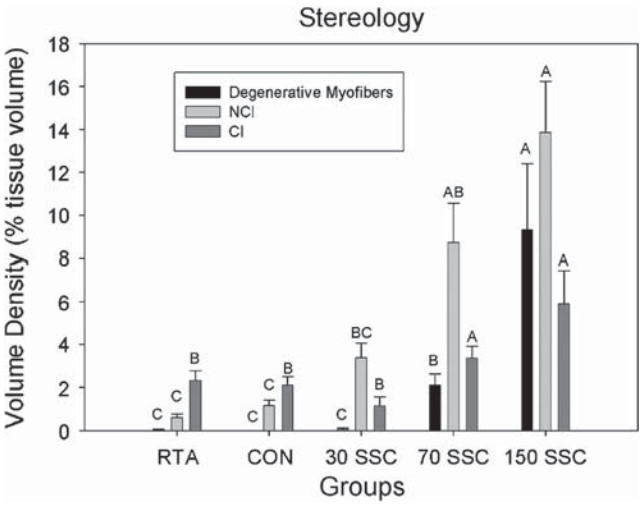

FIGURE 4—Percent volume density of degenerative myofibers, noncellular interstitium, and cellular interstitium of muscles exposed to SSC of varying repetition numbers. Different letters denote significance (*A, B, C*) and are reported at the $P < 0.05$ level. Group ($N = 6$) data shown are mean values ± SEM.

volume density of normal myofibers between the 70- and 150-SSC groups, with 150 SSC resulting in a decreased percentage of volume density of normal myofibers ($P < 0.05$, Fig. 3).

Stereological analyses of degenerative myofibers. SSC exposure did result in an increased percentage of volume density of degenerative myofibers in the 70- and 150-SSC groups ($P < 0.0001$, Fig. 4). There also was a significant difference in the percentage of volume density of degenerative myofibers between the 70- and 150-SSC groups, with 150 SSC resulting in a significantly greater percentage of volume density of degenerative myofibers ($P < 0.05$, Fig. 4). Representative images illustrate that none of the contralateral control muscles exhibited stereological indices for degenerative myofibers (Fig. 5A). In addition, no degenerative myofibers were observed in the isometric control muscles (Fig. 5B). Surprisingly, there were no significant levels of degenerative myofibers in the 30-SSC group compared with the control groups (Fig. 5C). As reported above, myofiber degeneration is present in histological images from both the 70- and 150-SSC groups (Fig. 5D and E, respectively).

Stereological analyses of inflammation. Noncellular and cellular interstitium (indicative of edema and infiltration of inflammatory cells, respectively) was quantified to determine the overall inflammatory changes occurring after acute exposure. The percent volume density of noncellular interstitium was not different in the contralateral control, isometric control, or 30-SSC groups (Fig. 5A–C). Conversely, 70 and 150 SSC did result in increases in the percent volume density of noncellular interstitium compared with the control groups ($P < 0.05$, Figs. 4 and 5D and E). There was no difference in percent

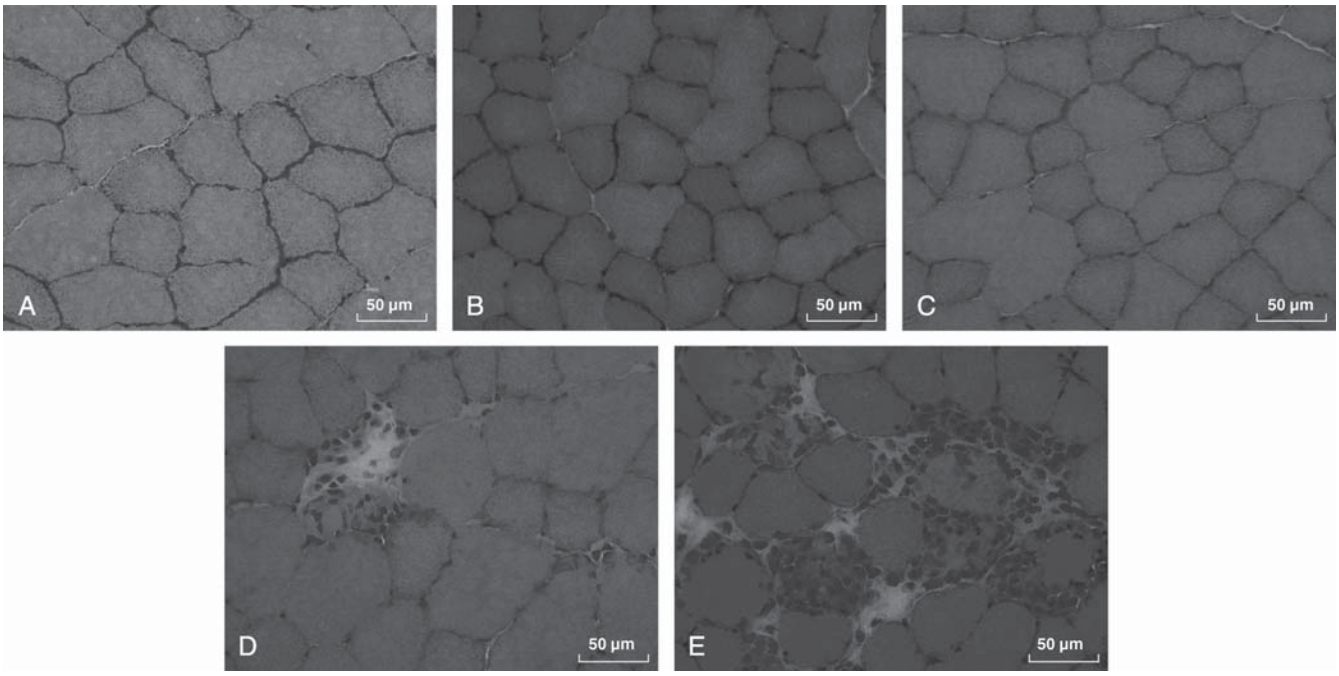

FIGURE 5—Hematoxylin- and eosin-stained sections of the nonexposed tibialis anterior muscle (*A*, RTA (contralateral control)) and exposed tibialis anterior muscle (*B*, CON (isometric control); *C*, 30-SSC group; *D*, 70-SSC group; *E*, 150-SSC group), respectively. Micrographs shown are representative of the overall groups. The micrograph from the RTA, CON, and 30-SSC groups (*A–C*) depict normal morphology. The micrograph from the 70- and 150-SSC groups (*D, E*) depict myofiber degeneration as well as cellular and noncellular infiltrates. The 150-SSC group (*E*) exhibits a significant increase in both degenerative myofibers and inflammation. All micrographs are captured at 40× magnification.

volume density of cellular interstitium in the control groups or the 30-SSC group, yet an increase in the percentage of volume of cellular interstitium was evident in the 70- and 150-SSC groups compared with the control groups ($P < 0.05$, Figs. 4 and Fig. 5D and E).

DISCUSSION

The most significant finding of the current study was that increasing numbers of SSC did produce increasing indices of myofiber degeneration and inflammation and, additionally, paralleled the decrease in functional deficit exhibited by the decline in isometric force production in the SSC groups. The morphological results were obtained using a systematic sampling technique (stereology) that is rapid, sensitive (a major advantage), and quite distinct from the approaches previously used to quantify muscle injury (2,3). Furthermore, a clear division was evident with respect to the number of SSC repetitions required to induce the subsequent inflammatory cascade and degenerative response, thus surpassing the TA's safety threshold at 70 SSC repetitions. In contrast, no myofiber degeneration or inflammatory response was observed in the contralateral control group, the isometric control group, or the 30-SSC group. Additionally, the isometric control group displayed a significant recovery of isometric force 48 h after exposure, whereas no such recovery was observed in the SSC-exposed groups. Our results are in agreement with previous results reported by Geronilla and colleagues (13), furthering their initial observations that myofiber necrosis and myositis increased with increasing repetition number. Additionally, we observed an increasing quantity of degeneration and inflammation with increasing SSC number, and this increase clearly exhibits a dose–response finding (this is consistent and corroborates the findings of Hesselink and Colleagues (15).

As with previous reports (15), our findings are not consistent with those reported previously by others, most notably McCully and Faulkner (21,22). These authors have reported that skeletal muscle damage levels off after a specified number of repetitions. Many likely explanations may be attributed to these data, which conflict with our present findings. First, the exposure protocols that were employed in the previous studies used forced lengthening contractions, whereas we used stretch-shortening cycle contractions to induce myofiber injury. In addition to the type of contraction involved is the active or passive state of the contraction. A number of previous investigations have used only active lengthening contractions, which have been immediately followed by passive shortening contractions. Although the aim of previous studies was to ascertain the involvement of lengthening contractions on skeletal muscle injury, this paradigm may not account for the physiological status of an active shortening-phase contraction. Realizing the physiological relevance of this feature is important because the results reported in previous works may overestimate the safety threshold for skeletal muscle in an *in vivo* preparation. Alternatively, the current protocol may not have

used enough repetitions to achieve the ceiling effect that has been reported. However, our focus was not on attaining a ceiling effect but, rather, on ascertaining when the initiation of myofiber injury might first appear with respect to increasing repetition number. Secondly, as noted by Hesselink and colleagues (15), the target muscle investigated (tibialis anterior vs extensor digitorum longus) may contribute to variations in the response observed. Finally, the animal species used (e.g. mouse, rabbit, and rat), or even the strain of rat used, might influence the observed results.

In summary, we have observed increases in the stereological indices for myofiber degeneration, noncellular interstitium (edema), and cellular interstitium (cell infiltrates) with increasing SSC repetitions. These measures became significant and continued to increase at the 70-SSC repetition number, suggesting a clear threshold for the target muscle's safety threshold with increasing numbers of repetitions. Conversely, we did not see any measures increase in the isometric control group or in the 30-SSC group. Moreover, functional capacity, assessed by isometric force production, was decreased in both the isometric control group and the SSC groups. However, the decrease in force production was only observed at 48 h after exposure in the SSC groups, with the isometric control group showing a full recovery. Thus, identifying the safety threshold of skeletal muscle with respect to increasing numbers of repetitions is of major importance when designing preventative strategies in vocational and recreation arenas, as well as for understanding the etiology of acute loading injuries. These data strongly suggest that there is a level of exposure at which the capacity to withstand the initial injury is compromised, hence exceeding the muscle's safety threshold.

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