Importance of Satellite Cells in the Strength Recovery After Eccentric Contraction-induced Muscle Injury

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Abstract

The purpose of this study was to determine if the elimination of satellite cell proliferation using γ-irradiation would inhibit normal force recovery following eccentric contraction-induced muscle injury. Adult female ICR mice were implanted with a stimulating nerve cuff on the common peroneal nerve and assigned to 1 of 4 groups: 1) irradiation and eccentric contraction-induced injury, 2) eccentric contraction-induced injury only, 3) irradiation only, and 4) no intervention. Anterior crural muscles were irradiated with a dose of 2500 rads and injured with 150 in vivo maximal eccentric contractions. Maximal isometric torque was determined weekly through 35 d post-injury. Immediately following injury, maximal isometric torque was reduced by ~50% and had returned to normal by 28 d post-injury in the non-irradiated injured mice. However, torque production of irradiated injured animals did not recover fully, and was 25% less than that of injured non-irradiated mice 35 d post-injury. These data suggest that satellite cell proliferation is required for approximately half of the force recovery following eccentric contraction-induced injury.

keywords: contractile protein, irradiation, proliferation, myogenic precursor cells
Introduction

Exercise with eccentric contractions causes injury in skeletal muscle evidenced by a prolonged loss of strength (4, 12, 16, 28). Arguably, strength loss is the most important functional consequence and the most reliable indicator of injury (6, 27). Reductions in strength may be $\geq 50\%$ immediately after the injury (13, 18), and recovery may take 28 d (10) or longer (9). The mechanisms underlying this marked loss and prolonged recovery of force have not been fully resolved. Satellite cells, myogenic precursor cells located between the basal lamina and plasmalemma of mature muscle fibers (14), have been shown to proliferate following eccentrically biased exercise (3, 22), but whether or not satellite cell proliferation is required for the strength recovery following eccentric contraction-induced injury is not known.

Satellite cells are involved in the replacement and/or repair of damaged fibers following other forms of traumatic injury (19), and Smith et al. (22) provided data suggesting that satellite cells may aid in the repair of fibers following injurious eccentric contractions. Thus, it seems likely satellite cells contribute to the strength recovery after eccentric contraction-induced injury. However, a clear dissociation exists between the amount of histological damage incurred and the strength lost in eccentric contraction-injured muscles (27). As one example, McCully and Faulkner (16) reported 37% of the muscle fibers showed signs of degeneration due to eccentric contraction-induced injury when the reduction in maximal isometric tetanic force ($P_o$) was proportionally larger (78%). This discrepancy between histopathology and strength loss may be partially explained by limitations associated with histological measurements of eccentric contraction-injured muscle (27) and/or the fact that excitation-contraction (E-C) coupling
failure is responsible for a large part of the strength decrement (28). Because relatively few fibers degenerate when the strength loss is large (13, 15, 16), it is not unreasonable to suggest that elimination of satellite cell proliferation would have a minimal effect on the recovery of strength following eccentric contraction-induced injury.

Unlike the dissociation between histopathology and force production, the restoration of contractile protein and strength following eccentric contraction-induced injury are closely related in the later stages of recovery (10). In fact, the restoration of contractile protein content in muscles injured with eccentric contractions parallels strength recovery from 14 d until the muscle has functionally recovered, i.e., 28 d post-injury (10). Indications that satellite cells may play a role in the restoration of contractile protein in eccentric contraction-injured muscle are provided in models of skeletal muscle regeneration and hypertrophy, which require the restoration and/or addition of contractile protein (1, 7). The importance of satellite cell proliferation in these adaptations is evident when these models are used in conjunction with irradiation. Irradiation prior to the onset of a stimulus inducing regeneration or hypertrophy inhibits the normal adaptive response, attributable to the inhibition of satellite cell proliferation (8, 21).

Since satellite cells fuse to form new myofibers and/or fuse with damaged or overloaded tissue to replace or add force-generating structures, i.e., contractile protein, it seems likely satellite cells would also contribute to the strength recovery that follows eccentric contraction-induced injury. However, to our knowledge, this has not been previously demonstrated. Therefore, the purpose of this study was to test the hypothesis that the elimination of satellite cell proliferation with irradiation would prevent normal strength recovery following eccentric contraction-induced injury.
Methods

Animals

Female ICR mice (n = 40), 8-12 wk old, were purchased from Harlan Laboratories. They were housed 4 to 6 per cage at 20-23°C with a 12:12 h dark:light cycle. The animal care procedures and experimental protocol met the guidelines set by the American Physiological Society and were approved by the Texas A&M University Laboratory Animal Care and Use Committee.

Experimental design

To test if satellite cells are necessary for muscle strength recovery following eccentric contraction-induced injury, a stimulating nerve cuff was implanted on the common peroneal nerve in the left leg of mice, and the animals were subjected to 1 of the following 4 treatments: 1) irradiation and eccentric contraction-induced injury (IRR/INJ, n = 11), 2) eccentric contraction-induced injury only (INJ, n = 10), 3) irradiation only (IRR, n = 11), or 4) no intervention (CON, n = 8). Strength of the anterior crural muscle group was assessed by measuring in vivo maximal isometric torque production at the ankle. The primary muscle in this group is the tibialis anterior (TA), which contributes about 89% of the torque (24). Two rounds of experiments had to be completed to obtain sufficient numbers of replicates to test the hypothesis. In both rounds, torque was measured at 300 Hz 7 times after the irradiation procedure: pre-injury, immediately post-injury (0 d), and 7, 14, 21, 28, and 35 d post-injury (Fig. 1). After the first round of measurements, the data suggested that E-C coupling restoration might be different between INJ and IRR/INJ groups. Therefore, to estimate E-C coupling recovery, in vivo isometric torque was measured at a low frequency (40 Hz) in animals in the 2 INJ groups.
of the second round in addition to the 300 Hz torque measurements. Muscle wet weights of the TA and extensor digitorum longus (EDL) muscles from some (n ≥ 8) animals of the 4 groups were determined 49-56 d after the injury induction.

Surgical Procedures

A stimulating nerve cuff was implanted on the left common peroneal nerve of the mice in all 4 groups while the animals were under sodium pentobarbital anesthesia (100 mg · kg⁻¹ ip) as described previously (25). Briefly, the nerve cuff was constructed from two Teflon-coated, multi-stranded 90% Pt -10% Ir wires 0.15 mm diameter (Medwire-Sigmund Cohn Corp. 10Ir9/49T; Mt. Vernon, NY, USA). An incision was made through the biceps femoris muscle and loops formed from 2.5 mm deinsulated segments of the 2 wires were placed around the common peroneal nerve. The proximal end of the nerve cuff was externalized in the dorsal cervical region.

Irradiation

Between 19 and 36 d after implantation of the nerve cuff, mice were anesthetized with fentanyl citrate (0.33 mg · kg⁻¹), droperidol (16.7 mg · kg⁻¹), and diazepam (5 mg · kg⁻¹), and the left legs of mice in the 2 IRR groups were exposed to γ-irradiation from a cobalt-60 source (model NPTT-Series, Neutron Products). A dose of 2500 rads was applied over 13.1 min. This dose has previously been shown to be effective in inhibiting satellite cell proliferation (1, 17, 20). The effectiveness of the irradiation was verified in cross sections cut from TA muscles excised 49-56 d after performing eccentric contractions. Irradiation effectiveness was further verified using the traumatic injury model described previously (24). Briefly, a metal rod pre-cooled in dry ice was held to
the superficial aspect of the TA muscle for 5 s. Freeze-injured TA muscles were
removed at 5 or 10 d post-injury and studied histologically.

Isometric torque measurements

*In vivo* maximal isometric torques produced by the anterior crural muscles at the
ankle were measured in animals of all 4 groups as described previously (13, 23). The left
foot of the anesthetized mouse (fentanyl citrate, 0.33 mg · kg⁻¹; droperidol, 16.7 mg · kg⁻¹;
and diazepam, 5 mg · kg⁻¹) was positioned in the foot plate controlled by a servomotor
(Cambridge Technology 300B). The isometric contractions were performed with the
plantar surface of the foot perpendicular to the tibia. Peak torque production was
optimized by varying stimulation voltage in a series of 5-8 isometric contractions; the
stimulations were 45-60 s apart, and consisted of 200 ms trains of 0.1 ms pulses at 300
Hz. As stated above, IRR/INJ and INJ mice in the second round of experiments also
performed an isometric contraction at a low frequency (40 Hz) 90 s after the
determination of peak torque. Low to high frequency torque ratios have previously been
interpreted as an indicator for E-C coupling failure (5).

Eccentric contraction injury induction

Two min after optimization of peak torque, the left anterior crural muscles were
injured as described previously (11, 13). The foot was first passively dorsi-flexed 20°
from the perpendicular position about the ankle. The muscles were then stimulated using
120 ms trains of 0.1 ms pulses at 300 Hz. An isometric contraction was performed for
the first 100 ms of the stimulation, followed by 20 ms plantar flexion movement
(eccentric contraction) over a 40° arc at 2000 °/s. The foot was then passively returned to
the perpendicular position. There were 12 s between contractions, so the duration of the
protocol was ~30 min. Two min after the 150th contraction, a post-protocol isometric contraction at 300 Hz was performed. The animals in the second round also performed a post-protocol isometric contraction at 40 Hz following the 300 Hz contraction as described above.

Tissue collection and processing

At 49 or 56 d post-injury, animals were anesthetized with sodium pentobarbital and TA and EDL muscles were dissected free of connective tissue, blotted dry, and quickly weighed using an analytical balance. Following removal of the muscles, animals were euthanized with an overdose of sodium pentobarbital (200 mg · kg⁻¹ ip).

To estimate possible differences in fiber cross-sectional areas and numbers of centrally located nuclei in the injured muscles, several TA muscles from IRR/INJ (n = 3) and INJ (n = 3) mice taken 49-56 days after injury were coated in OCT embedding medium, immersed in isopentane chilled in liquid nitrogen, and stored at −80°C until use. Three cross sections (10 μm thick) approximately 250 μm apart were cut in a cryostat at −20°C and stained with hematoxylin and eosin. The perimeters of 6 separate groups of 50 fibers each (i.e., 300 fibers) were traced in each of the 3 muscle cross-sections from each mouse, and the total fiber cross-sectional area for the 50 fibers was measured using an Olympus BX-60 microscope with image analysis software (BioQuant, version 3.0; R & M Biometrics, Inc.). Interstitial tissue area was excluded in this analysis using thresholding of staining intensity. Thus, from this analysis the average fiber cross-sectional area for each mouse was determined from a total of 900 fibers. Also, the total number of fibers with centrally located nuclei was counted in each of the 3 muscle cross-
sections from each animal, and from these counts the average number of fibers with central nuclei per muscle was calculated.

In preliminary experiments to determine the effectiveness of the irradiation procedure, hematoxylin and eosin-stained sections of freeze-injured TA muscles from irradiated and non-irradiated mice were inspected at 5 and 10 d after injury. One TA muscle was processed from each of the 2 groups at each time.

Statistical Analysis

The effects of irradiation, injury, and time on in vivo maximal isometric torque production were evaluated using an irradiation (irradiated, non-irradiated) x injury (injured, non-injured) x time (pre-injury, immediately post-injury, 7, 14, 21, 28, and 35 d) ANOVA with repeated measures over time. The effects of irradiation, injury, and time on body weight were evaluated using an irradiation x injury x time (pre-irradiation through 35 d post-injury) ANOVA with repeated measures over time. Analysis of low (40 Hz) to high (300 Hz) frequency torque ratios were conducted using an irradiation x injury x time (pre-injury, immediately post-injury, 7, 14, and 21 d) ANOVA with repeated measures over time. Differences among TA and EDL muscle wet weights, respectively, were analyzed with a one-way ANOVA. A General Linear Model was applied to account for unequal cell sizes. Fiber CSA was analyzed with a Student’s t-test. Student-Newman-Keuls post hoc tests were used where appropriate. An \( \alpha \) level of 0.05 was used for all analyses. Results are presented as means ± SE.
Results

Effectiveness of irradiation

Forty-nine to 56 days after eccentric contraction-induced injury, non-irradiated TA muscle had many fibers with centrally located nuclei (91 ± 19 fibers per cross-section, n = 3) indicative of regeneration and satellite cell proliferation. In contrast, muscles irradiated prior to the injurious bout of eccentric contractions had few fibers with centrally located nuclei (6 ± 2 fibers per cross-section, n = 3), providing evidence the irradiation was effective in attenuating satellite cell proliferation (Fig. 1, B and D). Furthermore, freeze-injured TA muscles that were not irradiated showed signs of regeneration evidenced by small fibers with central nuclei in the area of freeze injury 5 d post-injury, and relatively larger fibers with central nuclei 10 d post-injury, whereas freeze-injured TA muscles that were irradiated had few fibers with central nuclei at these time points (Fig. 1, A and C).

Torque measurements

Prior to the protocol of 150 eccentric contractions, the peak isometric tetanic torques produced by the anterior crural muscles were not different among the groups (3.09 ± 0.06 N · m x 10⁻²; n = 40) (Fig. 2). Immediately following the eccentric contractions, IRR/INJ and INJ peak isometric tetanic torques had decreased by 49 and 51 % from pre-injury, respectively, and there was no difference between the 2 groups. At 7 d post-injury the torques in the 2 INJ groups were not different and were 31-35 % lower than pre-injury levels. However, between 7 and 14 d there was a marked difference between groups in their recovery, with the INJ and IRR/INJ mice recovering 21 and 6%
of their pre-injury strength, respectively. IRR/INJ torque was 16-25% less than INJ between 14 and 35 d after the injury.

Maximal isometric torque production by INJ mice that were not irradiated had recovered to control values by 28 d post-injury. The immediate strength loss and prolonged strength recovery by INJ animals were similar to previous reports of *in vivo* (13, 26), *in situ* (2), and *in vitro* (10, 11, 13) measurements of force recovery following eccentric contraction-induced injury.

*Low to high frequency torque ratio*

The low (40 Hz) to high (300 Hz) frequency torque ratios for the IRR/INJ and INJ animals were depressed to similar extents immediately (~ 5-6 fold) and 7 d (~ 2 fold) post-injury (Fig. 3). There were no differences in the low-to-high torque frequency ratios between the 2 injured groups at any time.

*Body weight, muscle wet weights, and fiber cross sectional area*

The body weights of all groups were unchanged throughout the experimental period, and there were no differences in body weights among the groups at any time point studied. The initial and final body weights of the mice are shown in Table 1. There were no differences 49-56 days post-injury in wet weights of TA or EDL muscles among INJ, IRR, CON, and contralateral control muscles (Table 1). However, IRR/INJ TA and EDL muscles weighed 16-21% less than INJ, IRR, CON, and contralateral control muscles. At 49-56 days post-injury, fiber CSA was 9% less in IRR/INJ muscles compared to INJ TA muscles (2894 ± 338 vs. 3193 ± 187 µm²), though this difference was not statistically significant. It is emphasized that these are estimates only because of the small number of animals included in the fiber size measurements.
Discussion

The purpose of this study was to determine if prevention of satellite cell proliferation by $\gamma$-irradiation would attenuate recovery of strength in muscles injured by eccentric contractions. The data show this to be the case. Loss of strength from injury was similar in irradiated and non-irradiated muscles through 7 d, but thereafter recovery of torque was markedly less in the irradiated muscles, and still was not restored by 35 d after injury. The critical importance of satellite cells in the recovery process is apparent when one considers the numerous times the muscles are injured over a lifetime as indicated by delayed onset soreness and/or a loss in strength (18). Accumulation of strength losses every time a muscle was injured by overexertion would have disastrous consequences. Thus, the results indicate that one essential role of satellite cells is to restore muscle strength after this common form of injury accruing from normal physiological activity.

The similarities in strength recovery between the injured groups through 7 d post-injury suggest that the recovery of muscle strength during the first week after injury does not require satellite cell proliferation. Similarly, Mitchell and Pavlath (17) recently reported that recovery of muscle mass in mice during the first week following hind limb suspension occurs in the absence of satellite cell proliferation. We have shown that the recovery of strength during the first week post-injury occurs independently of contractile protein accretion in the injured EDL muscle (10). In fact, contractile protein content in the injured muscle is decreasing over this time period even as strength is recovering (10). This apparently contradictory observation can be explained by our observations that E-C
coupling failure explains most of the strength loss in the first few days following injury, and that the E-C coupling failure is resolved by 2 weeks post-injury (11, 28). The data from the present study would indicate that satellite cell proliferation is not required for resolution of the E-C coupling failure. In addition, the low-to-high torque frequency ratios were similarly affected in the IRR/INJ and INJ groups, suggesting that the induction and resolution of the E-C coupling failure was unaffected by irradiation.

Loss of contractile protein in the EDL muscle after injury is maximal (~20%) at 2 wk post-injury (10). Thereafter, the rate of protein synthesis exceeds that of degradation and both contractile protein content and force increase in parallel and are restored by 28 d after injury (10). Therefore, the recovery of contractile protein appeared to be the limiting factor in the later stages of force recovery (14-28 days) following eccentric contraction-induced injury in the EDL muscle. In the present study, the INJ muscle torque also recovered by 28 d after the injury, but was still depressed 21% in the IRR/INJ mice. In light of the similarities in torque decrements and recoveries between this study and those reported by Ingalls et al. (10), it is tempting to speculate that the 21% lower torque that was observed 28 days post-injury in the INJ/IRR muscles was due to the failure to restore the previously reported 20% loss of contractile protein. Indirect support for this is provided by the observation that the mean weight of the irradiated injured muscles was ~20% less than that of the non-irradiated injured muscles (Table 1).

Whether the decreased wet weight, and presumably, contractile protein loss, in irradiated injured muscles is due to fiber loss and/or or the loss of protein from surviving fibers is not known. McCully and Faulkner (16) reported both a decrease in fiber number and cross sectional area following eccentric contraction-induced injury. Conversely, Ingalls
et al. (10) reported no change in EDL fiber number, but a ~20% loss of contractile protein employing the model of eccentric contraction-induced injury used in this study. The fact that there was only a 9% decrease (p > 0.05) in TA muscle fiber cross sectional area in irradiated injured muscles (that lost ~20% of their mass and strength) in this study suggests there may have been a failure to replace lost fibers. Regardless of the mechanisms of muscle repair, inability of satellite cells to proliferate apparently deprives the injured muscles of the genetic apparatus required for restoration of normal functional capacity. It seems reasonable to assume that the failure of the IRR/INJ animals to return to normal strength results from attenuated resynthesis of proteins essential to the development and transmission of contractile force.

In conclusion, inhibition of satellite cell proliferation with γ-irradiation prevents approximately half of the normal torque recovery following eccentric contraction-induced injury. The satellite cell-dependent portion of the strength recovery occurs 1-2 wk post-injury and most likely results from a restoration of contractile protein that requires satellite cell to proliferate, and presumably to mature and fuse with injured myofibers.
Acknowledgements

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References


Table 1. Body weights and muscle wet weights.

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<td>Initial body, g</td>
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<td>Final body, g</td>
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<td>30.8 ± 0.9</td>
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<td>(n = 10)</td>
<td>(n = 11)</td>
<td>(n = 8)</td>
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<tr>
<td>Experimental TA, mg</td>
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<td>65.0 ± 2.6</td>
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*Different than control
(p ≤ 0.05)
Figure Legends

Fig. 1. Photomicrographs of tibialis anterior (TA) muscle cross-sections 10 d after freeze-injury (A and C) or 49-56 d after eccentric contraction-induced injury (B and D). Non-irradiated muscles (A and B) showed signs of regeneration evidenced by many fibers with centrally located nuclei, whereas irradiated muscles (C and D) had few. Bar = 50 µm.

Fig. 2. In vivo maximal isometric torque production (means ± SE) in irradiated injured (IRR/INJ, n = 11), non-irradiated injured (INJ, n = 10), irradiated non-injured (IRR, n = 11), and non-irradiated non-injured (CON, n = 8) mice at 300 Hz stimulation over time following injury. Values with the same letters are not different from each other among groups at each time point (p < 0.05).

Fig. 3. Low (40 Hz) to high (300 Hz) frequency torque ratios (means ± SE) in irradiated injured (IRR/INJ, n = 8) and non-irradiated injured (INJ, n = 6) mice over time after eccentric contraction-induced injury. There were no differences between the 2 groups at any time (p > 0.05).
Figure 1
Figure 2
Figure 3

![Graph showing time after injury versus torque ratio for IRR/INJ and INJ conditions.]