Voluntary running protects against neuromuscular dysfunction following hindlimb ischemia-reperfusion in mice

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Endurance exercise training reduces hindlimb ischemia-reperfusion injury

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Abstract

Ischemia-reperfusion (IR) due to temporary restriction of blood flow causes tissue/organ damages under various disease conditions, including stroke, myocardial infarction, trauma and orthopedic surgery. In the limbs, IR injury to motor nerves and muscle fibers causes reduced mobility and quality of life. Endurance exercise training has been shown to increase tissue resistance to numerous pathological insults. To elucidate the impact of endurance exercise training on IR injury in skeletal muscle, sedentary and exercise-trained mice (5 weeks of voluntary running) were subjected to ischemia by unilateral application of a rubber band tourniquet above the femur for 1 hour followed by reperfusion. IR caused significant muscle injury and denervation at neuromuscular junction (NMJ) as early as 3 hours after tourniquet release as well as depressed muscle strength and neuromuscular transmission in sedentary mice. Despite similar degree of muscle atrophy and oxidative stress, exercise-trained mice had significantly reduced muscle injury and denervation at NMJ with improved regeneration and functional recovery following IR. Together, these data suggest that endurance exercise training preserves motor nerve and myofiber structure and function from IR injury and promote functional regeneration.

New and Noteworthy

This work provides the first evidence that preemptive voluntary wheel running reduces neuromuscular dysfunction following ischemia-reperfusion injury in skeletal muscle. These findings may alter clinical practices in which a tourniquet is used to modulate blood flow.
Key Words

Ischemia reperfusion, endurance exercise training, mitochondria, oxidative stress, skeletal muscle, motor nerve, neuromuscular junction
**Introduction**

Ischemia-reperfusion (IR) injury due to reestablishment of blood flow after a temporary lapse is common to many debilitating diseases and a corollary to some clinical procedures. Skeletal muscle as an organ is particularly relevant since, as a common practice in certain types of surgery or as a first response to traumatic injury, a tourniquet is often used to prohibit hemorrhage, exsanguination, or provide a bloodless operating field (4, 38). The negative consequences of this procedure include muscle weakness, atrophy as well as temporary or permanent nerve damage, all of which hinder the functional recovery (11, 18, 37, 52, 54). For example, ~26% of patients recovering from total knee arthroplasty in which a tourniquet was used reported complications, including profound limb swelling, numbness and weakness (52, 53). Severe cases will require amputation. As recently as 2008, it was reported that among ~140 million patients with peripheral arterial disease who suffer an acute ischemic event, ~10-30% required amputation within 30 days (21). Thus, limb IR injury poses a significant clinical problem, and despite its prevalence, there is no reliable intervention (51, 64, 67, 75).

The compound cellular alterations accrued during ischemia and reperfusion determine the extent of pathology. This includes intracellular ion imbalance (27), destabilization of the plasma membrane (81), and accumulation of metabolic intermediates (16) during ischemia, as well as excessive generation of ROS, plasma membrane rupture (30), activation of inflammatory cascades (12, 79) and necroptosis (46) during reperfusion. Given the diversity of deleterious pathways activated by IR, the best intervention(s) is likely to be the one that could assuage multiple pathologies rather than one component. Indeed, remote pre-conditioning, which involves repeated short bouts of ischemia in an organ/tissue other than the target organ/tissue prior to the prolonged ischemic event, has been found to attenuate IR injury in experimental
models in a multi-faceted manner (1, 13). However, the efficacy of direct pre-conditioning of himdlimb is far from optimal for full functional protection, and the ideal timing and duration of pre-conditioning events are yet to be elucidated (17, 23, 68). Thus, it is of the upmost importance to develop alternative therapeutics that target multiple components of IR injury, which may allow compound therapies in the future to achieve maximal protection.

Exposure to repeated, low-grade stress provokes adaptations that enhance cellular resistance to future and/or more potent insults, a phenomenon called hormesis (35, 47, 62). In line with this biological phenomenon, endurance exercise training involves transient energetic, oxidative and mechanical stresses that elicit favorable adaptations both locally and systemically (3, 19, 62). Indeed, endurance exercise training has been shown to lessen IR injury in the heart (7–9, 20, 26, 60), liver (69) and lungs (19), whereas the underlying mechanisms may vary and include enhancement of antioxidant (34, 65, 72, 76, 80) and repair enzyme activity and expression (25, 41, 45, 66), increased Ca\(^{2+}\) buffering capacity (43, 63, 82), and improved mitochondrial quality (22, 42, 82). However, there have not been studies investigating the impact of endurance exercise training on the susceptibility of the adapted skeletal muscle to IR injury. If endurance exercise training promotes skeletal muscle resistance to IR, the next question would be whether the protection occurs during ischemia or reperfusion phase or as a continuation between the two. Additionally, it is not known whether exercise-mediated protection is predominantly motor nerve fibers or myofibers. In the present study, we tested the hypothesis that endurance exercise training is sufficient to protect motor nerve fibers, the neuromuscular junction (NMJ) and/or myofibers against IR injury through a mechanism by reducing oxidative stress. The findings would significantly improve our understanding of the utility and underlying
mechanism(s) of endurance exercise training as a therapeutic intervention to attenuate/prevent IR injury.

**Material and Methods**

**Animals:** All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Virginia. Male mice were housed in temperature-controlled (21°C) cages in a pathogen-free room with a 12:12-h light-dark cycle, and free access to water and normal chow (Bar Harbor, ME). Inducible whole-body MitoTimer transgenic mice (CAG-CAT-MitoTimer) were generated as previously described (78). To induce MitoTimer expression, tamoxifen (40 mg/kg, i.p.) was administered daily for 7 days in CAG-CAT-MitoTimer mice of 9-12 weeks of age followed by 3 days of recovery prior to the experimental procedures.

**Voluntary Running:** Voluntary running was set as described previously (41). Briefly, mice in the exercise group were individually housed in cages equipped with running wheels for 5 weeks, and sedentary mice were housed in cages not equipped with running wheels. Daily running was recorded via a computerized monitoring system. Running wheels were locked for 24 hours prior to the subsequent experimental procedures to minimize the effect of acute exercise.

**Hindlimb Ischemia-reperfusion:** Hindlimb IR injury was induced as previously described with minor modifications (5, 77). Briefly, under anesthesia (isofluorane in oxygen), a 4.0-oz, 1/8-in orthodontic rubber band (DENTSPLY GAC International Inc; 11-102-03) was applied above the greater tronchanter of the femur using a McGivney Hemorrhodial Ligator to block the blood
flow. Mice were conscious and monitored during the 1-hour ischemic period before the
tourniquet was removed to induce reperfusion.

**Creatine Kinase Activity:** Serum creatine kinase activity was measured by using a
commercially available kit following the manufacturer’s instructions (Sigma Aldrich; MAK116).
For sample preparation, blood was collected from the tail vein before and 3 hours after IR,
incubated at room temperature for 30 minutes and then spun at 1,500 x g at 4°C for 30 minutes.
The supernatant was saved (serum), aliquoted and stored at -80°C until further analysis.

**In vivo muscle function:** Maximal isometric torque of the plantar flexor muscles was assessed
as previously described (10, 77) before and 24 hours, 72 hours and 7 days after IR injury.
Briefly, mice were placed on a heated stage in the supine position under anesthesia (1%
isofluorane in oxygen), and the right foot was secured to a foot-plate that was attached to a
servomotor at 90° relative to the immobilized knee (Model 300C-LR; Aurora Scientific, Ontario,
Canada). For nerve-stimulated contractions (Nerve Stim), a pair of Teflon-coated electrodes
were inserted percutaneously on both sides of the sciatic nerve ~1 cm proximal to the knee joint.
For direct muscle stimulation (Muscle Stim), electrodes were inserted into the proximal and
distal ends of the GA muscle. Peak isometric torque (mN●m), which is referred to as strength,
was achieved by varying the current delivered to the nerve or muscle and keeping the following
parameters constant: 10 Volts electric potential, 200 Hz stimulation frequency, 300 ms
stimulation duration and 0.3 ms pulse duration. The force-frequency relationship was determined
by incrementally increasing stimulation frequency with 45 seconds resting period between two
contractions (10, 20, 30, 40, 60, 80, 100, 125, 150 Hz). To account for differences in body size
among mice during longitudinal studies torque was normalized by body mass (g), which did not change over the experimental time period. Specific torque was calculated by dividing absolute torque by plantarflexor muscle (gastrocnemius, plantaris and soleus) wet weight (mg).

**Morphological and immunohistological analysis:** Morphological and immunohistological analyses of plantaris muscle were performed as previously described (77)(49). Transverse muscle sections (5 μm) were stained with hematoxylin and eosin (H&E) (49) or immunostained using primary and fluorophore-conjugated secondary antibodies. Primary antibodies against Ncam (Abcam, ab9018) and laminin (Chemicon MAB1928) were both diluted 1:100. Percentage of centralized nuclei and myofibers positive for Ncam in the cytosol were calculated by dividing the number of fibers with aforementioned markers (counted by a blinded investigator) divided by the total number of fibers in 3 random fields of view per muscle.

**MitoTimer Analysis:** MitoTimer is a mitochondria targeted reporter gene that serves as a sensor of mitochondrial oxidative stress. When MitoTimer is oxidized it shifts emission wavelength from green fluorescent protein (GFP, excitation/emission 488/518 nm) to Discosoma sp. red protein (DsRed excitation/emission 543/572 nm). Ratiometric analysis of MitoTimer (red:green ratio) is a quantifiable metric of mitochondrial oxidative stress (40, 42, 55, 78). Imaging of MitoTimer in plantaris muscle and sciatic nerve using Olympus Fluoview FV1000 was conducted as previously described (40, 42, 77, 78). Fluorescent intensity of MitoTimer red and green fluorescence was quantified using a custom MatLab based algorithm from which MitoTimer red:green ratio was calculated. Identical acquisition parameters were used for every sample of the same tissue type.
NMJ Analysis: NMJ morphology and occupancy were assessed as previously described (58, 59, 77). Immediately upon harvest, plantaris muscles were fixed in 4% paraformaldehyde for 20 minutes, washed 3x in PBS, blocked in 5% normal goat serum and incubated with primary antibodies against Tubulin β-III (Tuj1, Covance; 801201) 1:100 and synaptic vesicle 2 (SV2, Abcam; ab32942) at 4°C overnight. The muscles were then washed with PBS and incubated with fluorescently conjugated secondary antibodies and Alexa 647-conjugated α-bungarotoxin (Thermo Scientific; B35450) diluted 1:100 in PBS for 30 minutes (31, 77). Images were acquired using Olympus Fluoview FV1000. To assess all the NMJs, Z-stacks were acquired using both 20x and 60x objectives. Only NMJs complete en face acquired at 60x were analyzed as previously described (59, 77). Maximum intensity Z-stacks were reconstructed in ImageJ (National Institutes of Health) and underwent the following corrections in the order listed: background subtraction (50.0 pixels), despeckling, application of a Gaussian blur (2.0 radius) and conversion to binary. Occupancy was determined by dividing the area of the presynaptic structures by the area of post synaptic structures (pre μm²/post μm² x100). Denervation is defined as the percentage of total NMJs in which the occupancy is <5%. A minimum of 30 NMJs were analyzed per muscle.

Immunoblotting: Immediately after harvesting, proteins were extracted from tissues, and immunoblotting was performed as previously described (40, 71). Briefly, tissues were homogenized in 2x sample Laemmli sample buffer containing protease and phosphatase inhibitors (1:10 g tissue:μL buffer), boiled at 95°C for 5 minutes and spun at maximum speed for 5 minutes. The supernatant was transferred to a clean tube, and protein concentration was
determined using RC-DC assay (Bio-Rad). Equal amounts of protein were separated using SDS-page electrophoresis. Proteins were transferred to nitrocellulose membrane and then blocked with 5% milk in TBST. Membranes were incubated with the following primary antibodies: SOD1 (Abcam; ab16831), SOD2 (Abcam; ab13534), SOD3 (Upstate; 07-704), Catalase (Abcam; ab15834), 4-Hydroxynonenal (Abcam; 48506), Actin (Sigma-Aldrich; A2066).

**Statistical Analysis:** Statistical analyses were performed using GraphPad Prism software, and values are presented as means ± standard deviation (SD). Two-tailed t-test was used for comparisons between sedentary and exercise-trained mice. One-way analysis of variance (ANOVA) was used for comparisons among sham, sedentary and exercise-trained mice. Two-way ANOVA was used to compare torque produced between sedentary and exercise trained groups pre- and post-injury. A significant interaction of 0.05 was required to perform a between-variable post-hoc analysis, in which case Tukey’s honestly significance difference test was performed. $p < 0.05$ is considered statistically significant for all the analyses described above.

**Results**

**Long-term voluntary running preserves muscle contractile function following IR.** To ascertain if endurance exercise training leads to protection against IR injury in skeletal muscle, we subjected sedentary and exercise-trained mice (following 5 weeks of voluntary running) to IR injury with sham operated mice serving as controls. Myofiber and motor nerve fiber functions were assessed based on total strength of plantar flexor muscles following direct muscle or sciatic nerve stimulation, respectively. These approaches provide insight into muscle...
contractile function and neuromuscular transmission, indicative of myofiber and motor nerve function, respectively (23).

Prior to the injury, body weight (27.4 ± 1.4 g in sedentary mice and 27.0 ± 1.18 g in exercise-trained mice), serum creatine kinase (378 ± 54.5 units/L in sedentary mice, and 328 ± 77 units/L in exercise-trained mice), and muscle strength by direct muscle (Figure 1a) and nerve stimulation (Figure 1b) were indistinguishable between sedentary and exercise-trained mice. Exercise-trained mice had significantly greater strength, as shown by greater torque by either direct muscle (Figure 1a) or nerve (Figure 1b) stimulation at 24, 72 hours and 7 days following IR. At 7 days, gastrocnemius muscle mass from sedentary and exercise-trained mice was reduced by 22% and 29% (p > 0.05 between these two groups), respectively, compared to the sham control mice, suggesting an equal level of myofiber atrophy (Figure 1c). Next, we evaluated the torque-frequency relationship at 7 days. Interestingly, we observed a left shift in the torque-frequency relationship after IR injury, in which 50% of maximal strength of injured muscles was reached at a lower frequency (~30 Hz) than the sham control (~60 Hz). This suggests that surviving fibers are either predominantly slow-twitch, or there was altered Ca^{2+} handling following IR injury. However, we found that exercise-trained mice had greater strength than sedentary mice at submaximal frequencies by direct muscle (Figure 1d) and nerve (Figure 1e) stimulation. Together, these findings suggest that exercise training preserves both myofiber and motor nerve function.

Long-term voluntary running does not prevent IR-induced oxidative stress.

Oxidative stress and consequent damage to cellular components is a hallmark of IR injury. Reduction in the production of oxidants or enhanced detoxification of oxidants has been
found to reduce IR injury across a number of tissues (39, 48). Endurance exercise training has been reported to promote antioxidant defense systems in skeletal muscle (29, 34), which might lead to increased resistance to IR injury. Indeed, we found that expression of superoxide dismutase isoforms 1, 2 and 3 as well as catalase were significantly increased following 5 weeks of voluntary running in skeletal muscle (Figure 2a), but not in sciatic nerve (Figure 2b), prompting us to hypothesize that exercise training-mediated protection against IR injury was through at least a reduction in oxidative stress in myofibers.

To test this hypothesis, we first evaluated mitochondrial oxidative stress in vivo by using a novel transgenic mouse model with a globally induced expression of the mitochondria reporter gene MitoTimer (MitoTimer-Tg). MitoTimer encodes a mitochondrial targeted green fluorescent protein that irreversibly switches to Discosoma sp. red fluorescent protein upon oxidation (42, 73). Computer-assisted ratiometric analysis of MitoTimer red:green fluorescence ratio provides a quantifiable measure of mitochondrial oxidative stress (41, 42, 55, 78). We subjected sedentary and exercise-trained MitoTimer-Tg mice to IR and collected tissues at 3 hours. MitoTimer red:green ratio in myofibers (Figure 2c) and motor nerve exons (Figure 2d) was indistinguishable between sedentary and exercise-trained mice and higher than the sham control mice, indicating that exercise training does not attenuate IR-induced mitochondrial oxidative stress. Next, we measured 4-hydroxynoneal (4HNE), a stable product of lipid peroxidation (56, 57), in whole cell lysates. Similarly to the findings of MitoTimer, we observed significant increases of 4HNE in myofibers (Figure 2e) and motor nerve (Figure 2f) 3 hours after IR in sedentary and exercise-trained mice when compared to the sham control. Together, these data suggest that the main protective effect of endurance exercise training against IR may not be through an enhanced antioxidant defense with reduced oxidative stress.
Long-term voluntary running attenuates myofiber damage following IR.

To assess injury to myofibers, we performed morphological analysis on transverse sections of the plantaris muscle by H&E staining. At 3 hours post-IR, skeletal muscle from sedentary mice displayed many rounded myofibers with increased interstitial space, indicative of edema and structural disruption, which was absent in exercise-trained mice (Figure 3a). To further validate the protection by exercise, we measured the activity of creatine kinase (CK) in the serum, a clinically relevant marker for IR induced muscle damage (15, 32, 33). Compared to the sham control mice, serum CK activity increased 6-fold in sedentary mice, which was attenuated to 3.5-fold in exercise-trained mice (Figure 3b). Taken together, the reductions in morphological changes and serum CK are indicative of reduced myofiber damage. We then assessed muscle morphology 7 days following IR. Sedentary mice displayed a significant increase of myofibers with centralized myonuclei, a marker for ongoing muscle regeneration, when compared to the sham control (Figure 3d). While there was a trend of increased number of myofibers with centralized myonuclei in exercise-trained mice, it was not statistically significant. In sum, morphological and biochemical analysis of markers of myofiber damage suggests exercise training improves myofiber resistance to IR induced injury.

Long-term voluntary running preserves innervation at NMJ following IR.

Patients with tourniquet usage may have temporary or permanent motor nerve damage, which contributes to post-procedure muscle weakness and delayed functional recovery (44, 52, 70). Neuromuscular junction (NMJ) is a specialized chemical synapse formed between motor nerve and myofiber that serves as the nexus of neuromuscular transmission. Previous studies
have revealed that NMJ is vulnerable to IR injury (74); therefore, we asked whether endurance exercise training could preserve NMJ integrity. We quantified the fluorescent overlap of the presynaptic neuron-specific class III β–tubulin (Tuj1) with the postsynaptic acetylcholine receptors (AchR) in plantaris muscle as a parameter of innervation at NMJ. At 3 hours after IR, Tuj1 fluorescence that overlaps with AchR was profoundly decreased compared to the sham control (Figure 4a). However, significantly fewer NMJ showed this change in skeletal muscle of exercise-trained mice. To further ascertain long-term impact of IR on innervation, we measured intramuscular expression of neuronal cell adhesion marker (Ncam), a marker of denervation and muscle regeneration (14, 28, 36). At day 7 following IR, sedentary mice, but not exercise-trained mice, showed a clear trend of increased cytosolic expression of Ncam compared to the sham control (p = 0.053) (Figure 4b). These data collectively demonstrate that exercise training attenuates denervation at NMJ following IR.

Discussion

Impairment of neuromuscular function is an inherent risk in procedures that employ a tourniquet to block blood flow. The clinical manifestations of IR injury in this context are myofiber atrophy, weakness, limb numbness, and temporary or permanent paralysis, all of which jeopardize the quality of life and amplify the incidence of morbidity and mortality. Although we have recently demonstrated IR injury to NMJ can be attenuated by targeted enhancement of mitochondrial protein S-nitrosation(77), there remains a need to develop an effective and accessible physiological intervention that also protects myofibers. Endurance exercise training is one of the most feasible candidates in this regard. Endurance exercise has been shown to improve myocardial tolerance to IR injury in a manner that is analogous to pre-conditioning (8,
In fact, during strenuous isotonic contractions, such as those elicited during exercise, arterial blood flow to skeletal muscle is arrested and is only restored when the muscle relaxes, effectively causing brief rounds of IR (2). However, whether endurance exercise training confers such benefits in skeletal muscle remained unaddressed. This study has provided the first evidence that endurance exercise training attenuates IR-induced neuromuscular derangement on the functional, morphological, cellular and molecular levels.

In this study, we assessed neuromuscular function by measuring and comparing muscle tetanic torque produced via muscle and motor nerve stimulations. Impairments in muscle contraction in response to direct muscle stimulation reveal reduced intrinsic muscle contractile capacity perhaps as a result of myopathies, including, but not limited to, abnormalities in protein degradation/synthesis, cross-bridge cycling and/or excitation-contraction coupling. We observed clear biochemical evidence of injury as well as concurrent muscle edema and rounding of fibers by IR, which was attenuated in exercise-trained mice. These findings suggest that endurance exercise training substantially reduces IR injury to myofibers. Moreover, the percentage of myofibers with centralized nuclei was significantly increased 7 days after IR in sedentary mice, whereas this increase was not statistically significant in exercise-trained mice. Considering these findings in sum, we conclude that endurance exercise training resulted in fewer damaged myofibers by IR. Alternatively, the same number of myofibers were affected, but to a lesser degree in exercise-trained mice, or a mixture of both. Future studies are necessary to determine which phenomena predominate.

Assessment of muscle contraction in response to sciatic nerve stimulation and innervation at NMJ provide insight into motor nerve function. The former assesses neuromuscular transmission whereby nerve impulses initiate muscle contraction, and the latter reveals the
structural integrity underlying this important function. We observed a dramatic decrease in
nerve-stimulated muscle tetanic torque concurrent with denervation at NMJ, supporting the
notion of compromised neuromuscular transmission following IR. This functional parameter
was significantly preserved in exercise-trained mice following IR accompanied by attenuated
denervation at NMJ. These data suggest that exercise training preserves motor nerve function, at
least in part, by preserving innervation at NMJ.

Acute bout(s) of exercise causes transient oxidative stress in skeletal muscle and other
remote tissue/organs, which may trigger adaptive responses and ultimately render the effected
tissues/organs more resistant to ensuing future stresses (please see reviews (6, 22, 24)). A
seemingly important adaptation induced by endurance exercise training is increased expression
of enzymes in the antioxidant defense system. Consistent with the findings by our and other
groups, we found that long-term voluntary running led to modest increases of antioxidant
enzymes in myofibers (34, 50). However, IR-induced cytosolic and mitochondrial oxidative
stresses assessed by a fluorescent reporter for mitochondrial oxidative stress as well as 4-HNE
mitochondrial protein adducts were not attenuated in myofibers of exercise-trained mice. The
most straightforward explanation is that endurance exercise training-induced increases in
antioxidant enzymes are not sufficient to prevent oxidative stress induced by IR.

We have shown clear evidence of muscle injury and degeneration/regeneration following
IR in sedentary mice as indicated morphological disruptions and appearance of centralized
myonuclei, respectively. Exercise-trained mice had significantly attenuated increases of these
parameters, consistent with the notion that myofibers from exercise-trained mice are more
resistant to IR injury despite the fact that they endure similar oxidative stress following IR. It is
equally intriguing that despite the similar degree of oxidative stress in the motor nerve, exercise-
trained mice showed protected neuromuscular transmission and innervation at NMJ. The underlying mechanisms for endurance exercise training-induced resistance to IR injury in motor nerve and skeletal muscle remains a mystery and warrants further investigations.

In conclusion, this study has provided the first evidence that endurance exercise training is sufficient to attenuate IR injury in motor nerves and myofibers, thus preserving neuromuscular function and promote functional regeneration from IR. This exercise training-induced protection may not be through reduced oxidative stress in the myofibers and motor nerve. Collectively, our findings support a new application of endurance exercise training with strong clinical implications where endurance exercise regime could be prescribed in preparation for surgeries or procedures that will employ a tourniquet. Whether injury and/or recovery could be augmented by exercise training after injury or coupling exercise training with other interventions, such as the aforementioned augmentation of mitochondrial protein S-nitrosation, is a compelling question, worthy of investigation. Finally, these discoveries provide a foundation for future studies to elucidate the precise mechanism(s) of exercise training-mediated protection against IR injury, which may be relevant to other IR-related injuries or diseases.
Author Contributions

R.J.W. designed the study, conducted experiments, analyzed and interpreted data, and wrote the manuscript. J.C.D. designed the study, interpreted data, and edited the manuscript, D.C., M.L.R., Y.G. M.Z., and L.M.L. performed experiments, analyzed data, provided technical support, and edited the manuscript. J.A.C., interpreted data and edited the manuscript. A.G. edited the manuscript. Z.Y. designed the study, interpreted data, and wrote the manuscript.

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Conflicts of interest

The authors have no conflict of interest to declare.
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Figure Legends

**Fig. 1. Long-term voluntary running preserves neuromuscular function following IR.** To determine whether endurance exercise training provides protection against IR injury-mediated loss of neuromuscular function, sedentary (Sed) and exercise-trained (Ex) mice were subjected to IR followed by measurements of muscle weight and muscle and nerve function 7 days after IR. (a) Peak isometric torque elicited by direct muscle stimulation prior to and during recovery from IR (* and *** denote $p<0.05$ and $p<0.001$; n = 6), and only statistical differences between sedentary and exercise are indicated. (b) Peak isometric torque of plantar flexors elicited by nerve stimulation prior to and during recovery from IR (* and *** denote $p<0.05$ and $p<0.001$; n = 6). For simplicity, only statistical differences between sedentary and exercise are indicated. (c) Gastrocnemius muscle wet weight (mg) normalized to tibia length (mm) to account for differences in body size (**$p < 0.001$; n = 6); (d) Torque-frequency relationship of muscle contractions elicited by direct muscle stimulation 7 days following IR (n=6); and (e) Force-frequency relationship of muscle contractions elicited by nerve stimulation 7 days following IR (n=6). Data are represented as mean ± SD.

**Fig. 2. Long-term voluntary running does not attenuate IR-induced oxidative stress in myofibers and motor nerve.** We measured mitochondrial and whole cell markers of oxidative stress in sedentary and exercise-trained mice following IR. (a) Representative immunoblots and quantification of expression of antioxidant proteins SOD1, SOD2, SOD3 and Catalase normalized by actin in skeletal muscle (* denotes $p < 0.05$, n = 5); (b) Representative immunoblot images and quantification of expression of antioxidant proteins SOD1, SOD2, SOD3 and Catalase normalized by actin in sciatic nerve (* denotes $p < 0.05$; n = 5); (c)
Representative confocal images and quantification of MitoTimer red:green ratio in skeletal muscle 3 hours after IR, scale = 25 μm, (** denotes p < 0.01; n = 4-7); (d) Representative confocal images and quantification of MitoTimer red:green ratio in sciatic nerve 3 hours after IR, scale = 25 μm, (** denotes p < 0.01; n = 4-7); (e) Representative immunoblot images and quantification of 4HNE in skeletal muscle (** denote p < 0.01; n = 6); and (f) Representative immunoblot images and quantification of 4HNE in sciatic nerve (*, **, and *** denote p < 0.05, p < 0.01, and p < 0.01, respectively; n = 6). Data are represented as mean ± SD.

Fig. 3. Long-term voluntary running renders myofibers resistant to IR. Morphological and biochemical evaluations of muscle damage were conducted following IR. (a) Representative images of H&E-stained transverse sections of skeletal muscle 3 hours after IR. Scale bar = 50 μm; (b) Serum creatine kinase activity 3 hours after IR (*, **, and *** denote p < 0.05, p < 0.01, and p < 0.001, respectively; n = 6); (c) Representative images of H&E-stained transverse sections of skeletal muscle 7 days after IR. Scale bar = 50 μm; and (d) Percentage of total myofibers with centralized nuclei (* denote p < 0.05; n = 6). Data are represented as mean ± SD.

Fig. 4. Long-term voluntary running attenuates denervation of skeletal muscle following IR. To elucidate the consequence of endurance exercise training on skeletal muscle innervation, muscles were collected from sedentary and exercise-trained mice following IR and innervation at NMJ and muscle denervation were measured using immunofluorescent techniques. (a) Representative confocal images of presynaptic motor neurons identified by Tuj1 (green) and postsynaptic acetylcholine receptors detected with α-bungarotoxin (red) and quantification of denervated NMJs 3 hours after injury. Scale bars = 20 μm (top panels) and 5 μm (bottom panels).
panels), respectively (*, *** denote $p < 0.05$ and 0.001, respectively; n=8); (b) Representative confocal images of transverse sections of plantaris muscle expressing cytosolic Ncam (red) and laminin (green), and DAPI staining (blue) and quantification of percentage of cytosolic Ncam$^+$ myofibers 7 days following IR. Mice in the sedentary group had a trend of increase toward significant ($p = 0.053$). Scale bar = 100 μm (n=5). Data are represented as mean ± SD.
Fig. 1

a. Muscle Stim

b. Nerve Stim

c. Sed Ex

d. Specific Torque (mN*m/mg)

e. Specific Torque (mN*m/mg)
Fig. 2

Skeletal Muscle

a. 

b. 

Sciatic Nerve

c. 

Skeletal Muscle

Sham          Sed          Ex

[Images of protein bands]

Red/Green ratio

**

[d.]

Sciatic Nerve

Sham          Sed          Ex

[Images of tissue images]

Red/Green ratio

**

e. 

Skeletal Muscle

[Images of protein bands]

4HNE

50kD          37kD

Red/Green ratio

**

f. 

Sciatic Nerve

[Images of protein bands]

4HNE

50kD          37kD

Red/Green ratio

**

*
Fig. 3

3h Post IR

a. Sham | Sed | Ex

b. Serum Creatine Kinase (units/L)

** *** *

7d Post IR

c. Sham | Sed | Ex

d. Centralized Nuclei (% Total)

*
Fig. 4

a. 3h Post IR
   Sham  Sed  Ex
   [Images of nerve terminal morphology]
   [Graph showing denervation ratio: Sham, Sed, Ex]

b. 7d Post IR
   Sham  Sed  Ex
   [Images of nerve terminal morphology]
   [Graph showing NCAM+ fibers: Sham, Sed, Ex]