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Treatment of Tourniquet-Induced Ischemia Reperfusion Injury with Muscle Progenitor Cells

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Background. **Acute ischemia reperfusion injury (IRI) results in muscle atrophy and functional loss. Although studies have shown that stem cells can improve muscle function in chronic ischemia caused by vascular diseases, none investigated whether stem cells can improve muscle function following acute IRI. The primary purpose of this study was to determine whether transplantation of muscle progenitor cells (MPCs) improves recovery of muscle function after tourniquet (TK) induced IRI.**

Methods. **IRI was induced in rat hind limb muscles with a pneumatic TK (250 mmHg) for 3 h. Rats were then divided into two groups; receiving either intramuscular injection of MPCs or vehicle control into the injured tibialis anterior muscle 48 h after tourniquet application. Muscle mass, isometric contractile properties, and selected histologic properties were evaluated at 2 wk after ischemia.**

Results. **IRI resulted in significant reductions in absolute muscle force (N) and specific muscle force (N/cm^2). MPC treatment significantly prevented the loss in muscle specific force compared with vehicle controls. The mass and cross sectional areas of the muscles were similar between treatment groups. Histologic results showed that a small number of transplanted cells differentiated and formed muscle fibers, which could potentially contribute to force generation. IRI caused significant fibrosis and inflammation, both of which could affect muscle-specific force, of which inflammation was reduced by MPCs treatment.**

Conclusions. **Intramuscular injection of MPCs may provide a beneficial treatment for improving functional recovery following IRI, and the beneficial effects are mainly through improving muscle quality (specific force) but not quantity (mass).** Published by Elsevier Inc.

Key Words: **muscle function; satellite cells; cell tracking; stem cells; skeletal muscle.**

INTRODUCTION

Extremity trauma constitutes the majority of war wounds [1], both historically, and during the current wars in Iraq and Afghanistan [2], and is a significant problem in civilian medicine [3]. Most of these wounds include muscle trauma, often involving acute ischemia reperfusion injury (IRI) due to vascular injury, emergency tourniquet (TK) application, and/or acute extremity compartment syndrome.

IRI is a complex process involving a cascade of events. Initially, ischemia results in muscle necrosis due to energy depletion and ion imbalance, and the subsequent reperfusion results in edema, inflammation, and excessive release of reactive oxygen and nitrogen species that leads to further muscle damage, causing muscle atrophy and weakness [4]. In the past two decades, numerous intervention strategies have been proposed aiming at reducing IRI. These strategies include thermomodulation [5], ischemia preconditioning [6], controlled reperfusion[7], reperfusion with various resuscitation fluids [8], and infusion or injection of agents aimed at reducing oxidative stress, inflammation, vascular injury, and to provide energy supply [9–11]. Many of these strategies have shown some level of success in reducing the extent of IRI when applied prior to, during, or immediately after ischemia [12–14], but few have shown benefits when applied at delayed time points [15]. This is an important distinction because

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in a trauma setting, pretreatments are impractical and treatments applied during, or immediately after ischemia are rarely feasible [16]. Conversely, a more appropriate approach may involve treatment at later time points with therapies designed to hasten and increase the magnitude of healing. To this end, cell-based therapies may provide a treatment option.

Studies using animal models of chronic partial ischemia treated with mesenchymal stem cells (MSCs) have demonstrated benefits [17, 18]. Additionally, a small number of clinical trials have reported some success in treating peripheral arterial disease with autologous bone marrow derived stem cells (BMSCs) [19, 20]. The majority of cell-based therapies have concentrated on BMSCs and, to a lesser extent, endothelial precursor cells [18]. Another potential source of progenitor cells is skeletal muscle. Transplantation of muscle progenitor cells (MPCs) has been shown to improve muscle function in animal models of muscular diseases, denervation, toxins, cryo-injuries, and volumetric muscle loss [21–24], and have been used to treat Duchenne dystrophy and cardiovascular diseases in clinical trials [25, 26]. Advantages of MPCs as an autologous cell source for transplantation include their abundance in skeletal muscles, high proliferative potential under culture conditions, commitment to myogenic lineage, and high resistance to ischemia [27]. In addition, recent observations suggest that MPCs may be beneficial for muscle repair based on their ability to support angiogenesis and neurogenesis [28, 29]. Despite the promising beneficial effects of MPCs in muscle repair in other animal models and in *in vitro* studies, their effects for treating acute IRI are untested. Therefore, the purpose of the current study was to determine whether the functional outcome of acute IRI could be improved by delayed treatment with MPCs.

METHODS

Animals

This study was conducted in compliance with the Animal Welfare Act, Implementing Animal Welfare Regulations, and in accordance with the principles of the Guide for the Care and Use of Laboratory Animals. All animal procedures were approved by the United States Army Institute of Surgical Research Animal Care and Use Committee. Adult Male Lewis rats weighing 400–450 g (Harlan Laboratories, Indianapolis, IN, USA) were housed in a vivarium accredited by the American Association for the Accreditation of Laboratory Animal Care, provided with food and water *ad libitum*.

Muscle Progenitor Cell Isolation, Culture

Muscle progenitor cells were isolated similar to that described by Lees *et al.* [30]. Briefly, soleus, plantaris, and gastrocnemius muscles of adult male Lewis rats were isolated, minced, and digested with 1.25 mg/mL pronase (Sigma, Saint Louis, MO, USA) in PBS at 37°C for 1 h. The pronase and tissue debris were then removed through differential centrifugation, and cells were preplated on 150 mm tissue culture-treated dishes in DMEM medium with 10% FBS for 2 h to remove contaminating fibroblasts. After preplating, cells were seeded onto matrigel-coated (0.1 mg/mL) 100 mm tissue culture-treated dishes in growth medium consisting of F-10 medium supplemented with 20% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 40 μg/mL gentamicin. Forty-eight hours after seeding, cells were infected with adenovirus as described below. After infection, when cells reached 70% confluence, they were trypsinized and used for transplantation. To verify the isolation procedure, MPCs were characterized by MyoD and desmin expression, and myogenicity was also confirmed by the ability to form myotubes and myosin heavy chain (MHC) expression when confluent cultures were treated with differentiation media (data not shown).

Labeling of MPCs

Adenoviral vector encoding LacZ gene was purchased from Vector BioLabs (Philadelphia, PA). MPCs were infected with adenoviruses at a multiplicity of infection (MOI) of 500 plaque-forming units (PFU)/cell in 10 mL F-10 medium containing 5% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 40 μg/mL gentamicin overnight.

Lentiviruses encoding luciferase and GFP were purchased from Targeting Systems (El Cajon, PA). MPCs were infected with lentiviruses at a MOI of 50 PFU/cell in 10 mL F-10 medium containing 20% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 40 μg/mL gentamicin for 24 h.

Ischemia Reperfusion

To induce injury, a tourniquet was applied to induce ischemia as previously described [31]. Briefly, animals ($n = 6$) were anesthetized with 1.5% to 2.5% isoflurane, a pneumatic digit tourniquet attached to a tourniquet regulation system was placed around either the right or left thigh (determined randomly) and inflated to a pressure of 250 mm Hg for 3 h. Forty-eight hours after TK application, animals were then randomly assigned into two treatment groups, receiving injections with either 0.1 mL saline containing MPCs (MPC) or 0.1 mL saline alone as vehicle control (Veh) per injection site. MPC injections containing a total of 10^6 MPCs were injected at a perpendicular angle into the injured tibialis anterior (TA) muscle at three sites for each muscle. The contralateral TA muscle was used as a noninjured control.

In *Vivo* Imaging

At 2, 7, and 14 d after MPC injection, animals ($n = 6$) were injected with luciferin at 100 mg/kg i.p. 15 min after injection, and animals were imaged in an IVIS imaging system (Caliper, Hopkinton, MA) under anesthesia at an exposure time of 30 min. The data were collected using Living Image 3.2 software (Caliperls, Hopkinton, MA, USA).

Muscle Contractile Properties

Two weeks after tourniquet application, *in situ* muscle contractile property measurements were performed as described previously [31]. Briefly, while animals were under anesthesia (1.5% to 2.5% isoflurane), a cuff electrode was placed around the peroneal nerve adjacent to the TA muscle for stimulation. Experimental limbs were stabilized by a transverse Steinmann pin drilled through the femur. Both sides of the pins were held securely onto a frame that was secured to a marble table. The distal TA tendon was dissected, cut, and secured to the lever of a dual-mode muscle lever system (Aurora Scientific, mod. 309b, Ontario, Canada). The muscle lever was controlled and data were acquired with a PC using a custom designed LabView-based program (National Instruments, Austin, TX, USA). The nerve was stimulated using a physiologic stimulator (A-M

Systems, model 2100 Isolated Pulse Stimulator, Carlsborg, WA, USA) at stimulus intensity of 2× the voltage required to elicit maximal peak twitch tension (Pt) and a pulse width of 500 μs. The muscle length was adjusted until maximum twitch tension was obtained, and all following measurements were made at this muscle length (Lo). Pt was determined from an average of two twitches (2 min between each twitch); peak tetanic force (Po) was determined from an average of 2 tetani separated by 2 min (150 Hz stimulation frequency; 300 ms train width). The optimal length was measured, and muscle was weighed, the physiologic cross sectional area (PCSA) was calculated using the following formula:

$$\text{PCSA} = \frac{M \cdot \cos\theta}{\rho \cdot L_f}$$

where M is the wet weight of the muscle (in g); θ is the angle of fiber pinnation (12.8 for TA); Lf is the mean fiber length (57% of TA muscle length); and ρ is muscle density (1.067 g cm^{-3}) [32]. Muscle-specific force was calculated by dividing Po by PCSA.

Tissue Processing and Histology

Following assessment of muscle function, the TA was snap-frozen in isopentane submerged in liquid nitrogen. Serial transverse sections of 10 μm thickness were collected at the proximal, medial, and distal part of the TA muscle using a cryostat. The sections were then stained with H&E, collagen, CD68, desmin, and X-gal. For collagen, CD68 and desmin stain tissue sections were blocked in PBS containing 0.5% Triton X-100 and 4% goat serum, and incubated in primary antibody solutions for desmin (1:100 dilution; BD Pharmingen, San Diego, CA, USA), CD68 (1:500 dilution; Chemicon, Temecula, CA, USA), and collagen (1:500 dilution; Millipore, Billerica, MA, USA) at 4°C overnight, then rinsed in PBS and incubated in corresponding Alexaflour 488 or 596 labeled secondary antibodies (1:500 dilution; Invitrogen, Carlsbad, CA, USA) at RT for 1 h. The sections were then dried and mounted in Fluoromount (Fisher Scientific, Pittsburgh, PA, USA).

Imaging and Quantitative Analysis

To quantify collagen and CD68 immunoreactivity in TA muscle, tissue samples were batch-stained and imaged using a fluorescent microscope under the same exposure time. Four 10× images, randomly selected and dispersed through each tissue section, were taken for each stain. Image-pro Plus software was used for analyses. A threshold was set to filter out the background nonspecific staining, and the same threshold value was used to filter all images. The areas that were stained for collagen I and CD68 were then normalized as a percentage of the area of the entire image.

Statistical Analysis

SPSS software (SPSS Inc., Chicago, IL) was used for all statistic analysis. ANOVA followed by Tukey *post hoc* analysis were used to determine differences among non-injured controls, Veh, and MPCs. Difference is considered significant when $P < 0.05$. All values are presented as mean ± standard error of the mean (SEM).

RESULTS

At 2 wk after the tourniquet application, there was a similar decrease in muscle mass and PCSA in both MPC-treated animals and the vehicle controls compared with noninjured controls (Fig. 1A, B). The maximal twitch force and tetanic force were not different between MPC and Veh (Fig. 1C, D) ($P = 0.345$). The average maximum twitch forces are 1.81 ± 0.75 N and 2.28 ± 0.36 N in Veh and MPC treated muscle, respectively (Fig. 1C). The average maximum tetanic forces are 3.27 ± 0.82 N and 4.32 ± 0.65 N in Veh and MPC treated muscle, respectively (Fig. 1D). The specific force (N/PCSA) (Fig. 1E) for MPC treated muscle was 39% higher than vehicle controls.

Luminescent signal was detected in injured leg at 48 h after MPC injection (Fig. 2A), suggesting acute engraftment. When cultured *in vitro*, the luminescent signal from luciferase labeled MPCs increases over time in accordance with cell proliferation (data not shown). In contrast, the *in vivo* luminescent signal decreased and disappeared by between d 2 and 7, which suggests the MPCs became apoptotic during this time. Histologic sections co-stained for LacZ and desmin indicate that all the LacZ positive cells are desmin-positive (Fig. 2B, C), suggesting the transplanted MPCs formed myocytes or fused with host myocytes. However, the number of lacZ positive myocytes represents a small portion of all of the muscle fibers.

There were a large number of centrally located nuclei that were similar between Veh and MPC, suggesting that there is extensive tissue regeneration at 2 wk in both groups (Fig. 3).

Immunohistochemical analysis showed that there is a similar increase in collagen deposition in the TA from Veh and MPC at 2 weeks (Fig. 4). In normal muscle, type I collagen constitutes less than 17% ± 2 % of tissue cross sectional area. However, the amount of type I collagen increased to 28% ± 5% and 26% ± 2% of cross sectional area in Veh and MPC treated animals, respectively. CD68 immunoreactivity, indicative of macrophage infiltration, was only found in the interfiber area, and was higher than noninjured controls in Veh, but not MPC (Fig. 5). There was a significant correlation between inflammation and collagen deposition ($r^2 = 0.72$; $P = 0.01$).

DISCUSSION

Previous studies by others showed that by using variety types of treatments, muscle injury caused by 3 h TK could be reduced at acute time points of 1, 2, 3, 4, 24, 48 h, and 7 d [9, 33–41]. However, there is a lack of a study showing beneficial effects of any treatment that lasts for a longer time period. Moreover, few treatments improved muscle functional recovery (Backer, 2005, Salm 1996). Our results showed that application of TK for 3 h impacts both muscle mass and function at 2 wk, which is consistent with previous findings by our laboratory and by others [31, 42]. MPC treatment improved muscle function at 14 d. To the best of our knowledge, this is the first study that showed

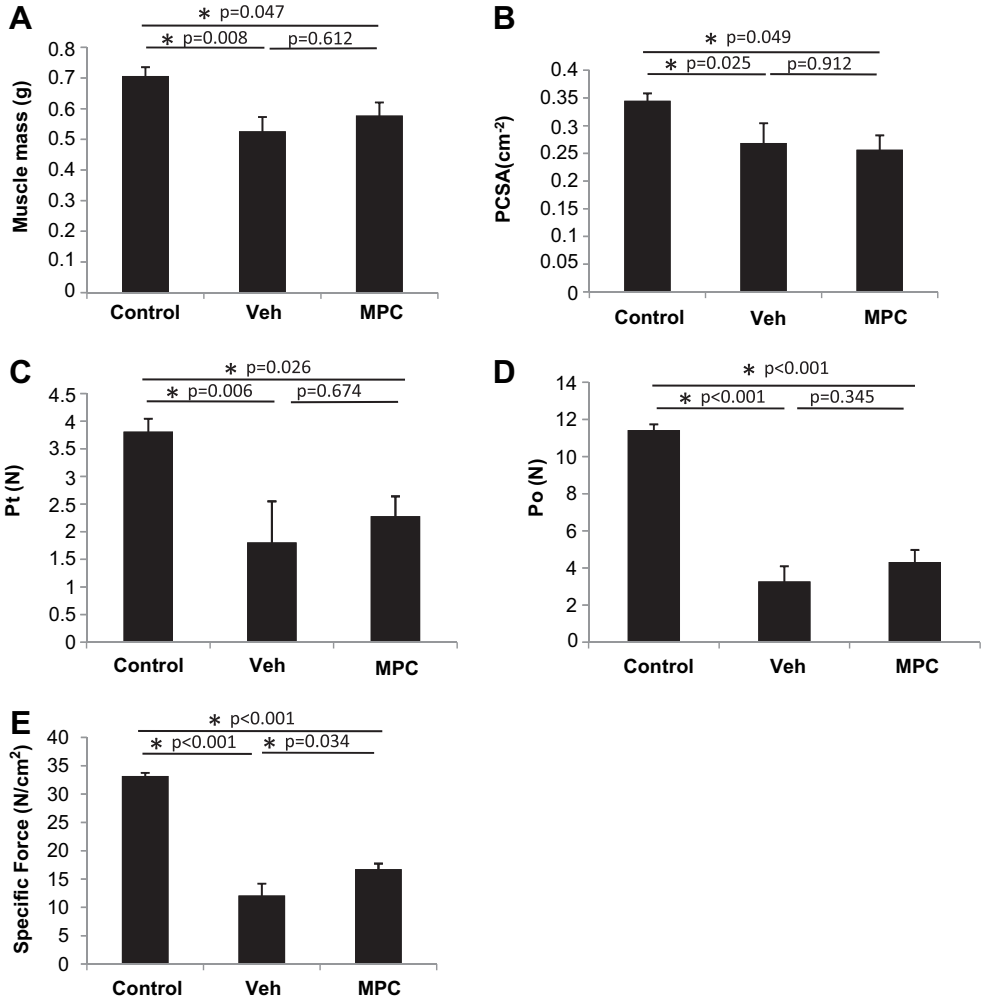

FIG. 1. Muscle mass (A), physiological cross sectional area (PCSA) (B), maximum twitch force (Pt) (C), Maximum tetanic force (Po) (D), and muscle specific force (E) at 2 wk after tourniquet application. Values are expressed as mean ± SEM. *Significance at $P < 0.05$.

a treatment improved muscle recovery following 3 h TK at a prolonged time point, and this is also the first study that investigated the potential for MPCs to improve muscle function, as well as other indices of regeneration including collagen deposition and macrophage infiltration following acute IRI.

Our decision to apply MPC treatment 48 h post-TK was based on the rationale that this would correspond to the earliest time at which such a treatment could be administered clinically. This is based on the current battlefield trauma scenario, which involves stabilization in theatre, followed by air transport for definitive care at Landstuhl Regional Medical Center, Germany. The optimal time points for MPC delivery needed future investigation. Nonetheless, this is the first paper showing that a delayed treatment improved muscle recovery following IRI.

Functional loss is a combined effect of loss in muscle mass (quantity) and specific force (quality). Others have reported a decrease in specific force following acute IRI [31, 43]. In our study, the 70% loss in absolute muscle force in Veh controls resulted from the 20% loss in muscle mass and 60% loss in muscle-specific force; thus the observed deficits in force production are primarily attributable to the loss of specific force. While Po was not statistically different in the MPC group, specific force was significantly less reduced. Although the mechanism of loss in muscle-specific force following IRI is unclear, studies in aging and sports injury models show that muscle-specific force can be reduced by (1) compromised excitation-contraction coupling; (2) disruption and/or loss of proteins involved in force generation and transmission; and (3) increase in non-contractile element in muscle tissue [44–46]. It is therefore likely that MPC transplantation in our study resulted in improved specific force by influencing one or a combination of these factors. Regardless of the mechanism, the relative improvement in specific force with MPC transplantation indicates an improvement in the quality or health of the muscle compared to the Veh treated group.

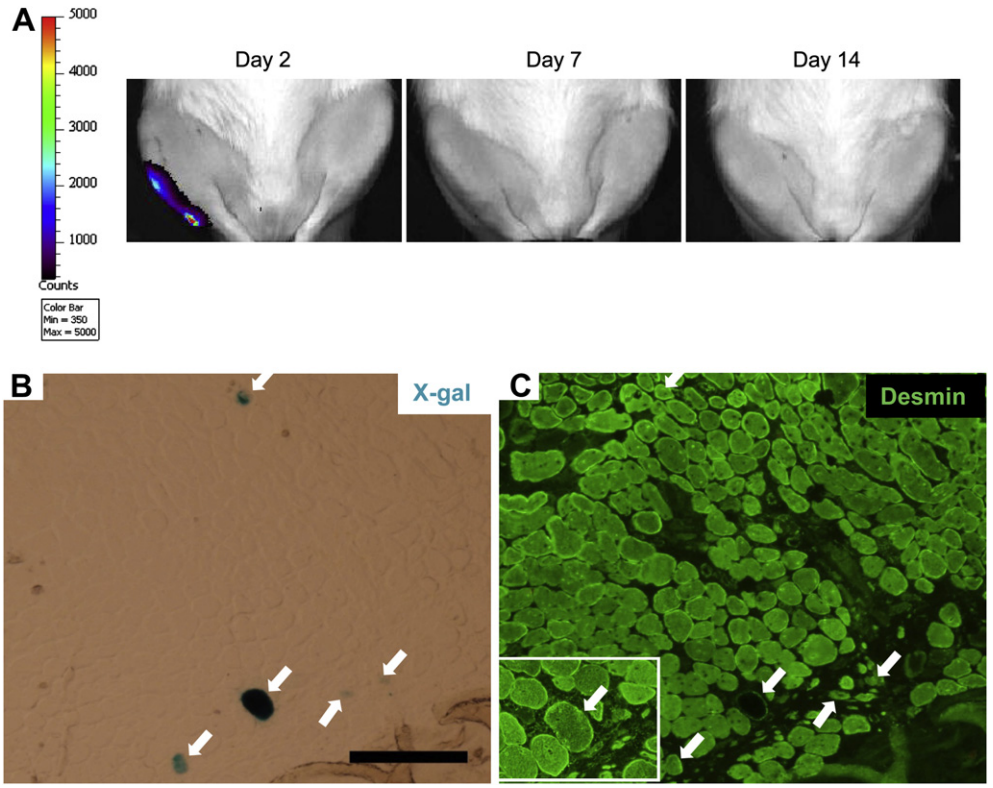

FIG. 2. Tracing of transplanted MPCs. (A) *In vivo* luminescent signal at 2, 7, and 14 d after MPC injection. (B), (C) A muscle tissue section double-stained with X-gal (B) and desmin (C). The white arrows point at myocytes that are X-gal positive (B). Note these cells are also desmin-positive (C). The lack of desmin stain in the myofiber labeled with asterisk is due to heavy x-gal substrate deposition, blocking the access of immunostaining reagent. The insert showed an adjacent section stained with desmin only, proving the fiber is desmin-positive. Scale bar = 250 μm.

The contribution of the few myofibers generated from transplanted MPCs on improving force generation is likely to be minimal since the majority of injected MPCs died by d 7 based on the *in vivo* cell tracing results. It is unlikely that the cell death is due to labeling with virus since muscles treated with MPCs labeled with virus have similar functional recovery as those treated with MPCs without labeling (unpublished data from collaborating lab). The beneficial effect of MPCs on functional recovery is more likely through secretive factors during the acute phase. For example, MPCs' beneficial effect on muscle recovery of function could be through encouraging host myocyte regeneration and preventing apoptosis by secreting trophic factors and anti-apoptotic molecules, which is a known property of transplanted precursor cells [47, 48]. We did not observe difference in the number of myofibers with central nuclei at 14 d, possibly because (1), the number of regeneration fiber in MPC treated group is higher at earlier time points, and (2), the reduced apoptosis played a role, which will be investigated in future studies. The beneficial effect of MPCs could also be through reducing inflammation. Inflammation can reduce muscle-specific force by interfering with contractile protein synthesis and degradation, altering contractile protein force generation capacity, and affect E-C coupling by reducing calcium sensitivity and causing mitochondria dysfunction through secretion of inflammatory cytokines and reactive oxygen and nitrogen species [49–51]. In addition, inflammatory cells can induce fibrosis through secretion of TGF- β1 and MCP-1 [52, 53], thus increasing non-contractile elements in muscle tissue. Therefore, inflammation and collagen deposition, both of which significantly increased at 2 wk after TK application, may provide a potential explanation to the loss of muscle specific force. Our observation of decreased CD68 immunoreactivity with MPC transplantation suggests that the MPCs may have reduced inflammation. Several lines of evidence support the ability of mesenchymal stem cells to reduce inflammation [54, 55]; however, whether or not transplanted MPCs have the same capacity in the context of IRI remains to be determined. Support for this idea is drawn from the observation that transplantation of MPCs together with decellularized muscle derived ECM reduced inflammatory response and fibrotic tissue formation compared with ECM implanted alone [56], suggesting that MPCs have anti-inflammatory and anti-fibrotic effects. Although we did find statistical difference in fibrosis between MPC treated group and the saline controls, there is a trend that less fibrotic tissue was present in

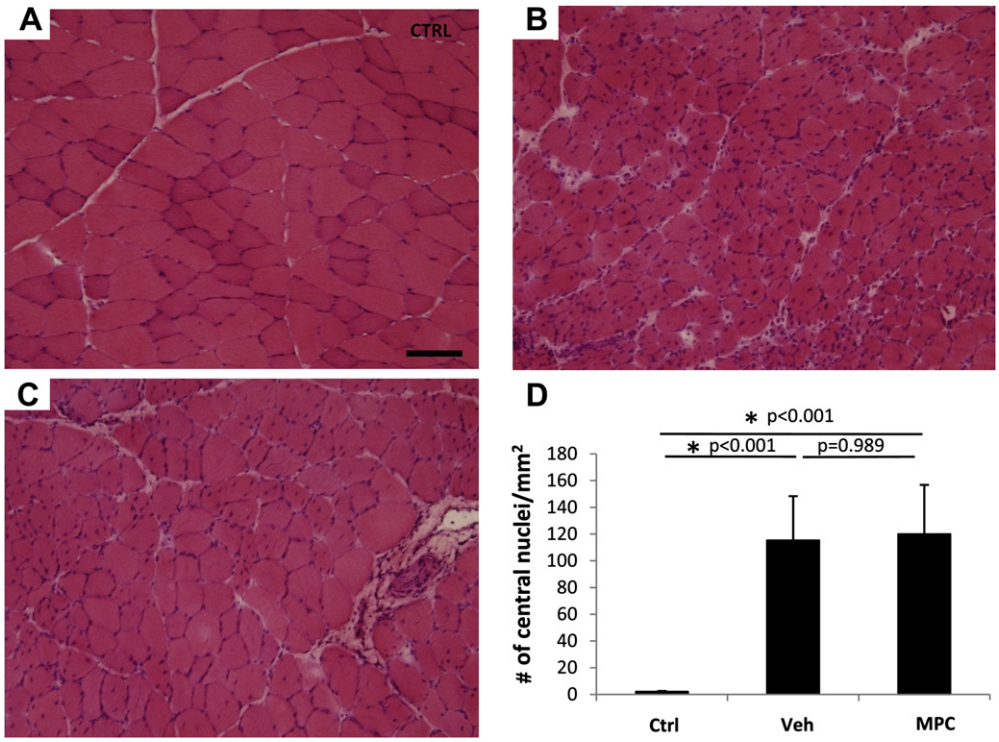

FIG. 3. Representative bright field images of muscle tissue sections stained for hematoxylin (cytoplasm) and eosin (nuclei) in noninjured control (A), Veh (B), and MPC (C) treated muscle 2 wk after tourniquet application. (D) Quantitative comparison of amount of myofibers with central nuclei between groups. *Significance at $P < 0.05$ compared with non-injured controls. Scale bar = 100 μm.

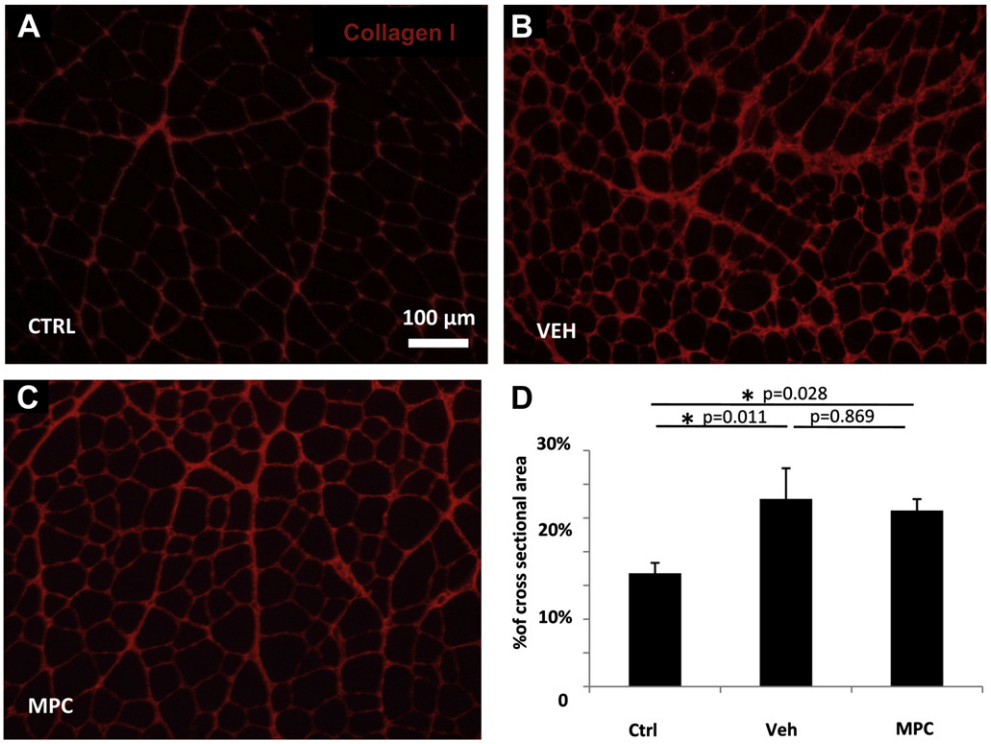

FIG. 4. (A–C) Representative fluorescent images of muscle tissue sections stained for type I collagen. (D) Quantitative comparison of collagen deposition between groups. Values are expressed as mean ± SEM. *Significance at $P < 0.05$ compared with non-injured controls.

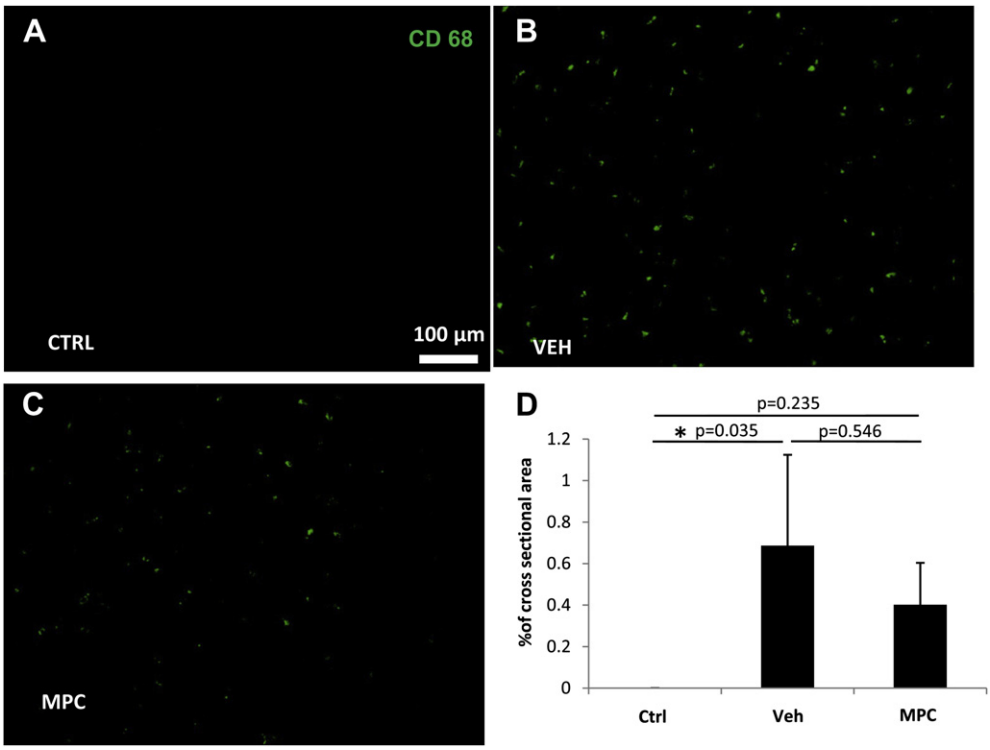

FIG. 5. (A–C) Representative fluorescent images of muscle tissue sections stained for CD68. (D) Quantitative comparison of CD68 immunoreactivity between groups. Values are expressed as mean ± SEM. *Significance at $P < 0.05$ compared with non-njured controls.

MPC treated muscles. More animals will be needed to increase statistic power for future studies.

The presence of denervated muscle fibers can also increase the proportion of non-contractile elements and decreased specific force [22]. However, it is unlikely that nerve injury contributes to the loss of specific force, as results from our previous study and others showed that nerve injury under similar ischemic condition is only acute and force generated through direct muscle stimulation is similar to that generated through nerve stimulation at 2 wk after TK application, suggesting a lack of nerve injury at this time point [57].

The presence of a large quantity of muscle cells with central nuclei in IRI muscles suggests intensive and incomplete muscle regeneration at 2 wk post-injury. The number of regenerating cells is similar between Veh and MPC. Thus, it is not possible to determine if the improved function in the MPC muscles represents only accelerated healing, or will also contribute to improved healing at the completion of the regeneration process. A long-term study is needed to answer this question.

It is common practice to expand cell numbers through multiple passages, primarily because it is believed that injecting a higher number of cells results in improved outcomes. To the contrary, it has been shown that stem cells derived from skeletal muscle may lose their reparative potential with multiple passages [58]. We therefore chose to perform all experiments using cells that were passaged once. Our data suggested that the transplanted MPCs formed myotubes improved muscle function, but did not improve muscle mass. Whether the loss of muscle mass can be reduced when a larger number of cultured MPCs [22] or whether freshly isolated cells are injected [59] remains to be determined.

In conclusion, our data showed that acute IRI injury caused a loss in muscle quantity and most importantly muscle quality as reflected by decrease in specific force. Inflammation and fibrosis accompanied the loss in specific force. Delayed intramuscular transplantation of muscle MPC cells improved muscle function after acute IRI injury by improving muscle quality, possibly through forming functional myofibers and effects on non-myofiber related components. Our results suggested that cell therapy with MPCs could potentially be used as a new intervention strategy for delayed treatment of acute IRI.

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