Satellite cell proliferation and myofiber cross-section area increase after electrical stimulation following sciatic nerve crush injury in rats

Hua-Yi Xing, Nan Liu, Mou-Wang Zhou

Department of Rehabilitation Medicine, Peking University Third Hospital, Beijing 100191, China.

Abstract

Background: Electrical stimulation has been recommended as an effective therapy to prevent muscle atrophy after nerve injury. However, the effect of electrical stimulation on the proliferation of satellite cells in denervated muscles has not yet been fully elucidated. This study was aimed to evaluate the changes in satellite cell proliferation after electrical stimulation in nerve injury and to determine whether these changes are related to the restoration of myofiber cross-section area (CSA).

Methods: Sciatic nerve crush injury was performed in 48 male Sprague-Dawley rats. In half (24/48) of the rats, the gastrocnemius was electrically stimulated transcutaneously on a daily basis after injury, while the other half were not stimulated. Another group of 24 male Sprague-Dawley rats were used as sham operation controls without injury or stimulation. The rats were euthanized 2, 4, and 6 weeks later. After 5-bromo-2'-deoxyuridine (BrdU) labeling, the gastrocnemia were harvested for the detection of paired box protein 7 (Pax7), BrdU, myofiber CSA, and myonuclei number per fiber. All data were analyzed using two-way analysis of variance and Bonferroni *post-hoc* test.

Results: The percentages of Pax7-positive nuclei ($10.81 \pm 0.56\%$) and BrdU-positive nuclei ($34.29 \pm 3.87\%$) in stimulated muscles were significantly higher compared to those in non-stimulated muscles ($2.58 \pm 0.33\%$ and $1.30 \pm 0.09\%$, respectively, Bonferroni $t = 15.91$ and 18.14, $P < 0.05$). The numbers of myonuclei per fiber (2.19 ± 0.24) and myofiber CSA (1906.86 ± 116.51 μm^2) were also increased in the stimulated muscles (Bonferroni $t = 3.57$ and 2.73, $P < 0.05$), and both were positively correlated with the Pax7-positive satellite cell content ($R^2 = 0.52$ and 0.60, $P < 0.01$). There was no significant difference in the ratio of myofiber CSA/myonuclei number per fiber among the three groups.

Conclusions: Our results indicate that satellite cell proliferation is promoted by electrical stimulation after nerve injury, which may be correlated with an increase in myonuclei number and myofiber CSA.

Keywords: Skeletal muscle satellite cells; Peripheral nerve injuries; Electric stimulation therapy; Pax7 transcription factor; Cell proliferation

Introduction

The denervation of skeletal muscle results from the injury of the peripheral nerve and is observed in various diseases and types of trauma. Denervated muscles undergo irreversible atrophy, which is characterized by a reduction of the myofiber cross-section area (CSA) and strength.[1] Several studies have found that the loss of myonuclei is proportional to the reduction of myofiber CSA, such that the ratio of myofiber CSA/myonuclei number per fiber is maintained after denervation.[2,3] This ratio is defined as the myonuclear domain (MND). In functionally overloaded and exercised rat muscles, an increase in the myonuclei number is associated with an increase in the myofiber CSA, resulting in a stable MND of the hypertrophic muscle, which does not change.[4] The principle of the MND hypothesis suggests that each myonucleus is responsible for a defined volume of cellular territory within a myofiber.[5,6] The gene transcription and protein synthesis of myofibers are controlled by the myonuclei in each individual MND. As a result, changes in myofiber CSA and myonuclei content are coupled with each other.[7]

It is generally accepted that satellite cell proliferation is the main source of new myonuclei for mature muscle fibers.[8,9] Once stimulated in pathologic situations or by exogenous factors, such as myofiber damage and passive stretching, quiescent satellite cells can be activated and begin to proliferate and fuse with the fibers adjacent to them.[10] Paired box protein 7 (Pax7), a transcription factor, marks both quiescent and activated satellite cells.[11,12] Interestingly, although there is severe reduction in the myonuclei

Access this article online

Quick Response Code:

Website: www.cmj.org

DOI: 10.1097/CM9.0000000000000822

Correspondence to: Prof. Mou-Wang Zhou, Department of Rehabilitation Medicine, Peking University Third Hospital, 49 North Garden Road, Beijing 100191, China
E-Mail: zhoumouwang@163.com

Chinese Medical Journal 2020;133(16)

Received: 05-03-2020 **Edited by:** Ning-Ning Wang

number and myofiber CSA after nerve injury, satellite cells in denervated muscles always fail to proliferate appropriately and cannot compensate for the loss of myonuclei.[13-15] Furthermore, in long-term denervated skeletal muscles in adult rats, both the population and local density of satellite cells are progressively reduced due to the exhaustion of the satellite cell pool.[16,17] According to the MND hypothesis, the decrease of myofiber CSA in denervated muscles may be partly attributed to the defect of satellite cell proliferation and, consequently, the decrease of the number of myonuclei.

Electrical stimulation has been recommended as an effective therapeutic tool for patients with nerve injury.[1] It has been previously demonstrated that electrical stimulation can be used to maintain the force and mass of skeletal muscles.[18-21] The myofiber CSA of the denervated muscles can also be gradually restored by electrical stimulation.[22,23] However, the underlying mechanisms of these therapeutic effects are not yet fully understood. Previous studies have found that satellite cell proliferation is improved by electrical stimulation and loading exercise, both in healthy animals and animals with disuse atrophy/metabolic syndrome.[10,24-27] Thus, we hypothesized that electrical stimulation may promote satellite cell proliferation and increase the total myonuclei number after nerve injury. As a result, the myofiber CSA could proportionally increase to allow for the MND to remain stable.

The purpose of this study was to determine whether electrical stimulation increases the satellite cell content and proliferation after nerve injury. Moreover, we evaluated how the myonuclei content, myofiber CSA, and MND changed after nerve injury with or without electrical stimulation and investigated whether changes in myofiber CSA were correlated with enhanced satellite cell proliferation.

Methods

Ethical approval

The protocol of this study was approved by the Committee on the Ethics of Animal Experiments of Peking University (No. LA2013-78). All animal procedures were carried out with strict accordance to the recommendations of the Chinese Laboratory Animal Requirements of Environment and Housing Facilities.

Animals

Seventy-two adult male Sprague-Dawley rats (supplied by Department of Laboratory Animal Science, Peking University Health Science Center, China) weighing 280 to 300 g were randomly separated into three groups using a random number table (each $n = 24$): (1) sham operation controls (Sham), (2) sciatic nerve crush injury models (Injury), and (3) sciatic nerve crush injury + daily electrical stimulation (Injury + Stim). This method ensured an adequate sample size for histologic study, flow cytometry analysis, and Western blotting ($n = 8$ for each).

Sciatic nerve crush injury procedure

Surgery was performed as previously described.[28] Briefly, animals were anesthetized with intra-peritoneal sodium pentobarbital (5 mg/100 g body weight). The skin on the lateral side of the left hindlimb was shaved and prepared with 75% alcohol. For animals in the Injury and Injury + Stim groups, the left sciatic nerve was exposed and crushed 10 mm above the bifurcation for 30 s with a serrated clamp which exerted a force of about 100 N. For animals in the Sham group, the same procedures were performed without crushing the nerve.

Electrical stimulation

For animals in the Injury + Stim group, electrical stimulation began from the first day post-injury and was applied daily thereafter until the endpoint. To this end, the animals were anesthetized lightly with intra-peritoneal sodium pentobarbital (3 mg/100 g body weight). The left gastrocnemius was stimulated using two surface electrodes connected with a stimulator (ZS Dichuang, Beijing, China) for 30 min each day. The stimulation parameters were follows: pulse rate = 2 Hz; pulse width = 300 ms; volts = 25 V; current = 1 mA. Similar procedures and parameters of stimulation have been previously used in other studies and in our own research.[29-31] The animals in the Sham and the Injury groups were anesthetized but not stimulated.

5-Bromo-2'-deoxyuridine labeling and animal endpoint

Animals were randomly chosen from each of the three groups and euthanized at 2, 4, and 6 weeks post-injury (wpi), respectively (for each group at each time point, $n = 8$). Three days before being euthanatized, the animals were injected intra-peritoneally with 5-bromo-2'-deoxyuridine (BrdU; Sigma-Aldrich, St. Louis, MO, USA; 50 mg/kg body weight) dissolved in sterile physiologic saline (10 mg/mL) every 12 h for 3 days. One hour after the sixth injection, the animals were sacrificed by anesthetizing with intra-peritoneal sodium pentobarbital (10 mg/100 g body weight), followed by cervical dislocation. The left gastrocnemius of each animal was harvested for hematoxylin-eosin (HE) staining, immunofluorescence, flow cytometry, and Western blotting analyses.

HE staining

Regular HE staining was performed on 5-μm-thick paraffin-fixed cross-sections of muscle samples, as previously described.[32] The measures of the myofiber CSA (μm^2) and the myonuclei number per fiber were obtained using Image Pro Plus (Media Cybernetics, Rockville, MD, USA). The MND was calculated as follows: myofiber CSA/myonuclei number per fiber in μm^2.

Immunofluorescence for laminin, myonuclei, and Pax7

About 8-μm-thick frozen cross-sections of the muscle samples were permeabilized in 0.2% Triton X-100/phosphate-buffered saline (PBS) and blocked with 1% bovine serum albumin (BSA)-PBS. The sections were then

co-incubated with primary antibodies targeting laminin (chicken polyclonal antibody; 1:500; Abcam, Cambridge, UK) and Pax7 (rabbit polyclonal antibody; 1:200; Abcam), followed by secondary antibodies (goat anti-chicken immunoglobulin G [IgG]-conjugated tetramethylrhodamine and goat anti-rabbit IgG-conjugated tetramethylrhodamine (TRITC) and goat anti-rabbit IgG-conjugated daylight 488; Abcam). Sections were mounted with Hoechst 33342 (Sigma-Aldrich) for nuclei identification. The percentage of Pax7-positive satellite cell nuclei to the total nuclei was calculated using a fluorescence microscope (Leica, Oskar-Barnack-Straße, Solms, Germany).

Flow cytometry for BrdU detection

The muscle sample was digested with 0.2% type II collagenase for 1 h, followed by digestion with 0.01% type I collagenase and 0.05% dispase for 30 min. This sequential digestion approach has been performed in previous studies to yield a preparation of satellite cells with a high purity (about 95%, as reported in previous studies).[33,34] The purity of this satellite cell preparation was confirmed via flow cytometry using a mouse anti-rat Pax7 monoclonal antibody (1:100; Abcam) and a goat anti-rat IgG-conjugated Alexa Flour 488 (1:500; Molecular Probes, Carlsbad, CA, USA). Since skeletal muscle nuclei are post-mitotic, any BrdU-positive nuclei were considered as proliferated satellite cells.[25] For intra-nuclear BrdU staining, the satellite cell suspension was washed with cold PBS, then fixed and permeabilized using Foxp3/transcription factor staining buffer set (eBioscience, San Diego, CA, USA), blocked with 0.5% bovine serum albumin-PBS, and incubated with a mouse monoclonal antibody against BrdU labeled with FITC (1:60; Abcam) for 1 h at room temperature. The cell suspension was then analyzed using a fluorescence-activated cell sorting system (BD, Franklin Lakes, NJ, USA). The percentage of BrdU positivity was recorded as an index of satellite cell proliferation.

Western blotting

Western blotting was performed as previously described.[18] Briefly, the total-tissue protein of each muscle sample was extracted, and the protein concentration was determined using the bicinchoninic acid method. Protein samples were analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (CWBIO, Beijing, China) (30 μg/lane), followed by Western blotting. The antibodies used for protein detection included mouse anti-rat Pax7 polyclonal antibody (1:1000; Abcam), mouse anti-rat glyceraldehyde 3-phosphate dehydrogenas (GAPDH) monoclonal antibody (1:500; Abcam) as a loading control, and goat anti-mouse IgG-conjugated IRdye 800 (LI-COR, Lincoln, NE, USA). The resulting bands were densitometrically scanned and quantitatively evaluated using the Odyssey system (LI-COR). The ratio of the Pax7 band density to that of the GAPDH (relative $A \times$ area) was calculated using ImageJ (National Institute of Health, Bethesda, Maryland, USA). The average density ratios of different groups could thus be compared. The samples from the Injury, Injury + Stim, and Sham-6 wpi groups were run simultaneously on the same gel, while the samples from the Sham-2 wpi and the Sham-4 wpi groups were run on another gel.

Statistical analysis

All the data were presented as the mean ± standard error of the mean and analyzed by two-way analysis of variance. Statistical analysis was performed using SPSS 16.0 (IBM, NY, USA). When a significant F ratio was found, Bonferroni *post-hoc* test was used to locate the differences. Linear regression analysis was performed between the percentage of Pax7-positive satellite cells and myofiber CSA, as well as between the percentage of Pax7-positive satellite cells and myonuclei number per fiber. Differences were considered significant when $P < 0.05$.

Results

Myofiber CSA, myonuclei content, and mean MND

Figure 1 shows typical images of HE staining obtained for the Sham, Injury, and Injury + Stim groups at 6 wpi. Data on myofiber CSA and myonuclei number per fiber are presented in Table 1. By 2 wpi, the myofiber CSA of the Injury group was 1357.79 ± 89.69 μm^2, which was significantly lower than that of the Sham group (2225.4 ± 91.8 μm^2, Bonferroni $t = 5.90$, $P < 0.01$). At 4 and 6 wpi, the myofiber CSA of the Injury group further decreased to 1105.9 ± 93.7 μm^2 and 1095.1 ± 115.9 μm^2, respectively. No significant differences were observed between 6 and 4 wpi (Bonferroni $t = 1.23$, $P > 0.05$). In the Injury + Stim group, myofiber CSA was also significantly reduced compared to the Sham group, but electrical stimulation produced a significant improvement in myofiber CSA at 2 wpi (1538.2 ± 92.1 μm^2) compared to the Injury

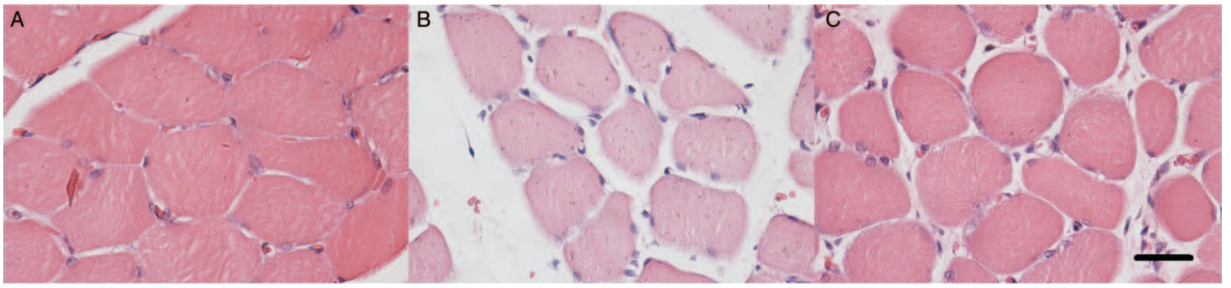

Figure 1: Typical images of HE staining for cross-section of left gastrocnemius from (A) Sham, (B) Injury, and (C) Injury + Stim groups at 6 wpi. Scale bar: 10 μm. HE: Hematoxylin-eosin; Stim: Stimulation; wpi: Weeks post-injury.

Table 1: The myofiber CSA and the myonuclei number per fiber of the Sham, Injury, and Injury + Stim group at 2, 4, and 6 wpi (each $n = 8$).

	Myofiber CSA (μm^2)			Myonuclei number per fiber		
Time	Sham	Injury	Injury + stim	Sham	Injury	Injury + stim
2 wpi	2225.4 ± 91.8	1357.8 ± 89.7[*]	1538.2 ± 92.1[*,†]	2.45 ± 0.37	1.39 ± 0.21[*]	1.73 ± 0.15[*,†]
4 wpi	2197.1 ± 122.0	1105.9 ± 93.7[*,‡]	1796.3 ± 102.0[*,†,‡]	2.63 ± 0.20	1.15 ± 0.08[*,‡]	1.92 ± 0.09[*,†,‡]
6 wpi	2309.1 ± 106.8	1095.1 ± 115.9[*,‡]	1906.9 ± 116.5[*,†,‡,§]	2.58 ± 0.28	1.10 ± 0.16[*,‡]	2.19 ± 0.24[*,†,‡,§]

Data are presented as mean ± SEM. [*] $P < 0.05$, significantly different from Sham at the same time point. [†] $P < 0.05$, significantly different from the Injury group at the same time point. [‡] $P < 0.05$, significantly different from the same group at 2 wpi. [§] $P < 0.05$, significantly different from the Injury + Stim group at 4 wpi. CSA: Cross-section area; SEM: Standard error of the mean; Stim: Stimulation; wpi: Weeks post-injury.

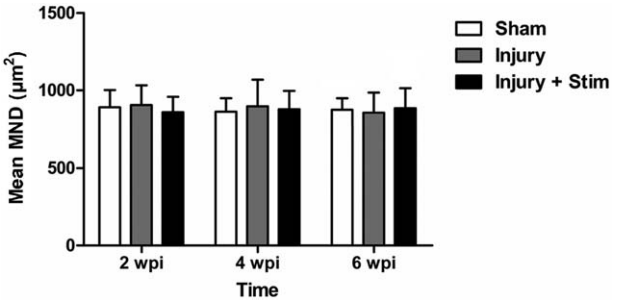

Figure 2: Data of the mean MND. No significant differences were found between the three groups or the different time points. Each $n = 8$. MND: Myonuclear domain; Stim: Stimulation; wpi: Weeks post-injury.

group (Bonferroni $t = 4.67$, $P < 0.01$), and further improvement were observed at 4 wpi (1796.3 ± 102.0 μm^2) and 6 wpi (1906.9 ± 116.5 μm^2), although it was still significantly lower than that in the Sham group at the same time point (Bonferroni $t = 2.72$ and 2.73, $P < 0.05$).

The myonuclei number per fiber of the Injury group was significantly reduced to 1.39 ± 0.21 at 2 wpi compared to the Sham group (2.45 ± 0.37, Bonferroni $t = 3.47$, $P < 0.01$), and obtained even less at 4 wpi (1.15 ± 0.08, Bonferroni $t = 4.85$, $P < 0.01$). No significant difference was observed between 6 wpi (1.10 ± 0.16) and 4 wpi (Bonferroni $t = 2.35$, $P > 0.05$). In contrast, the myonuclei number per fiber of the Injury + Stim group gradually increased over weeks. The myonuclei number per fiber in the Injury + Stim group was 1.73 ± 0.15 at 2 wpi, 1.92 ± 0.09 at 4 wpi, which was significantly higher than 2 wpi (Bonferroni $t = 2.52$, $P < 0.05$), and even higher at 6 wpi (2.19 ± 0.24, Bonferroni $t = 3.57$, $P < 0.01$), although still significantly lower than that in the Sham group.

Data on the mean MND are provided in Figure 2. The mean MND of the Sham group at 2 wpi was 908.34 ± 100.76 μm^2, representing a normal level in healthy adult rats. No significant differences were found between the three groups or different time points ($F = 0.16$, $P > 0.05$).

Content and proliferation of satellite cells

A typical image of a Pax7-positive satellite cell is provided in Figure 3A. In the Sham group, satellite cells accounted for 2.8 ± 0.47%, 2.96 ± 0.36%, and 2.73 ± 0.42% of total nuclei at 2, 4, and 6 wpi. No significant change was observed during the 6 weeks (Bonferroni $t = 0.24$, $P > 0.05$). Similar results were found in the Injury group (2.94 ± 0.39%, 3.16 ± 0.45%, and 2.58 ± 0.33% at 2, 4, and 6 wpi), and no significant differences were observed over time post-injury (Bonferroni $t = 0.33$, $P > 0.05$). Moreover, no significant differences were observed between the Sham group and the Injury group at each of the three time points. In contrast, the proportion of the satellite cells in the Injury + Stim group increased with time post-injury, with a percentage of 6.09 ± 0.32% at 2 wpi, which was about two-fold higher than that of the Sham group. At 4 and 6 wpi, this number increased further to 9.41 ± 0.44% and 10.81 ± 0.56%, respectively. This was significantly higher than at 2 wpi (Bonferroni $t = 10.75$ and 13.47, $P < 0.01$). The western blotting results for Pax7 detection revealed similar findings, showing statistical differences observed between the Sham and the Injury + Stim groups, the Injury and the Injury + Stim groups, but not between the Sham and the Injury groups at each time point [Figure 3B–D].

The results of flow cytometry analysis are shown in Figure 4. Compared to the negative control, 97.23% of the cells in the suspension prepared for BrdU detection were Pax7 positive, indicating a high purity of satellite cells [Figure 4A]. Figure 4B illustrated an example of two distinguishing flow cytometric curves from the Sham group-4 wpi and the Injury + Stim group-4 wpi. In the Sham group and the Injury group, the BrdU-positive satellite cells were hardly detectable, with an extremely low percentage of 1.28 ± 0.06% and 1.30 ± 0.09%, respectively, at 2 wpi and without significant changes up to 6 wpi ($F = 0.24$, $P > 0.05$), suggesting that the proliferation of satellite cells in these groups was very rare. On the contrary, the average percentage of the BrdU-positive satellite cells in the Injury + Stim group was 22.49 ± 2.85% at 2 wpi, and a significantly higher percentage of 43.51 ± 3.64% was observed at 4 wpi. These results were significantly different from those of the Sham and Injury groups. At 6 wpi, the BrdU positive satellite cells significantly decreased to 34.29 ± 3.87%, which was lower than the 4 wpi (Bonferroni $t = 15.88$, $P < 0.01$), but still visibly above the level of the 2 wpi (Bonferroni $t = 7.95$, $P < 0.01$) [Figure 4C].

Correlation between myofiber CSA, myonuclei content, and satellite cell content

Using linear regression analysis, we found a striking and significant correlation between the percentage of

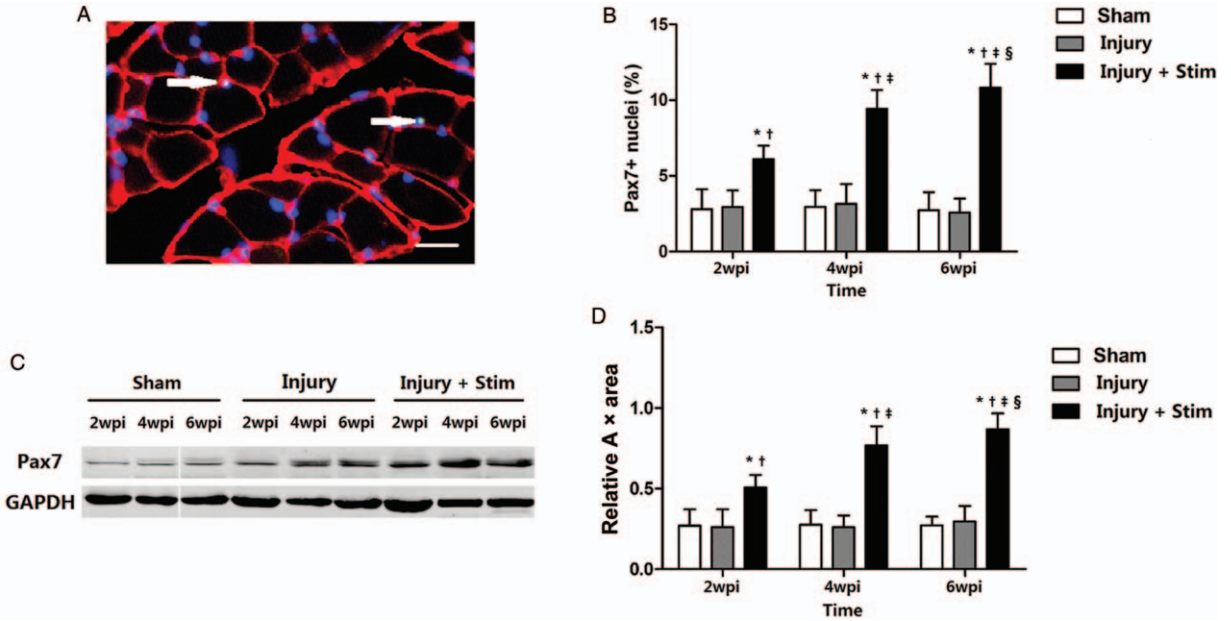

Figure 3: Representative image of the muscle cross-section, with Pax7 (green), myonuclei (blue; Hoechst 33342), and laminin (red) merged. The white arrows denote typical satellite cells; scale bar: 10 μm (A). The percentages of Pax7-positive nuclei to the total nuclei (B). Pax7 expression detected by Western blotting (C). Statistical results of band intensity (D). Each $n = 8$. $^{*}P < 0.05$, significantly different from the Sham group at the same time point; $^{\dagger}P < 0.05$, significantly different from the Injury group at the same time point; $^{\ddagger}P < 0.05$, significantly different from the Injury + Stim group at 2 wpi; $^{\S}P < 0.05$, significantly different from the Injury + Stim group at 4 wpi. GADPH: Glyceraldehyde-3-phosphate dehydrogenase; Stim: Stimulation; wpi: Weeks post-injury.

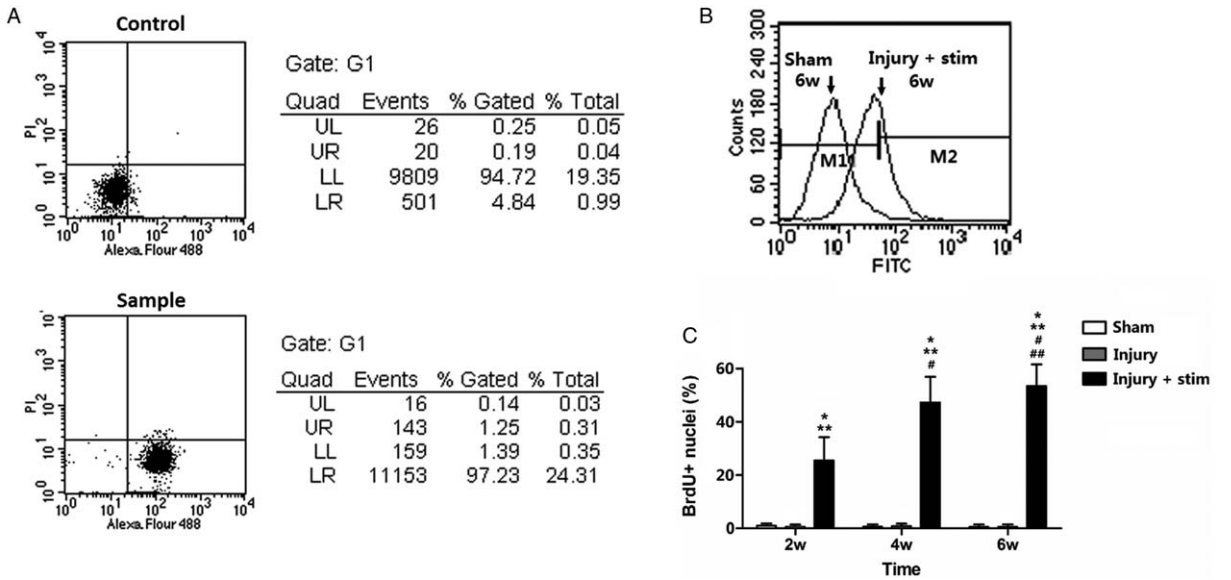

Figure 4: (A) Percentage of Pax7-positive cells in the suspension detected by flow cytometry. (B) The FITC-positive curve in the Injury + Stim group at 4 wpi shifted to a higher value compared to the Sham group at 4 wpi. (C) Percentages of BrdU-positive satellite cells. Each $n = 8$. $^{*}P < 0.05$, significantly different from the Sham group at the same time point; $^{\dagger}P < 0.05$, significantly different from the Injury group at the same time point; $^{\ddagger}P < 0.05$, significantly different from the Injury + Stim group at 2 wpi; $^{\S}P < 0.05$, significantly different from the Injury + Stim group at 4 wpi. BrdU: 5-Bromo-2'-deoxyuridine; FITC: Fluoresceine isothiocyanate; Stim: Stimulation; wpi: Weeks post-injury.

Pax7-positive nuclei and the myonuclei number per fiber ($R^2 = 0.52$, $P < 0.01$). Another significant correlation was observed between the percentage of Pax7-positive nuclei and the myofiber CSA ($R^2 = 0.60$, $P < 0.01$) [Figure 5A and 5B). Only the data of the Injury + Stim group were included in this analysis. These results confirmed a strong correlation between enhanced satellite cell proliferation and the improvement of myofiber CSA.

Discussion

This study addressed several questions regarding electrically stimulated muscles post-nerve injury. We demonstrated an increase in the satellite cell content and in the promotion of satellite cell proliferation induced by electrical stimulation after sciatic nerve injury in rats. The myonuclei number and the myofiber CSA were higher

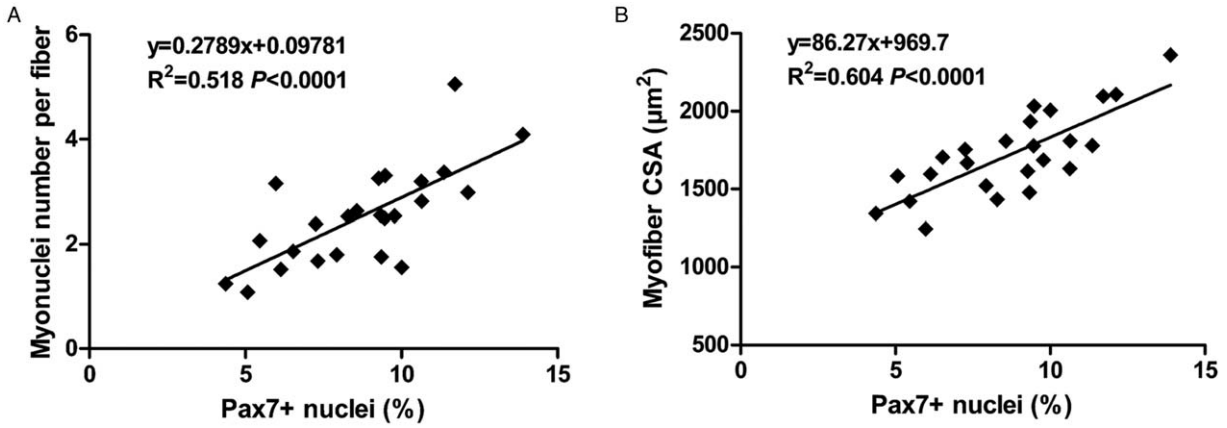

Figure 5: Linear regression analysis. A significant correlation was observed for the myofiber CSA (A) and myonuclei number per fiber (B) with the content of satellite cells in the Injury + Stim group. Each diamond represents the data of an animal. CSA: Cross-section area; Stim: Stimulation.

in the Injury + Stim group compared to the Injury group, and both were positively correlated with the satellite cell content. The sciatic nerve crush injury model used in this study is a consistent, reproducible strategy for obtaining an incomplete nerve lesion, which is most often seen in clinical work.[28,35-37] In our previous study, we also found that sciatic nerve function in this model was maintained at a low level in animals post-nerve injury during the 6 weeks of experimental period, and that the effect of self-recovery was minimized.[32]

To evaluate the change in the satellite cell content across different groups, we investigated the number of Pax7-positive satellite cells out of the total number of myonuclei. In order to exclude non-specific staining, the satellite cells were determined by their location beneath the basal lamina using triple staining of laminin, Pax7, and myonuclei.[38,39] The content of satellite cells in normal (Sham) and denervated (Injury) muscles was kept at a relatively stable level of 2% to 3% of the total nuclei, which was similar to that shown previously in the literature.[40] A fold-increase in the satellite cell number was dramatically induced by electrical stimulation in denervated muscle as early as 2 wpi. However, this increase tended to be less marked at 6 wpi. The significant correlation between satellite cell content and myonuclei number in the Injury + Stim group was consistent with the conclusions reported by previous studies, whereby satellite cell proliferation was found to be the main source of new myonuclei to mature muscle fibers.[4,8]

An increase in satellite cell content is attributed to the enhanced proliferation. In the present study, the proliferation of the satellite cells was reflected in the elevated number of BrdU-labeled nuclei. We used the percentage of BrdU-positive nuclei compared to the total number of satellite cell nuclei to assess the number of satellite cells that had undergone proliferation. This method has been widely used in the literature.[24,40-42] Being aware that cells other than those of myogenic origin in the muscle samples should be excluded to avoid any possible interference,[41] satellite cells were first isolated by sequential digestion, followed by flow cytometry analysis. The method we used to prepare the cell suspension has been previously demonstrated to produce concentrated satellite cells with a high purity (confirmed in previous studies).[33,34]

The proliferation of the satellite cells in the Sham and Injury groups was rare, which explained the stable content of satellite cells in these two groups. Interestingly, the rate of the satellite cell proliferation in the Injury + Stim group was highest at 4 wpi and lower at 6 wpi. These findings were in agreement with the changes in the satellite cell content. By 4 wpi, the proliferation of the satellite cells was rapid, resulting in an acute and continuous increase in the number of satellite cells. Between 4 and 6 wpi, the proliferation decreased. As a result, we found that the number of satellite cells increased significantly over time, whole the amplitude became smaller. This result indicated that the effect of electrical stimulation on satellite cell proliferation was most noticeable at 4 wpi. After the first 4 weeks, the proliferating potential of satellite cells began to decline. This decline of satellite cell proliferation after a period of electrical stimulation was also observed in rats with hypothyroidism.[40] Future studies will be needed to confirm whether a longer period of stimulation may cause chronic exhaustion of the satellite cell pool.

Under normal conditions, protein synthesis and degradation in the myofibers was kept at a dynamic balance.[43] Since the protein metabolism of the myofibers depended on the myonuclei, each myofiber required a specific number of myonuclei to maintain the production of necessary proteins. That is to say, the larger the fibers were, the more myonuclei they needed.[44] The concept of MND was suggested by researchers to describe the limited cytoplasmic territory that each myonucleus supports. The MND has been demonstrated to be maintained at a relatively stable value in adult individuals.[45,46] When the number of myonuclei was too small to supply such a large "domain," the protein degradation pathways, including the cytosolic Ca^{2+}-dependent calpain system and the lysosomal proteases, among others, were activated to break down proteins and reduce the myofiber size to return the MND to normal levels.[43] This theory could be used to explain the changes in myofiber CSA in the different experimental groups. After nerve injury, the loss of myonulei was severe,[5,18,47] and a reduction in the satellite

cells proliferation could not make up for the loss,[16,17,48] resulting in a reduction of the myonuclei number and a subsequent decrease in myofiber CSA in the Injury group. On the contrary, electrical stimulation promoted the proliferation of satellite cells in the Injury + Stim group, which consequently raised the myonuclei number and partly restored myofiber CSA. As expected, no significant differences were found among the mean MND of the Sham, Injury, and Injury + Stim groups. This result confirmed the existing reorganization about the MND.

Researchers have previously demonstrated that satellite cell proliferation is improved by electrical stimulation and loading exercise, both in healthy animals and animals with disuse atrophy/metabolic syndrome.[10,24-27] Electrical stimulation is believed to increase the proliferation of satellite cells, skeletal myoblasts, and mesenchymal stem cells.[49] Several underlying mechanisms are involved in this stimulation-induced increased in cell proliferation, including the promotion of the secretion of trophic factors, such as neural cell adhesion molecule and vascular endothelial growth factor,[50] interfering with the cell cycle, and the activation of proliferation signaling pathways, including PI3-Akt[26] and Wnt.[51] It is well known that Wnt is an essential pathway for the regulation of cell proliferation and myogenesis.[52,53] As an element in the Wnt pathway, the expression of Pitx2 is up-regulated by electrical stimulation in the muscle of rats with spinal cord transection. The activation of Wnt induced by electrical simulation is also found in neural stem/progenitor cells[54,55] and cardiomyocytes.[56] Thus, it is implied that the activation of Wnt signaling pathway may also be involved in the increase of satellite cell proliferation induced by electrical stimulation after nerve injury.

One limitation of this study is that the apoptosis of satellite cells was not detected. Presumably, the inactivity of satellite cells induced by nerve crushing could cause apoptosis or autophagy, as has been previously reported during denervation. Thus, it is unclear whether electrical stimulation prevented autophagy or apoptosis of the satellite cells. The other limitation is that electrical stimulation may influence the proliferation of satellite cells via several intra-cellular signaling pathway; however, this fell outside the scope of this study. Further research will be needed to elucidate the signaling activation, which is essential for understanding the underlying mechanisms of stimulation-induced effects.

Conclusion

Our data suggested that satellite cell proliferation was improved by electrical stimulation after nerve injury. The effect of electrical stimulation on satellite cell proliferation was most noticeable at 4 wpi. A significant increase in the myonuclei content and the restoration of myofiber CSA were also observed in stimulated muscles post-nerve injury. Furthermore, both the myonuclei content and the myofiber CSA were positively correlated to the satellite cell content, while the MND was unchanged. These results revealed that the enhanced proliferation of satellite cells induced by electrical stimulation after nerve injury was most likely responsible for the increased myonuclei number and myofiber CSA. These findings provide a preliminary mechanism responsible for the therapeutic effects of electrical stimulation on post-denervation muscle atrophy.

Funding

This study was supported by the grants from the National Natural Science Foundation of China (No. 81501940, No. 11472018, No. 81871851).

Conflicts of interests

None.

References

1. Salvini TF, Durigan JL, Peviani SM, Russo TL. Effects of electrical stimulation and stretching on the adaptation of denervated skeletal muscle: implications for physical therapy. Rev Bras Fisioter 2012;16:175–183. doi: 10.1590/s1413-35 552012005000027.
2. Schwartz LM. Atrophy and programmed cell death of skeletal muscle. Cell Death Differ 2008;15:1163–1169. doi: 10.1038/cdd.2008.68.
3. Ohira Y, Yoshinaga T, Nomura T, Kawano F, Ishihara A, *et al*. Gravitational unloading effects on muscle fiber size, phenotype and myonuclear number. Adv Space Res 2002;30:777–781. doi: 10.1016/s0273-1177(02)00395-2.
4. Roy RR, Monke SR, Allen DL, Edgerton VR. Modulation of myonuclear number in functionally overloaded and exercised rat plantaris fibers. J Appl Physiol 1999;87:634–642. doi: 10.1152/jappl.1999.87.2.634.
5. Guo BS, Cheung KK, Yeung SS, Zhang BT, Yeung EW. Electrical stimulation influences satellite cell proliferation and apoptosis in unloading-induced muscle atrophy in mice. PLoS One 2012;7:e30348. doi: 10.1371/journal. pone.0030348.
6. Aravamudan B, Mantilla CB, Zhan WZ, Sieck GC. Denervation effects on myonuclear domain size of rat diaphragm fibers. J Appl Physiol 2006;100:1617–1622. doi: 10.1152/japplphysiol.01277.2005.
7. McCall GE, Allen DL, Linderman JK, Grindeland RE, Roy RR, *et al*. Maintenance of myonuclear domain size in rat soleus after overload and growth hormone/IGF-I treatment. J Appl Physio 1998;84:1407–1412. doi: 10.1152/jappl.1998.84.4.1407.
8. Kawai M, Saitsu K, Yamashita H, Miyata H. Age-related changes in satellite cell proliferation by compensatory activation in rat diaphragm muscles. Biomed Res 2012;33:167–173. doi: 10.2220/biomedres.33.167.
9. Bareja A, Holt JA, Luo G, Chang C, Lin J, *et al*. Human and mouse skeletal muscle stem cells: convergent and divergent mechanisms of myogenesis. PLoS One 2014;9:e90398. doi: 10.1371/journal. pone.0090398.
10. Shefer G, Rauner G, Yablonka-Reuveni Z, Benayahu D. Reduced satellite cell numbers and myogenic capacity in aging can be alleviated by endurance exercise. PLoS One 2010;5:e13307. doi: 10.1371/journal.pone.0013307.
11. Bosnakovski D, Xu Z, Li W, Thet S, Cleaver O, *et al*. Prospective isolation of skeletal muscle stem cells with a Pax7 reporter. Stem Cells 2008;26:3194–3204. doi: 10.1634/stemcells.2007-1017.
12. Gnocchi VF, White RB, Ono Y, Ellis JA, Zammit PS. Further characterisation of the molecular signature of quiescent and activated mouse muscle satellite cells. PLoS One 2009;4:e5205. doi: 10.1371/journal.pone.0005205.
13. de Castro Rodrigues A, Andreo JC, de Mattos Rodrigues SP. Myonuclei and satellite cells in denervated rat muscles: an electron microscopy study. Microsurgery 2006;26:396–398. doi: 10.1002/micr.20258.
14. Borisov AB, Dedkov EI, Carlson BM. Differentiation of activated satellite cells in denervated muscle following single fusions in situ and in cell culture. Histochem Cell Biol 2005;124:13–23. doi: 10.1007/s00418-005-0012-1.
15. Isaacs J, Feher J, Shall M, Vota S, Fox MA, *et al*. Effects of nandrolone on recovery after neurotization of chronically denervated

muscle in a rat model. J Neurosurg 2013;119:914–923. doi: 10.3171/2013.5.JNS121837.
16. Jejurikar SS, Kuzon WM Jr. Satellite cell depletion in degenerative skeletal muscle. Apoptosis 2003;8:573–578. doi: 10.1023/A:1026127307457.
17. Liao H, Wang Q, Xu D, Qiu X, Yu L, *et al*. Pax7 and depletion of satellite cell pool in prolonged denervated skeletal muscles of adult rats [in Chinese]. Chin J Repar Reconstr Surg 2009;23:92–96. doi: 10.1021/acs.nanolett. 9b00363.
18. Lim JY, Han TR. Effect of electromyostimulation on apoptosis-related factors in denervation and reinnervation of rat skeletal muscles. Muscle Nerve 2010;42:422–430. doi: 10.1002/mus.21719.
19. Dow DE, Carlson BM, Hassett CA, Dennis RG, Faulkner JA. Electrical stimulation of denervated muscles of rats maintains mass and force, but not recovery following grafting. Restor Neurol Neurosci 2006;24:41–54. doi: 10.4103/1673-5374.266047.
20. Dirks ML, Wall BT, Snijders T, Ottenbros CL, Verdijk LB, *et al*. Neuromuscular electrical stimulation prevents muscle disuse atrophy during leg immobilization in humans. Acta Physiol (Oxf) 2014;210:628–641. doi: 10.1111/apha.12200.
21. Karlsen A, Cullum CK, Norheim KL, Scheel FU, Zinglersen AH, Vahlgren J, *et al*. Neuromuscular electrical stimulation preserves leg lean mass in geriatric patients. Med Sci Sports Exerc 2020;52:773–784. doi: 10.1249/MSS.0000000000002191.
22. Gargiulo P, Vatnsdal B, Ingvarsson P, Knútsdóttir S, Gudmundsdóttir V, *et al*. Restoration of muscle volume and shape induced by electrical stimulation of denervated degenerated muscles: qualitative and quantitative measurement of changes in rectus femoris using computer tomography and image segmentation. Artif Organs 2008;32:609–613. doi: 10.1111/j.1525-1594.2008.00610.x.
23. Gargiulo P, Reynisson PJ, Helgason B, Kern H, Mayr W, *et al*. Muscle, tendons, and bone: structural changes during denervation and FES treatment. Neurol Res 2011;33:750–758. doi: 10.1179/1743132811Y.0000000007.
24. Martins KJ, Gordon T, Pette D, Dixon WT, Foxcroft GR, *et al*. Effect of satellite cell ablation on low-frequency stimulated fast-to-slow fibre type transitions in rat skeletal muscle. J Physiol 2006;572:281–294. doi: 10.1113/jphysiol.2005.103366.
25. Peterson JM, Bryner RW, Alway SE. Satellite cell proliferation is reduced in muscles of obese Zucker rats but restored with loading. Am J Physiol Cell Physiol 2008;295:C521–C528. doi: 10.1152/ajpcell.00073.2008.
26. Wan Q, Yeung SS, Cheung KK, Au SW, Lam WW, *et al*. Optimizing electrical stimulation for promoting satellite cell proliferation in muscle disuse atrophy. Am J Phys Med Rehabil 2016;95:28–38. doi: 10.1097/PHM.0000000000000307.
27. Bosutti A, Bernareggi A, Massaria G, D'Andrea P, Taccola G, Lorenzon P, *et al*. A "noisy" electrical stimulation protocol favors muscle regeneration in vitro through release of endogenous ATP. Exp Cell Res 2019;381:121–128. doi: 10.1016/j.yexcr.2019.05.012.
28. Varejão AS, Cabrita AM, Meek MF, Bulas-Cruz J, Melo-Pinto P, *et al*. Functional and morphological assessment of a standardized rat sciatic nerve crush injury with a non-serrated clamp. J Neurotrauma 2004;21:1652–1670. doi: 10.1089/neu.2004.21.1652.
29. Pikov V, McCreery DB. Spinal hyperexcitability and bladder hyperreflexia during reversible frontal cortical inactivation induced by low-frequency electrical stimulation in the cat. J Neurotrauma 2009;26:109–119. doi: 10.1089/neu.2008.0584.
30. Dobsák P, Nováková M, Siegelová J, Fiser B, Vítovec J, *et al*. Low-frequency electrical stimulation increases muscle strength and improves blood supply in patients with chronic heart failure. Circ J 2006;70:75–82. doi: 10.1253/circj.70.75.
31. Xing H, Zhou M, Assinck P, Liu N. Electrical stimulation influences satellite cell differentiation after sciatic nerve crush injury in rats. Muscle Nerve 2015;51:400–411. doi: 10.1002/mus.24322.
32. Fujiya H, Ogura Y, Ohno Y, Goto A, Nakamura A, Ohashi K, *et al*. Microcurrent electrical neuromuscular stimulation facilitates regeneration of injured skeletal muscle in mice. J Sports Sci Med 2015;14:297–303. doi: 10.2147/IJN.S216365.
33. Cerletti M, Jurga S, Witczak CA, Hirshman MF, Shadrach JL, *et al*. Highly efficient, functional engraftment of skeletal muscle stem cells in dystrophic muscles. Cell 2008;134:37–47. doi: 10.1016/j.cell.2008.05.049.
34. Conboy IM, Conboy MJ, Smythe GM, Rando TA. Notch-mediated restoration of regenerative potential to aged muscle. Science 2003;302:1575–1577. doi: 10.1126/science.1087573.
35. Jiang M, Zhuge X, Yang Y, Gu X, Ding F. The promotion of peripheral nerve regeneration by chitooligosaccharides in the rat nerve crush injury model. Neurosci Lett 2009;454:239–243. doi: 10.1016/j.neulet.2009.03.042.
36. Gigo-Benato D, Russo TL, Geuna S, Domingues NR, Salvini TF, *et al*. Electrical stimulation impairs early functional recovery and accentuates skeletal muscle atrophy after sciatic nerve crush injury in rats. Muscle Nerve 2010;41:685–693. doi: 10.1002/mus.21549.
37. Baptista AF, Gomes JR, Oliveira JT, Santos SM, Vannier-Santos MA, *et al*. High- and low-frequency transcutaneous electrical nerve stimulation delay sciatic nerve regeneration after crush lesion in the mouse. J Peripher Nerv Syst 2008;13:71–80. doi: 10.1111/j.1529-8027.2008.00160.x.
38. Neal A, Boldrin L, Morgan JE. The satellite cell in male and female, developing and adult mouse muscle: distinct stem cells for growth and regeneration. PLoS One 2012;7:e37950. doi: 10.1371/journal.pone.0037950.
39. Putman CT, Düsterhöft S, Pette D. Changes in satellite cell content and myosin isoforms in low-frequency-stimulated fast muscle of hypothyroid rat. J Appl Physiol 1999;86:40–51. doi: 10.1152/jappl.1999.86.1.40.
40. Putman CT, Düsterhöft S, Pette D. Satellite cell proliferation in low frequency-stimulated fast muscle of hypothyroid rat. Am J Physiol Cell Physiol 2000;279:C682–C690. doi: 10.1152/ajpcell.2000.279.3.C682.
41. Smith HK, Maxwell L, Rodgers CD, McKee NH, Plyley MJ. Exercise-enhanced satellite cell proliferation and new myonuclear accretion in rat skeletal muscle. J Appl Physiol 2001;90:1407–1414. doi: 10.1152/jappl.2001.90.4.1407.
42. Enns DL, Tiidus PM. Estrogen influences satellite cell activation and proliferation following downhill running in rats. J Appl Physiol 2008;104:347–353. doi: 10.1152/japplphysiol.00128.2007.
43. Van der Meer SF, Jaspers RT, Degens H. Is the myonuclear domain size fixed? J Musculoskelet Neuronal Interact 2011;11:286–297. doi: 10.1002/bem.22207.
44. Degens H, Rittweger J. What is new in musculoskeletal interactions? Muscle oxygenation, myonuclear domain, acupuncture, titin and phosphate. J Musculoskelet Neuronal Interact 2010;10:245–248. doi: 10.1039/c9bm01203f.
45. Mackey AL, Andersen LL, Frandsen U, Sjøgaard G. Strength training increases the size of the satellite cell pool in type I and II fibres of chronically painful trapezius muscle in females. J Physiol 2011;598:5503–5515. doi: 10.1113/jphysiol.2011.217885.
46. Delhaas T, Van der Meer SF, Schaart G, Degens H, Drost MR. Steep increase in myonuclear domain size during infancy. Anat Rec (Hoboken) 2013;296:192–197. doi: 10.1002/ar.22631.
47. Arakawa T, Katada A, Shigyo H, Kishibe K, Adachi M, *et al*. Electrical stimulation prevents apoptosis in denervated skeletal muscle. Neuro Rehabilitation 2010;27:147–154. doi: 10.3233/NRE-2010-0591.
48. Dedkov EI, Kostrominova TY, Borisov AB, Carlson BM. Reparative myogenesis in long-term denervated skeletal muscles of adult rats results in a reduction of the satellite cell population. Anat Rec 2001;263:139–154. doi: 10.1002/ar.1087.
49. Love MR, Palee S, Chattipakorn SC, Chattipakorn N. Effects of electrical stimulation on cell proliferation and apoptosis. J Cell Physiol 2018;233:1860–1876. doi: 10.1002/jcp.25975.
50. Qi F, Wang Y, Ma T, Zhu S, Zeng W, Hu X, *et al*. Electrical regulation of olfactory ensheathing cells using conductive polypyrrole/chitosan polymers. Biomaterials 2013;34:1799–1809. doi: 10.1016/j.biomaterials.2012.11.042.
51. Wu Y, Collier L, Qin W, Creasey G, Bauman WA, Jarvis J, *et al*. Electrical stimulation modulates Wnt signaling and regulates genes for the motor endplate and calcium binding in muscle of rats with spinal cord transection. BMC Neurosci 2013;14:81. doi: 10.1186/1471-2202-14-81.
52. Brack AS, Conboy IM, Conboy MJ, Shen J, Rando TA. A temporal switch from notch to Wnt signaling in muscle stem cells is necessary for normal adult myogenesis. Cell Stem Cell 2008;2:50–59. doi: 10.1016/j.stem.2007.10.006.
53. Liu Q, Zhao H, Gao Y, Meng Y, Zhao XX, Pan SN. Effects of dandelion extract on the proliferation of rat skeletal muscle cells and the inhibition of a lipopolysaccharide-induced inflammatory reaction. Chin Med J 2018;131:1724–1731. doi: 10.4103/0366-6999.235878.

54. Liu HH, Xiang Y, Yan TB, Tan ZM, Li SH, He XK. Functional electrical stimulation increases neural stem/progenitor cell proliferation and neurogenesis in the subventricular zone of rats with stroke. Chin Med J 2013;126:2361–2367. doi: 10.3760/cma.j.issn.0366-6999.20130478.
55. Liu J, Zhu B, Zhang G, Wang J, Tian W, Ju G, *et al*. Electric signals regulate directional migration of ventral midbrain derived dopaminergic neural progenitor cells via Wnt/GSK3β signaling. Exp Neurol 2015;263:113–121. doi: 10.1016/j.expneurol.2014.09.014.
56. Martherus RS, Vanherle SJ, Timmer ED, Zeijlemaker VA, Broers JL, Smeets HJ, *et al*. Electrical signals affect the cardiomyocyte transcriptome independently of contraction. Physiol Genomics 2010;42A:283–289. doi: 10.1152/physiolgenomics.00182.2009.

How to cite this article: Xing HY, Liu N, Zhou MW. Satellite cell proliferation and myofiber cross-section area increase after electrical stimulation following sciatic nerve crush injury in rats. Chin Med J 2020;133:1952–1960. doi: 10.1097/CM9.0000000000000822