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

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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 myonuclear 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...
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.

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. 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. It has been previously demonstrated that electrical stimulation can be used to maintain the force and mass of skeletal muscles. The myofiber CSA of the denervated muscles can also be gradually restored by electrical stimulation. 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. 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. 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 as 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. 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. The measures of the myofiber CSA (μm²) 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 μm².

**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). 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 Fluor 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. 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. 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 dehydrogenases (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 × 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 µm², which was significantly lower than that of the Sham group (2225.4 ± 91.8 µm², 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 µm² and 1095.1 ± 115.9 µm², 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 µm²) compared to the 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).

| Time   | Sham | Injury | Injury + stim |
|--------|------|--------|---------------|
| 2 wpi  | 2225.4 ± 91.8 | 1357.8 ± 89.7 | 1538.2 ± 92.1 |
| 4 wpi  | 2197.1 ± 122.0 | 1105.9 ± 93.7 | 1796.3 ± 102.0 |
| 6 wpi  | 2309.1 ± 106.8 | 1095.1 ± 115.9 | 1906.9 ± 116.5 |

| Time   | Sham | Injury | Injury + stim |
|--------|------|--------|---------------|
| 2 wpi  | 2.45 ± 0.37 | 1.39 ± 0.21 | 1.73 ± 0.15 |
| 4 wpi  | 2.63 ± 0.20 | 1.15 ± 0.08 | 1.92 ± 0.09 |
| 6 wpi  | 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.

group (Bonferroni t = 4.67, P < 0.01), and further improvement were observed at 4 wpi (1796.3 ± 102.0 μm²) and 6 wpi (1906.9 ± 116.5 μm²), 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 μm², 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 ± 0.36%, 1.92 ± 0.24% 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
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...
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. 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.

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. 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. 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.

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. Being aware that cells other than those of myogenic origin in the muscle samples should be excluded to avoid any possible interference, 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).

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. 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. 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. 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. When the number of myonuclei was too small to supply such a large “domain,” the protein degradation pathways, including the cytosolic Ca²⁺-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. This theory could be used to explain the changes in myofiber CSA in the different experimental groups. After nerve injury, the loss of myonuclei was severe, and a reduction in the satellite content was observed. In our previous study, we also found 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. Future studies will be needed to confirm whether a longer period of stimulation may cause chronic exhaustion of the satellite cell pool.

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.
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.\textsuperscript{10,24-27} Electrical stimulation is believed to increase the proliferation of satellite cells, skeletal myoblasts, and mesenchymal stem cells.\textsuperscript{189} Several underlying mechanisms are involved in this stimulation-induced increased cell proliferation, including the promotion of the secretion of trophic factors, such as neural cell adhesion molecule and vascular endothelial growth factor,\textsuperscript{50} interfering with the cell cycle, and the activation of proliferation signaling pathways, including PI3-Akt\textsuperscript{26} and Wnt.\textsuperscript{51} It is well known that Wnt is an essential pathway for the regulation of cell proliferation and myogenesis.\textsuperscript{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 stimulation after nerve injury is implicated that Wnt is an essential pathway for the regulation of myogenesis and myonuclei content and the restoration of myofiber CSA in the Injury group. This result confirmed the existing reorganization about the MND.

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.

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Conflicts of interests
None.

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