

Correlation between Innervation of Skeletal Muscles and Myoblast Stem Cells

Correlación entre la Inervación de los Músculos Esqueléticos y las Células Precursoras Mioblásticas

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SUMMARY: In order to explore the change rule of myoblast stem cells (satellite cells, SCs) in the denervated and re-innervated muscle and to investigate the cellular mechanism of the morphological and functional changes of the muscle, denervated muscle atrophy and nerve regeneration models were established in one-month-old rats. Postoperative indexes such as muscle wet weight, cell section areas, content of collagen fibers and DNA, electrophysiology, numbers of SCs in the triceps muscle of calf were dynamically tested. After denervation, the muscle wet weight and cell area reduced rapidly, and the collagen fiber content increased slowly. The number of SCs increased at first, and then declined suddenly two months later. From 4 to 5 weeks after re-neuralization, muscle action potentials could be evoked, but the best innervation effect was found in the groups, which received re-neuralization at 2 months and 3 months after denervation. Denervation causes a progressive progress of muscle atrophy. SCs proliferate within 3 months after denervation, and then atrophy becomes irreversible from 4 months. At 4 or 5 weeks after re-neuralization, muscle action potentials can be evoked. Re-neuralization at 2 months and 3 months after denervation can achieve a good effect on the functional recovery of the atrophic muscle.

KEY WORDS: Satellite cells; Atrophy; Denervation; Innervations; Stem cells.

INTRODUCTION

Prevention and cure of denervated muscle atrophy has long been a tough problem in osteology. Until now, scholars have carried out a lot of empirical studies on this topic, and some measures to stop the process of denerved muscle atrophy have been raised, such as the application of clenbuterol, electric stimulation, Chinese drugs, and nerve homogenate and neuron implantation; although all these measures can relieve atrophy partially, none of them can address the problem of atrophic muscle functional recovery fundamentally (Bischoff & Heintz, 1994; Hall *et al.*, 2011; Paylor *et al.*, 2011; Sharifiaghdas *et al.*, 2011; Yablonka-Reuveni, 2011). With the rise of study on embryonic stem cells and adult stem cells in recent years, more and more scholars have realized that muscle satellite cells (SCs) play an important role in atrophic muscle regeneration, and SCs have been more and more extensively accepted as muscle adult stem cells (Schmalbruch & Lewis, 1994). SCs were first discovered by Mauro in 1961 when he conducted an observational study on the muscle fibers in frogs under an electron microscope. As these cells have a close relation with

position of muscle cells, they were named satellite cells (Ten Broek *et al.*, 2010). SCs are a group of mononuclear cells located between the basement membrane and the cell membrane. They adhere closely to the surfaces of muscle cells. SC has an oblate nucleus with sparse heterochromatin in it and a small amount of cytoplasm. Mauro presumed that SCs was correlated with skeletal muscle regeneration, which has been proved by subsequent studies. In a normal circumstance, SCs stay in a silent state. However, when an injury, necrosis or overload occurs to the muscle, SCs will be activated. They begin to proliferate, differentiate, bind into myotubes, and form muscle fibers finally.

Based on the aforementioned, it is necessary to make clear the dynamic change process of SCs in skeletal muscles after denervation and re-neuralization before studying muscle tissue engineering in treatment of denervated muscle atrophy. Therefore, in the present study, we explored the correlation between innervation of the skeletal muscle and the number and activity of SCs.

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MATERIAL AND METHOD

Denervation. Animal model establishment. A total of 27 rats at age of 1 month (ranging from 50 g to 70 g) were taken for denervation model establishment. Their right lower limbs were subjected to neurotomy, leaving the left lower limbs as the normal controls. Three rats were observed using histology and histochemistry every month from 1 month to 6 months after operation. Meanwhile, one rat underwent cell biological detection at 1 w, 2 w, 3 w, and 1 month after operation. Then, the biological detection was carried out once every month from 1 month to 6 months.

Detection indexes. Muscle wet weight. Bilateral triceps muscles were cut off, and weighed immediately using an electronic analytical balance. The weights of the bilateral triceps were recorded. After weighing, the middle 1/3 piece (that is, the muscle belly) was taken. It was fixed in 10% paraformaldehyde, dehydrated routinely, and imbedded in paraffin for section preparation. Then, the sections were deparaffinated.

Muscle cell section area Sections were stained with hematoxylin and eosin (HE), and then analyzed using the Q500MC image analysis system (Leica, Germany) for determination of the muscle cell section area. At least five visual fields were selected for each section, and the counted cell number was no less than 100. A mean value was calculated.

Collagen fiber determination. Collagen fibers were Van Gieson (VG)-stained. Five visual fields were randomly selected for each section. The collagen fiber content in each field was automatically determined. Then, the collagen fiber content in each cell was calculated based on the following formula: the collagen fiber content in each cell = the collagen fiber area/the muscle cell number in the same visual field.

DNA content determination. Nuclear DNA staining was performed using Feulgen-Rasenbeck modified DNA staining. The nuclear brilliance value was calculated using the image analytical technique. No less than 200 nuclei with a clear background of dispersed cells were selected from each section for brilliance determination.

SC isolation and culture. Two small pieces of muscles were taken from the middle parts of the bilateral triceps muscles in one rat every month from 1 month to 6 months after neurotomy. After weighing, the samples were used for SC isolation and culture. After differential adhesion, the cells were counted, and the ratio between the bilateral cell numbers (i.e., the ratio between the bilateral cell numbers per unit

weight) was calculated. Then, the cells were cultured in a CO₂ incubator. The morphologic change of the cells was observed, and cell proliferation and differentiation capacities were evaluated (the procedures were the same as the modified SC culture procedures (Shadrach & Wagers, 2011).

Re-neuralization. Thirty-five rats at age of 1 month (ranging from 50 g to 70 g) were subjected to tibial neurotomy for denervation model establishment. After that, five rats received immediate common peroneal nerve transposition implantation. Of the remaining rats, five received common peroneal nerve transposition implantation every month from 1 month to 6 months after denervation, respectively. All indexes were observed for 8 weeks after implantation. Rats in different groups were anesthetized on the expiration of an experiment. The dorsal skins of bilateral calves were cut open, and the great adductor muscles were bluntly dissected to expose the triceps muscles for morphological observations. Then, the bilateral triceps muscles were cut off for index detection.

Muscle electrophysiology. Rats were subjected to muscle electrophysiological detection immediately after operation using M2000 electromyography (Dantec, Denmark). Then, they were detected once every week. The electronic stimulus threshold was 3.3 mA. The stimulation adopted a manual manner, in which a concentric needle vertical in the muscle belly was used to detect whether there was an evoked muscle action potential. When there was an action potential, its emergence time, wave amplitude and latency period were recorded. Then, this action potential was compared with that on the opposite side evoked by the same electric current strength.

Morphologic observation. Bilateral triceps muscles were cut off at 8 w after nerve implantation. The samples were routinely fixed, and imbedded in paraffin for section preparation. The sections were HE-stained for morphologic observation. The results were compared with those obtained from the sections of the same atrophy period group.

Nerve staining. The triceps muscle on the experimental side was taken at 8 w after nerve implantation for section preparation. The sections were stained using the Bielschowsky modified method. Meanwhile, a small muscle tissue was taken for nerve regeneration observation under the light microscope using the formic acid-auri chloridium pellet method.

SC culture. SCs were isolated from the bilateral triceps muscles at 8 w after nerve implantation, and then cultured. The culture method was the same as the previously mentioned.

RESULTS

Denervation

The morphologic change. When the samples were drawn, the triceps muscle on the normal side was found rosy and fresh with a full configuration, whereas that on the experimental side was found pale, tarnished and loose and the muscle configuration showed a progressively shrinking trend with the prolongation of the atrophy time. In order to avoid a weight difference caused by age, the ratio between the bilateral muscle wet weights was adopted to measure the change in muscle-wet weight in the present study. The results showed that the ratio decreased rapidly within 3 months after denervation, and went on to decrease but a little less rapidly from 4 months to 6 months. HE staining showed that the muscle cells became thinner, the intercellular gap became wider gradually, more connective tissues appeared between muscle bundles, the sizes of the muscle cells between different muscle fascicles differed a lot, but those in the same fascicle were similar. At 4 months, only a few muscle fibers could be observed, around which abundant connective tissues existed. The muscle cell section area on the normal side stayed stable except for 1 month and 2 months when there was an increase, whereas that on the experimental side decreased with the prolongation of time showing a significant difference between months (one-way ANOVA, and an S-N-K test between groups; Table I).

Table I. Bilateral muscle cells sectional area every month after denervation surgery ($X \pm SD$).

| | Left (pp ²) | Right (pp ²) |
|-----|-------------------------|--------------------------|
| 1 M | 4093.51±54.32 | 3748.20±26.81** |
| 2 M | 5171.63±28.97 | 2495.91±54.21** |
| 3 M | 5984.71±76.31 | 1872.14±34.74** |
| 4 M | 6012.22±46.14 | 1029.86±71.64** |
| 5 M | 6347.45±24.31 | 819.33±41.25** |
| 6 M | 6296.51±67.35 | 502.42±28.05** |

**=Compared with unilateral of other months $p < 0.01$

The histological change. VG staining showed that more red stained collagen fibers appeared between muscle cells after denervation, and groups of collagen fibers were observed between muscle bundles (Fig. 1A); and the collagen fiber content per unit cell increased with the prolongation of denervation time but at a slow speed. DNA staining showed that the nucleus was prunosus stained with green cytoplasm as the background (Fig. 1B); the brilliance value was positively correlated with the nuclear staining and DNA content; and the percentage taken by the nucleus reached the peak between 3 months and 4 months, and then dropped quickly.

The number and growth of SCs. The number of SCs began to increase obviously at 3 w after denervation, but dropped rapidly from 2 months; and at 4 months, the number became very small. Figure 2 shows the ratio of the numbers of SCs per unit weight between the bilateral triceps muscles at the same observation time point. Cell growth activity reached the strongest at 2 w after operation (spindle cells could be observed at 4 h after adherence), after which it showed a decreasing trend. No significant difference in the cell proliferation or differentiation capacity was found among the early three-month groups. From 4 months, cell growth capacity was not detected due to a culture failure caused by a too small cell number.

Re-neuralization

Electrophysiology. After the tibial nerve in the right lower limb was cut off, the myoelectricity of the triceps muscle showed a denervated resting line. At 1 w to 2 w after re-neuralization, some fibrillation potentials were detected, but no muscle action potential was evoked by stimulating the transpositioned common peroneal nerve. At 4 w to 5 w, motor unit potentials began to appear successively in all the groups; and muscle action potentials at very low wave amplitude could be detected by stimulating the transpositioned nerve, but no significant differences were found among the different groups. At 8 w, the latency periods and wave amplitudes of bilateral evoked action potentials in different groups were compared (Table II). However, as evoked action potentials were not detected in five rats, comparisons of the recovery degrees were only made in the rest 30 rats (through the ratio between the bilateral wave amplitudes). The results showed that the recovery degrees of the rats receiving nerve transposition implantation at 2 months and 3 months were much better compared with other groups ($p < 0.05$; one-way ANOVA, and an S-N-K test for comparisons between groups).

The morphologic change. At 8 w after nerve implantation, all the sections from the 30 rats with evoked action potentials presented changes in muscle fibers: the intercellular gap in the muscle bundle was narrowed, and muscle fibers at difference sizes were distributed; after nerve staining, black stained tiny nerve branches and newly-regenerated motor end plates could be found between muscle fibers. However, such changes were not found in the lateral muscle fibers of the triceps. In the five rats without evoked action potentials, the muscle fibers still displayed a denervation change, in which a few residual muscle cells were surrounded by abundant connective tissues. Compared with the sections from the atrophic muscle at the same age, different sizes of muscle cells were found in some muscle fascicles, and these muscle fascicles were more concentrated in distribution; and

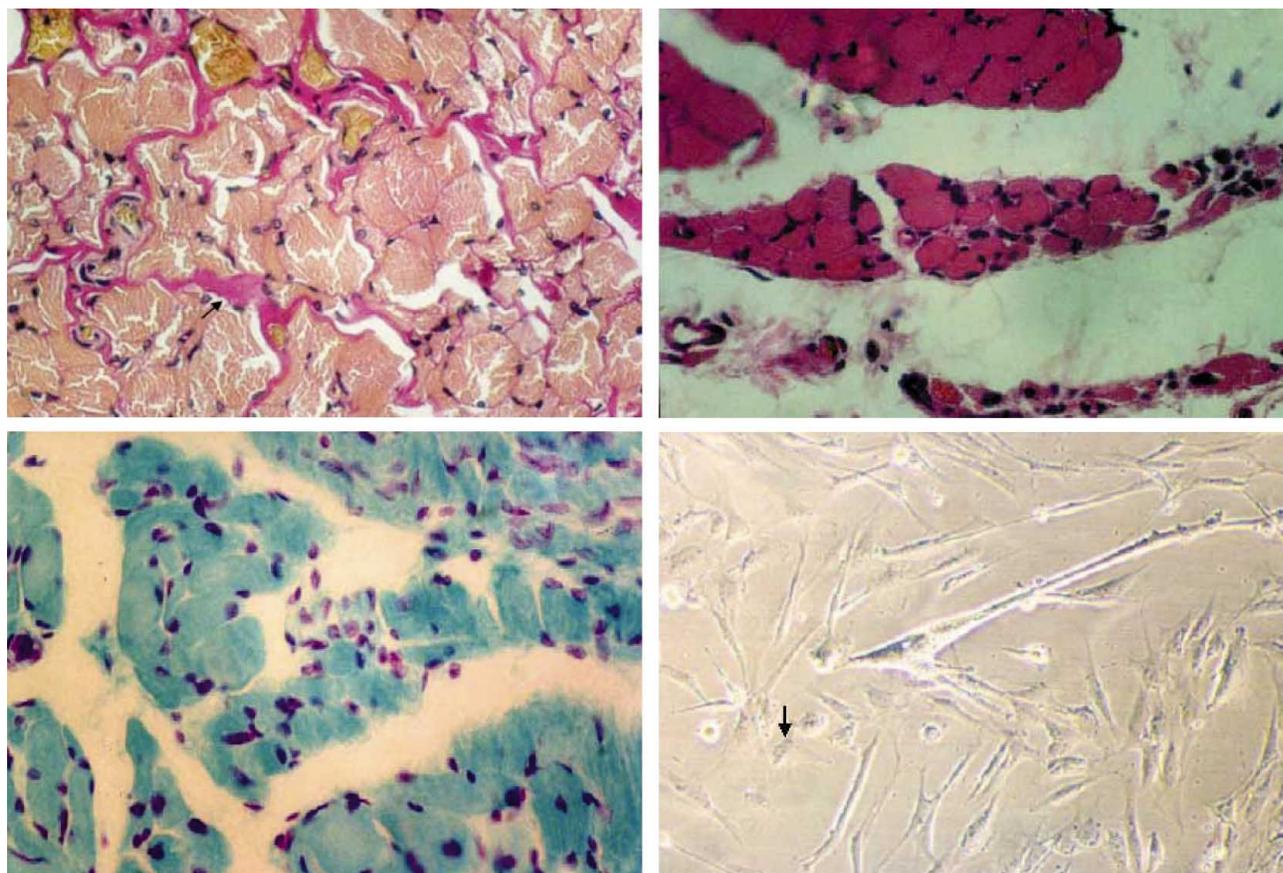


Fig. 1. Observation of the atrophic muscle at 2 months after denervation and the muscle re-neuralized at 2 months after atrophy. A: observation of the section at 2 months after denervation using VG staining (X150); B: observation of the section at 2 months after denervation using DNA staining (X150); C: observation the section re-neuralized at 2 months after denervation using HE staining (X150); and D: the morphology of the myotube-differentiating SCs (X150).

these changes were more obvious in the 2-month and 3-month groups (Fig. 1C).

The change in SCs. Compared with the atrophy group at the same observation time point, although SCs in the re-neuralization group did not show a significant difference in cell proliferation ability, the muscle tubularization rate, which can reflect cell differentiation ability, was increased significantly, and such a change was more obvious in the 2-month and 3-month groups (Fig. 1D). However, this change became uncertain from 4 months due to a cell culture failure.

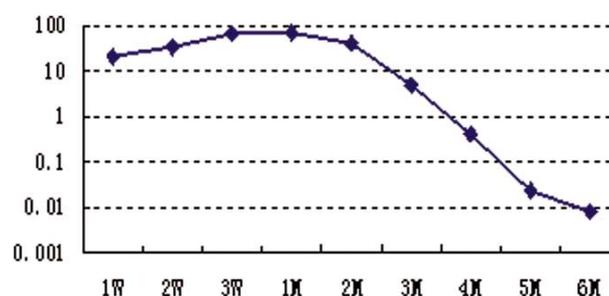


Fig. 2. Dynamic change in the ratio between the bilateral SC numbers after denervation.

Table II. Muscle evoked action potential results eight weeks after nerve implantation (X±SD).

| | Latent period (ms) | | Amplitude (mV) | |
|--------------------------------|--------------------|-----------|----------------|-------------|
| | Left | Right | Left | Right |
| Embedding immediately (4 rats) | 1.33±0.23 | 1.84±0.51 | 19.71±6.37 | 2.78±1.56 |
| Embedding after 1M (3 rats) | 1.35±0.44 | 1.91±0.66 | 27.12±11.30 | 2.49±0.87 |
| Embedding after 2M (5 rats) | 1.31±0.24 | 1.72±0.57 | 31.36±13.81 | 10.21±5.47* |
| Embedding after 3M (4 rats) | 1.24±0.33 | 1.71±0.64 | 34.44±11.64 | 9.50±5.61* |
| Embedding after 4M (5 rats) | 1.42±0.41 | 1.80±0.96 | 45.38±13.30 | 5.26±2.54 |
| Embedding after 5M (4 rats) | 1.37±0.29 | 1.81±0.53 | 49.62±10.51 | 4.60±2.06 |
| Embedding after 6M (5 rats) | 1.45±0.50 | 1.75±0.88 | 42.87±14.61 | 3.90±1.97 |

*= Compared with other five groups p<0.05

DISCUSSION

The nutrition and state of the skeletal muscle are mainly regulated by its controlling nerve. Once the controlling nerve is injured, the controlled muscle is doomed to show some histological changes such as a less weight, thinned muscle fibers and more collagen fibers (Nikolaou *et al.*, 2011). In the present study, the activity of SCs began to increase obviously at 2 w after denervation, and the number of SCs began to increase noticeably at 3 w; but such increase stopped at 2 months, and the number of SCs began to show a rapid decrease. Presumably, this phenomenon was caused by the fact that the SCs in the atrophic muscle made a stress reaction to denervation after a one-week lag phase, and they began to divide, proliferate and differentiate in a great quantity in order to relieve the cellular atrophy caused by denervation; however, as the tissue always stayed in a state of denervation, the SCs were unable to fend for themselves, and they began to die gradually before maturation, which could not stop the progressive atrophy trend; and at 4 months after denervation, the number of SCs dropped to a very low level, and muscle atrophy entered into an irreversible phase from then on. However, there is a puzzle during the process, that is, why the cell proliferation peak from the perspective of histology occurred two months later than that from the perspective of cytology. This time gap might stem from the following reasons: 1) the denervated muscle cells after muscle atrophy began to die gradually due to lack of nutrition from the nerve, and the number of the nuclei themselves decreased gradually; and 2) the isolated and cultured SCs which were counted in the current study did not include the myotubes which had already formed or the cells which had bound with muscle cells, but these cells were taken into consideration when the DNA staining method was adopted, in which the cell proliferation level was calculated taking the total number of nuclei (including the nuclei of the muscle cells and SCs) as the basis reference. Therefore, undoubtedly, the increased number of the numerator and the decreased number of denominator led to the delay of the cell proliferation peak from the perspective of histology.

Up to now, many studies on the morphologic change of an atrophic muscle have been released (Tamaki *et al.*, 2010; Sharifiaghdas *et al.*; Yoshimura & Harii, 1999). However, to the best of our knowledge, no report has focused on the change rule or the nature underlying the change from the perspective of a cellular level. Meanwhile, the present study suggests that, based on the aforementioned detection mechanism, the index of the SC number will be more accurate when a cell proliferation level is detected.

After re-neuralization, the residual SCs begin to proliferate and differentiate; under the innervation of the regenerated nerve, they form new muscle cells, or bind with the original muscle cells (the latter is the main mode of muscle regeneration after nerve regeneration, that is, hypertrophic muscle regeneration (Malatesta *et al.*, 2011); and this can be used to explain the morphologic change of muscle regeneration in which cells in the muscle fascicle are different in size as newly-generated muscle fibers can only come from the differentiated SCs rather than the differentiation of the residual muscle fibers (Yamada *et al.*, 1989; Dilworth & Blais, 2011; van der Meer *et al.*, 2011). In the present study, the 2-month and 3 month groups showed better neuromuscular function recovery, and they also showed stronger cell differentiation abilities compared to other groups. The underlying reasons might include: 1) muscle fibers begin to shrink and become thinner, and the gap between fibers begin to be widened after denervation, which may promote the growth of the nerve regeneration axons (Trendelenburg *et al.*, 2012; Mokalled *et al.*, 2012; Wang & Rudnicki, 2011); and 2) the activated SCs have a chemotaxis effect on nerve growth, and the well-regenerated nerves, in return, can promote the differentiation of the SCs to form new muscle fibers (Usas *et al.*, 2011; Yablonka-Reuveni).

In summary, the present study brings to light the cytological mechanism underlying the phenomenon in which the skeletal muscle enters into an irreversible phase due to lack of a sufficient SC number at 4 months after denervation. Further, re-neuralization between 2 months and 3 months after denervation can achieve a good effect on the functional recovery of the atrophic muscle.

WU, J.; SUN, X.; ZHU, M.; ZHU, C.; CHEN, B.; XU, Y.; OUYANG, J.; LIAO, H. & QUI, X. Correlación entre la innervación de los músculos esqueléticos y las células precursoras mioblásticas. *Int. J. Morphol.*, 30(4):1532-1537, 2012.

RESUMEN: Con el fin de explorar la regla de cambio de las células precursoras mioblásticas (células satélite, CSs) en el músculo denervado y re-inervado e investigar el mecanismo celular de los cambios morfológicos y funcionales del músculo, se establecieron, en ratas de un mes de edad, modelos de atrofia del músculo denervado y regeneración del nervio. Fueron examinados de manera dinámica índices postoperatorios tales como, el peso húmedo del músculo, áreas celulares de la sección, contenido de fibras de colágeno y ADN, electrofisiología, número de CSs en el músculo tríceps de las crías. Luego de la denervación, el peso del músculo húmedo y el área celular se redujeron rápidamente, mientras que el contenido de fibras colágenas aumentó lentamente. El número de CSs aumentó al inicio, pero más tarde, a los dos meses, disminuyó repentinamente. Entre las 4 a 5 semanas después de la reneuralización, los potenciales de acción muscular pudieron ser

evocados, pero el mejor efecto de inervación se encontró en los grupos que recibieron reneuralización a los 2 y 3 meses después de la denervación. La denervación causó un avance progresivo de la atrofia muscular. Las CSs proliferaron dentro de los primeros 3 meses post-denervación, y luego de los 4 meses la atrofia fue irreversible. A las 4 o 5 semanas después de la reneuralización, los potenciales de acción muscular pueden ser evocados. La reneuralización a los 2 y 3 meses después de la denervación puede lograr un buen efecto en la recuperación funcional del músculo atrofico.

PALABRAS CLAVE: Células satélite; Atrofia; Denervación; Inervación; Células precursoras.

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