Delay of Skeletal Muscle Atrophy after Transplantation of Mesenchymal Progenitor Cells into Transected Position

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ABSTRACT

Objective To study the delay of denervated skeletal muscle atrophy after transplantation of mesenchymal progenitor cells (MPC) into the transected position and the muscle. *Methods* MPC were taken from bones of hind limbs of GFP transgenic C57 mice for cultivation and identification. 48 C57 mice were divided into 4 groups evenly in random, group A (control group), group B (sham operated group), group C (MPC transplantation into transected position) and group D (MPC transplantation into muscle). 5μL MPC suspension were injected into the transected position of sciatic nerve and the gastrocnemius in group C and D, and 5μL Sodium Chloride was injected into the gastrocnemius in group B, while nothing was injected in group A. The locomotor ability of mice hind limbs was observed. The wet weight of gastrocnemius and the retain ratio of cross section area (CSA) of muscle fibers were measured and the ultrastructural structure was observed at 2 weeks and 4 weeks after the operation. The expressions of α-actin and myoglobulin (MHC) were detected with Western blot, and Myogenin and MyoD with RT-PCR. *Results* The wet weight of gastrocnemius and the retain ratio of muscle fibers CSA of group C and D were obviously higher than those of group B at 2 weeks and 4 weeks after the operation ($P < 0.01$); The degeneration level of muscle cell nucleus, mitochondria and endocytoplasmic reticulum and the degree of muscle fibrosis of group C and D were obviously lower than those of group B at 4 weeks after the operation ($P < 0.05$), while the expression level of α-actin, MHC, Myogenin and MyoD was obviously higher than that of group B ($P < 0.05$). *Conclusion* The transplantation of allogenic MPC in vivo is effective for the delay of denervated muscle atrophy.

Key Words

Mesenchymal progenitor cells, Muscle atrophy, Denervation, Peripheral nerve.

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It is found that neural stem cell can differentiate into neurons in vitro and can effectively delay the denervated skeletal muscle atrophy after transplanting it into impaired nerves or target muscle(1-2), while the way of obtaining sufficient neural stem cell is relatively limited and the amplification is also difficult, therefore, it is very important to find a new kind of transplanted cell(3). The new research has found that MPC can be induced to differentiate into neuron-like cell in vitro(4), and it is expected to become the seed cell instead of neural stem cell with the characteristics of easy access and growth, and strong reproductive activity, etc. (5). MPC can be induced to differentiate into neuron-like cell in vitro, whether it can delay the denervated muscle atrophy after transplanting it into transected position of nerves or target muscle or not? There are rare reports on it at present, and some related researches has done in this study on the basis of it.

1 Materials and Methods

1.1 Experimental animals

6 GFP transgenic C57 mice [Female, 3 years old, (10±1)g weight)]; 48 C57 mice [Male, 12 years old, (20±1)g weight)]; These animals are provided by the animal center of Third Military Medical University, Chinese People's Liberation Army. Animal use certificate: SCXK (Chongqing) 2007-0004; Environmental permit certificate: XYXK

(Yu) 2007-0004.

1.2 Main reagents

Rabbit antimouse α-actin, MHC, GAPDH antibody and the second antibody of goat anti-rabbit IgG/TRITC (Sigma Company, America); Protein extract (Pierce Company, America); RNAiso Reagent, RNA PCR Kit(AMV) ver.3.0 (TaKaRa Company, Japan); Myogenin amplification primers are 5′-TGGAGCTGTATGAGACATCCC-3′ and 5′-TGGACAATGCTCAGGGGT CCC-3′, GAPDH amplification primers are 5′-ACCACAGTCCATGCCATCA C-3′ and 5′-TCCACCACCCTGTTGC TGTA-3′, MyoD amplification primers are 5′-GCCCGCGCTCCAACTGCT CTGAT-3′, 5′-TCTTTTGGGCGTG AAGAACCAG-3′, and the primer is composed by Shanghai Sheng Gong biological engineering co., LTD.

1.3 Abstraction, cultivation and identification of MPC

Long bones of hind limbs in GFP transgenic C57 mice were obtained, and the mothods of abstraction, cultivation and identification of MPC could be seen in the literature(4).

1.4 Model preparation of the denervated gastrocnemius in mice

48 C57 mice were divided into 4 groups evenly in random, group A (control group), group B (sham operated group), group C (MPC transplantation into transected position) and group D (MPC transplantation into muscle). The model preparation ways of the denervated gastrocnemius in mice can be found in the literatures(6); Nothing was done in group A. 6 mice in each group were taken out and broken to death at 2 weeks and 4 weeks after operation, and the gastrocnemius extracted from bilateral legs was observed in the experiment, meanwhile, the muscle tissue of nerve transected position in group C was extracted.

1.5 MPC transplantation in vivo

The third generation of MPC was selected and adjusted its cell concentration to 5×105/μL with physiological saline. 5μL MPC suspension was slowly injected into the nerve transected position and gastrocnemius of group C and D respectively, and 5μL physiological saline was injected with the same method in group B; nothing was done in group A.

1.6 Observation of index

1.6.1 General condition

The locomotor activity of hind limbs in mice was observed.

1.6.2 Survival condition of MPC transplantation

The muscle surrounding the MPC injection site was cut to make tissue frozen section, and the survival condition of transplanted cells could be observed under fluorescence microscope with the spontaneous green fluorescence of body cells in GFP transgenic mice.

1.6.3 Measurement of wet weight retain ratio in gastrocnemius

The gastrocnemius at two sides was completely taken out and weighed, and its wet weight retain ratio could be calculated with the weight of right side divided by that of left side.

1.6.4 Measurement of retain ratio of muscle fibers CSA.

The tissue of middle muscle belly in two-side hind limbs was chipped to make frozen section and done HE stain, the muscle fibers CSA could be measured by VDSIII semi-automatic image analyzer (A.M.S Company, British), and the retain ratio could be calculated with the area of right side divided by that of left side.

1.6.5 Observation of ultramicro-structure

A small amount of tissue of the middle muscle belly in right hind limbs was chipped to make ultrathin section, and JEM-12OOEX transmission electron microscope was used to observe the degenerated myocyte nucleus, mitochondria, endoplasmic reticulum, shape of myofilament and myocomma, and the changes of collagen fibers.

1.6.6 Expression of α-actain and MHC was detected by Western blot

The total protein of muscle could be extracted following instruction, rabbit antimouse α-actin/MHC and goat anti-rabbit IgG with membrane were incubated under 37°C respectively after electrophoresis, semi-dry transfer membrane and blockage, and the membrane was discontinuously washed in this process. Chemiluminescence kit was used for visualization, fixing and photographs in the end, the results were analyzed with the gel imaging system, and the semiquantitative analysis was done with gray scanning.

1.7 Genetic expression of Myogenin and MyoD was detected by RT-PCR

The total RNA in muscle was extracted following the description and done reverse transcription, PCR and gel electrophoresis were done with conventional methods, and the image was scanned under ultraviolet transilluminator in the end, then the semiquantitative analysis was done with the Quantity one image analysis software.

1.8 Statistical treatment

Analysis was done with SPSS10.0 statistical package. The data was recorded as average ± standard deviation, and the comparison among groups was tested by pairing t.

2 Results

2.1 General condition

The locomotor activity of mice right hind limbs in group C and D was gradually recovered to the normal status along with the extension of treatment.

2.2 Survival condition of MPC transplantation in vivo

The cells with spontaneous green fluorescence of the muscles surrounding MPC injection site in group C and D were uniformly distributed into myocyte gap (Figure 1a and c); The same cells

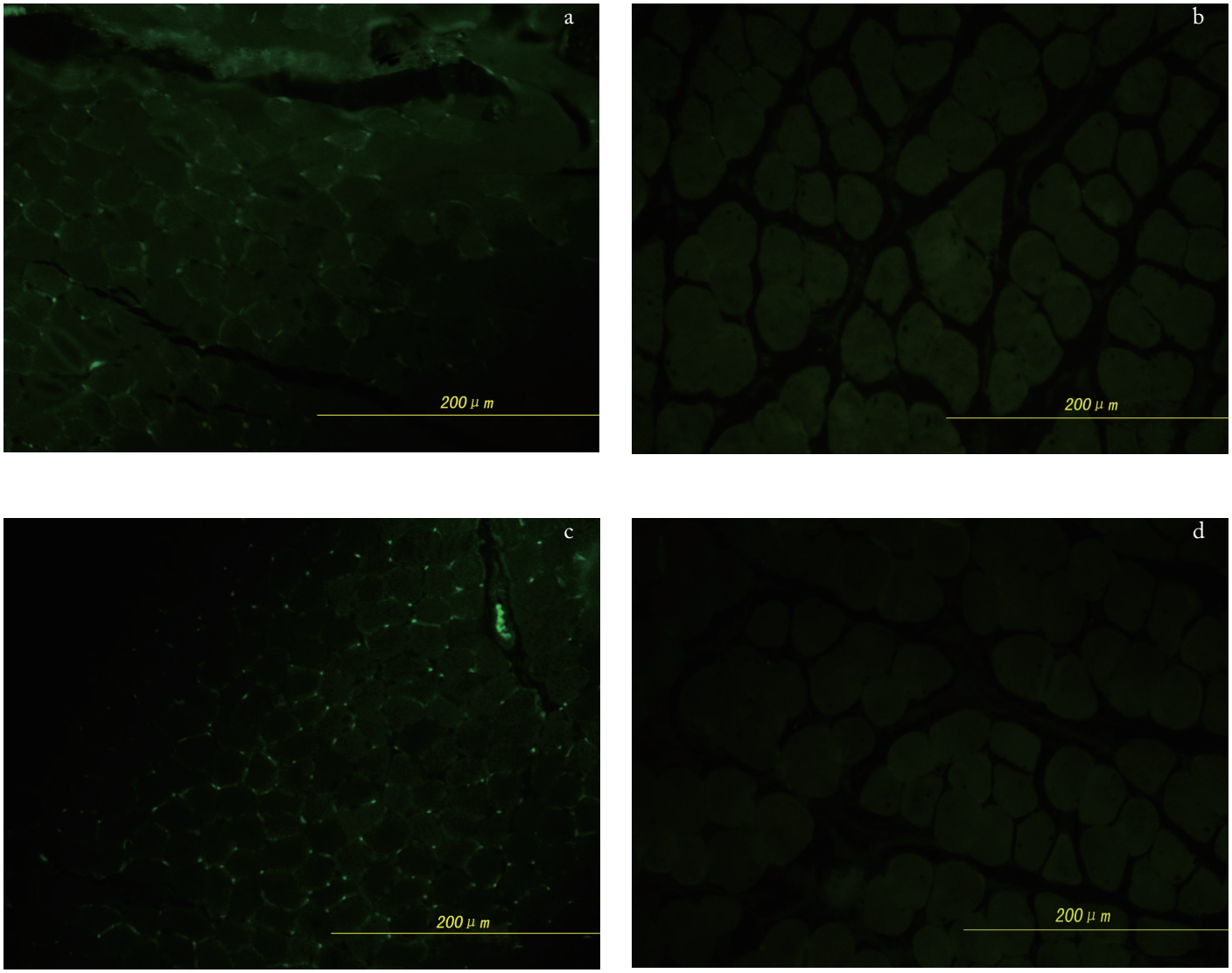

Fig 1. Survival condition of MPC in the muscle (×200). a. Group of MPC transplantation into transected position; b and d. Sham operated group; c. Group of MPC transplantation into muscle

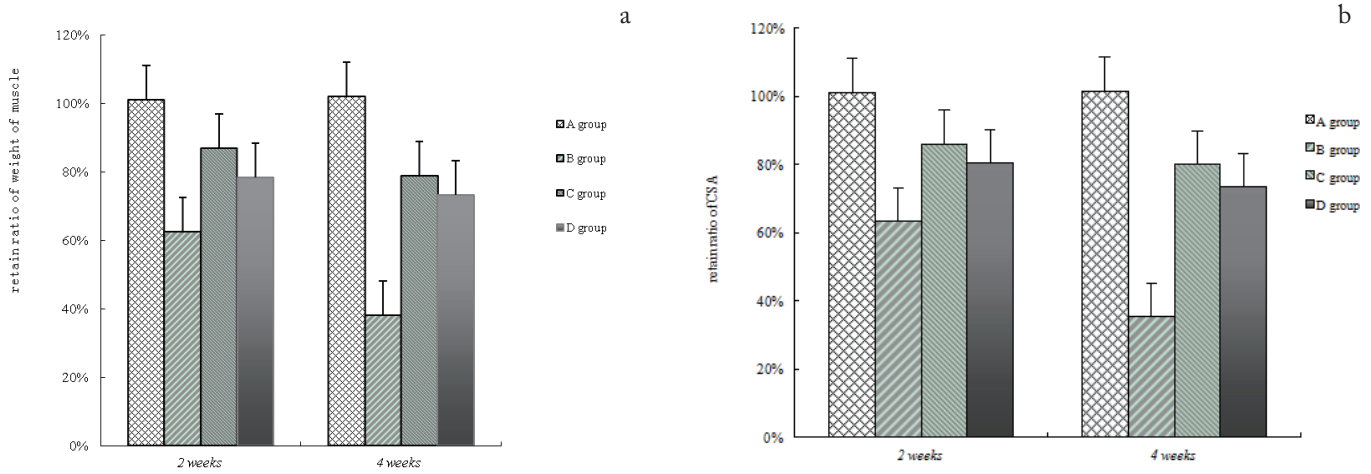

Fig 2. Changes of retain ratio of weight of muscle and cross section area of muscle fibers

P < 0.05, P < 0.01 compared with B group

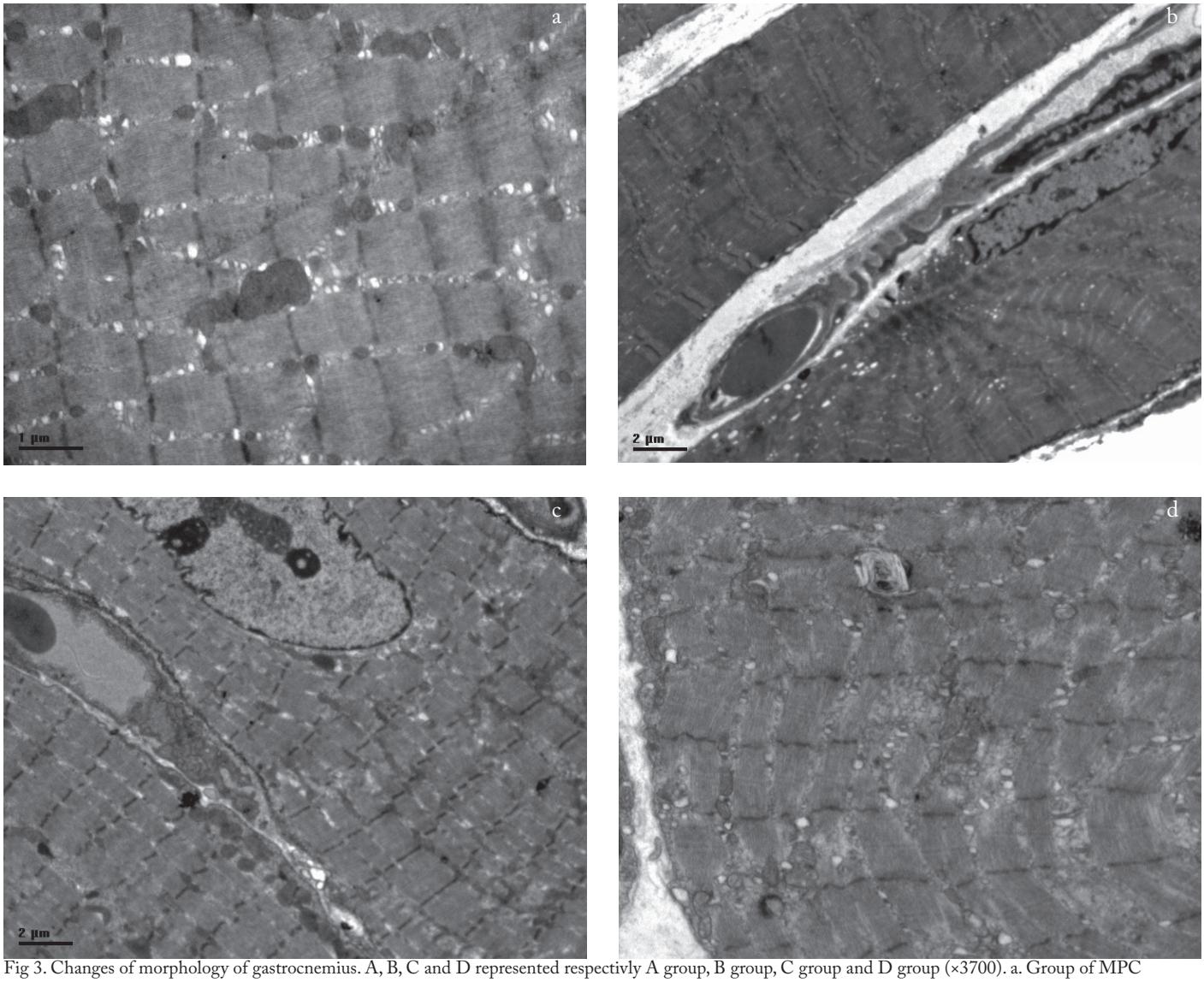

Fig 3. Changes of morphology of gastrocnemius. A, B, C and D represented respectivly A group, B group, C group and D group (×3700). a. Group of MPC transplantation into transected position; b and d. Sham operated group; c. Group of MPC transplantation into muscle

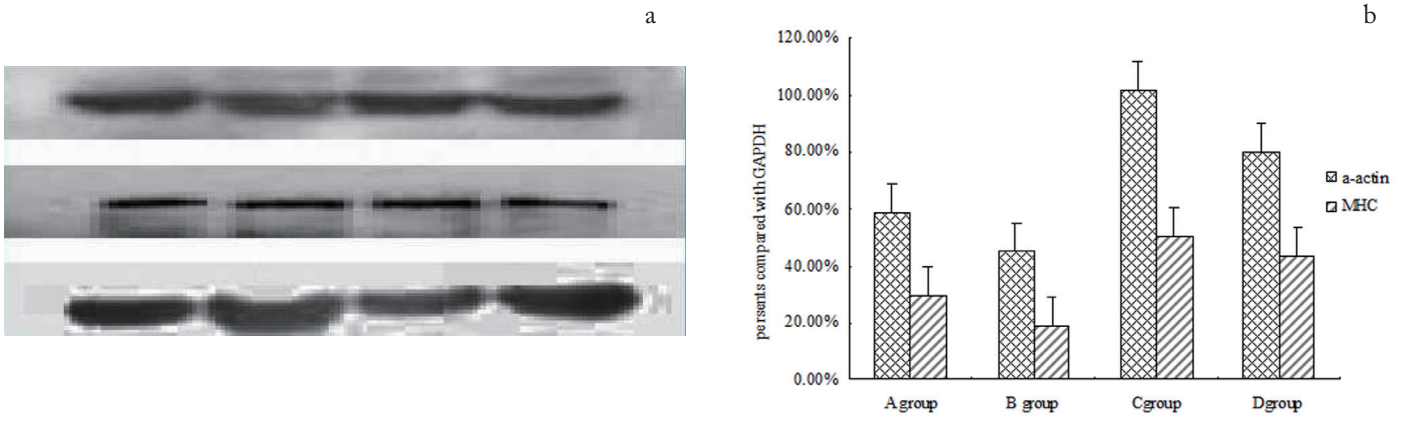

Fig 4. Expression of α-actin, MHC of gastrocnemius. a. represented the results of detection by western blot. b. represented the results of quantization.

$P < 0.05$, $P < 0.01$ compared with the A group.

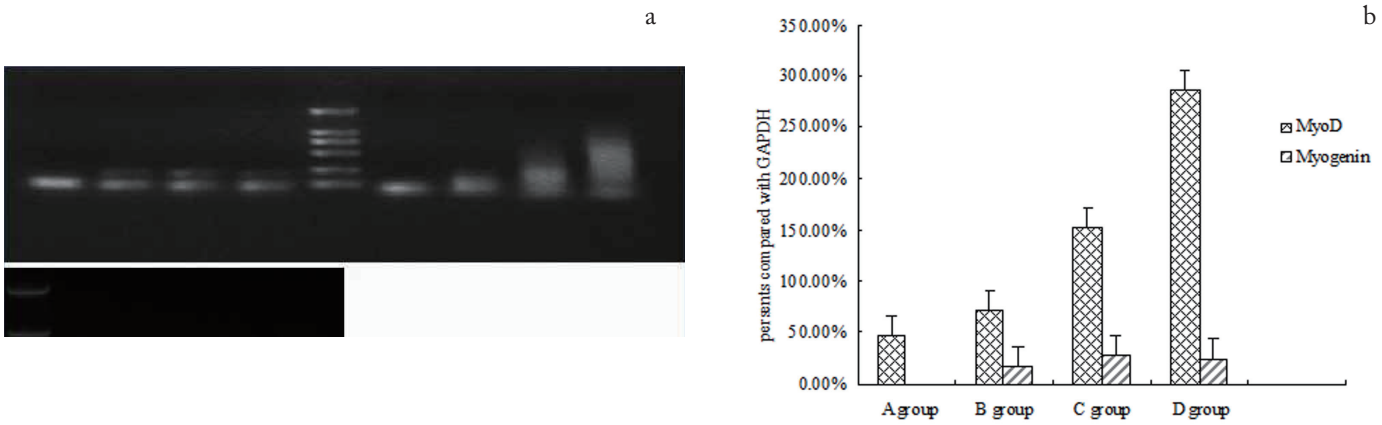

Fig 5. Expression of Myogenin and MHC of gastrocnemius. a. represented the results of detection by RT-PCR.1-4 represented respectivly A group B group C group and D group. b. represented the results of quantization.

P < 0.05, P < 0.01 compared with A group; P < 0.05 compared with the transected group.

were not found in group B (Figure 1b and d).

2.3 Changes of retain ratio of muscle wet weight and CSA

The wet weight of mice gastrocnemius and retain ratio of muscle fibers CSA were obviously higher than those of group B at 2 weeks and 4 weeks after operation (n=6) (Figure 2).

2.4 Observation of ultramicrostructure

The cytoblast increased obviously, Feulgen's stain was uniform and heterochromatin was developed at 4 weeks after operation in group C and D compared with group B; Mitochondrial quantity increased with unobvious swelling, mitochondria ridge was more and its shape was relatively normal; Endoplasmic reticulum expanded less; Myofilament and myocomma were laid in order, without obvious confluens; Interstitial collagen of muscle fibers was less (Figure 3).

2.5 Expression of actin and myoglobulin

The expression degree of actin and myoglobulin in group C and D was obviously higher than that of group A and B at 4 weeks after operation (n=6) (Figure 4).

2.6 Genetic expression of Myogenin and MyoD was detected by RT-PCR

The genetic expression degree of MyoD in group C and D was obviously higher than that of group A and B at 4 weeks after operation, and the genetic expression degree of Myogenin was obviously higher than that of group B; The genetic expression of Myogenin was not obviously found in group A (n=6) (Figure 5).

3 Discussion

It is very important to find a new kind of transplanted cells because the way of obtaining neural stem cells is relatively limited and the amplification is also difficult. Although the bone mesenchymal stem cells(BMSCs) have the characteristics of multi-directional differentiation potency, sufficient resource, convenient materials, low danger, no immunological rejection and directional differentiation into neuron-like cells in vitro(7-9), yet the proliferation may not be ideal because the culture of mice MPC in vitro is easy to be polluted by hematopoietic stem cells(10), therefore, the transplantation treatment in vivo of denervated skeletal muscle atrophy can be affected by hematopoietic stem cells. In recent years, the research has found that MPC has the same characteristics of BMSCs, and it can be the seed cells instead of neural stem cell because it comes from compact bone, and the pollution of hematopoietic stem cells can be avoided when culturing in vitro(11). Will the MPC transplantation in vivo survive and delay denervated skeletal muscle atrophy?

It is found that the locomotor activity of mice right hind limbs in group C and D is gradually recovered to the normal status along with the extension of treatment, transplanted cells can survive and evenly distribute into myocyte gap, and myofilament and myocomma are laid in order. Degeneration of myocyte nucleus, mitochondria and endoplasmic reticulum, and the muscle fibrotic degree are better than those of group B, and the descendent range of muscle wet weight and retain ratio of muscle fibers CSA, and the degradation speed of α-actin and MHC are obviously lower than those of group B, therefore, it shows that the MPC transplantation into nerve transected position or gastrocnemius can delay the denervated skeletal muscle atrophy. Muscle is still in a state of denervation because of nerve injury, and atrophy will be the last status of this kind of muscle without sufficient neurotrophy, but the transplantation of compact bone derived MPC will get more time for nerve regeneration, and can provide a better skeletal structure basis for the function recovery of muscle dominated by nerves.

In addition, the expression of myogenic regulating factors (MRFs) such as MyoD and myogenin, etc. after MPC transplantation in vivo has been researched in this study. Rodrigues, etc. (12-13) have found that the quantity of muscle satellite cells in denervated post-skeletal muscle decreases rapidly along with the time extension of denervation, therefore, the maintenance of normal morphology and structure of muscle cells, and the regeneration of damaged and atrophic muscle cells depend on the content of muscle satellite cells, while the MRFs play a decisive role in the proliferation of muscle satellite cells, and only with the role of those factors, can the regeneration of muscle satellite cells grow towards the desired goal. MyoD as the determinative factor for myogenic differentiation exists in the satellite cells of neonatal and regenerated skeletal muscle. The muscle satellite cells activate the early MyoD when skeletal muscle regenerates, and then the MyoD is expressed in all the proliferous muscle satellite cells, therefore, MyoD is regarded as the marked protein for activated muscle satellite cells(14).

A large number of animal studies have found that the expression level of Myogenin rises with the denervation in mature skeletal muscle, then the synthesis of specific embryonal receptors in a series of skeletal muscle and spectrin is launched, and the expression of embryonal protein is the prerequisite for the reinnervation of denervated skeletal muscle(15). Ekmark, etc.(16) have detected the expression of myogenin genes and MyoD protein through immunoblotting, and found those two things have different changing processes after denervation. The expression of the former increases quickly in 24h after denervation, while decreases quickly after 5d and reaches to the lower on the 7d; however, the expression of the latter starts to decrease gradually after denervation. Russo, etc.(17) have found that the expression of MyoD mRNA decreases obviously after denervation, and the expression of myogenin genes amplified with RT-PCR in skeletal muscle cells decreases obviously after denervation. The expression of myogenin genes and MyoD protein decreases obviously at the 8th week after denervation, and with obvious muscle atrophy, therefore, it indicates that the atrophic mechanism of skeletal muscle caused by the denervation is related to the decrease of myogenin genes and MyoD protein expression. It is found in this study that the expression level of MyoD in group C and D is obviously higher than that of group A and B, and the Myogenin genic expression is stronger than that of group B at the 4 weeks after operation. Therefore, the delay mechanism of skeletal muscle atrophy might be that the survival MPC secretes some neurotrophic factors, and they are brought to the gastrocnemius through axonal transport(MPC transplantation into nerve transected position) or diffusion(MPC transplantation into muscle), then they cause a large number of activated muscle satellite cells to differentiate and produce massive new muscle fibers or directly delay the atrophy of muscle cells, therefore, the wet weight, muscle protein level and muscle fibers CSA can be maintained, but its certain working mechanism needs a further study.

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