Int J Clin Exp Med 2018;11(8):7963-7970
www.ijcem.com /ISSN:1940-5901/IJCEM0044129

Original Article

Effects of one-time exhaustive exercise on skeletal muscle ultrastructure, satellite cells and hepatocyte growth factor in rats

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Received July 25, 2016; Accepted May 1, 2018; Epub August 15, 2018; Published August 30, 2018

Abstract: Skeletal muscle injuries are frequently occurred in various sports, among which satellite cells play a key role in the repair of skeletal muscle injury, and hepatocyte growth factors are the only growth factor that can activate satellite cells. This study aimed to investigate the effects of one-time exhaustive exercise on skeletal muscle ultrastructure, satellite cells and hepatocyte growth factor in rats. Samples were harvested from 24 rats in a quiescent state (control group) or at different time points after exhaustive exercise (E0, E24 and E48 groups) for observation of skeletal muscle ultrastructure change and determination of hepatocyte growth factor level in skeletal muscle and serum. Satellite cells harvested from the vastus lateralis of rats in control group and E0, E24 and E48 groups were cultured *in vitro*. Satellite cells harvested from the vastus lateralis of another six neonatal rats were also cultured *in vitro*. Rat skeletal muscle and serum hepatocyte growth factor levels in the exhaustive exercise (E0, E24 and E48) groups were significantly greater than that in the control group ($P < 0.01$). The number of satellite cells per unit volume of skeletal muscle in the E0, E24 and E48 groups was greater than that in the control group, but satellite cells in the E0, E24 and E48 groups proliferated faster with time going. Compared to adult rats, absolute satellite cell number in muscle tissue was less, but the number of satellite cells per unit volume of tissue was greater than that in the neonatal rats. Hepatocyte growth factor can activate satellite cells and accelerate repair of soft tissue and microvessels. The number of satellite cells per unit volume of skeletal muscle is greater in rats subjected to exhaustive exercise and these cells exhibit stronger proliferative ability than those in the control rats. Therefore, exhaustive exercise can activate quiescent satellite cells. The number of satellite cells per unit volume of tissue in neonatal rats is greater and their proliferative ability is stronger than that in adult rats.

Keywords: One-time exhaustive exercise, ultrastructure, satellite cells, hepatocyte growth factor

Introduction

Skeletal muscle injuries are extremely common, accounting for the highest proportion of all sport injuries [1, 2]. Long-term muscle injury and poor healing quality directly influence the normal exercise of professional sport teams and even terminate their career. There is evidence [3-5] that intensive exercise can result in skeletal muscle ultrastructural damage and the method to achieve tissue repair is to promote the proliferation and differentiation of satellite cells.

Satellite cells are a monocyte population located between the basal lamina and the plasmalemma of a myofibril and they are generally in a relatively quiescent state. Satellite cells play a key role in the repair of skeletal muscle injury. Some growth factors, such as insulin-like growth factor and epidermal growth factor, do not affect quiescent satellite cells, but they can strengthen the proliferative ability of activated satellite cells. However, hepatocyte growth factors are the only growth factor that can activate quiescent c-Met-expressing satellite cells from a G0 phase to enter S phase [6].

To better understand the repair process of skeletal muscle injury and the underlying mechanism, in this study we established rat models of skeletal muscle injury caused by one-time exhaustive downhill running. Ultrastructural changes in rat rectus femoris muscle at different time points after exhaustive exercise were observed and simultaneously rat skeletal mus-

cle and serum hepatocyte growth factor levels were determined. In addition, in vitro culture and proliferation of satellite cells at different time points after skeletal muscle injury were observed. These indices were compared across different groups.

Current related studies are mostly at the theoretical level and from the macroscopic perspective, and few studies have been performed from the microscopic biochemical perspective. The purpose of this study was to investigate the effects of one-time exhaustive exercise on hepatocyte growth factor level, to explore the role of hepatocyte growth factor in the activation of quiescent satellite cells and the possible mechanism underlying repair of skeletal muscle injury at different time points after one-time exhaustive exercise.

Animals and methods

Grouping

Thirty male Wistar rats of clean grade (24 rats aged 6 weeks and weighing 170 ± 11.94 g, 6 rats aged 3 days), were raised in separate cages and allowed free access to food and water.

Twenty-four adult rats were randomly divided into one control and three exhaustive exercise (E0, E24 and E48) groups, with 6 rats per group. Six neonatal rats were used for comparison. All included rats were used for cell culture.

All procedures involving animals in this study were approved by the Ethics Committee of Yangzhou University.

Modeling

Rat models of skeletal muscle injury caused by one-time exhaustive downhill running were established. Two days prior to formal experiments, all rats were subjected to 5-10 minutes of adaptive treadmill exercise at 5-10 m/min at 0° inclination. According to a previous method [7], rats were made to perform the exercise till exhausted based on the following schedule: first-level load: 0°, 8.2 m/min, 15 minutes (equivalent to 53% VO_2max); second-level load: 5°, 15 m/min, 15 minutes (equivalent to 64% VO_2max); third-level load: 10°, 19.3 m/min (equivalent to 76% VO_2max), until exhausted.

Sample harvesting

Exhaustive exercise groups: Rats in the exhaustive exercise and control groups were anesthetized by intraperitoneal injection of 20% urethane at corresponding time points after exhaustive exercise and in the quiescent state respectively. After sacrifice, 5 mL of blood was harvested from the abdominal aorta and serum level of hepatocyte growth factor was determined. Rat rectus femoris muscle on one side was rapidly fixed in 2.5% glutaral and then electron microscopic sections were made for observation of skeletal muscle ultrastructural changes under a transmission electron microscope. Rat rectus femoris muscle on the other side was preserved in liquid nitrogen for determination of hepatocyte growth factor level. Vastus lateralis muscle was used for cell culture. Immediately after that, separation, purification and culture of satellite cells were performed using a 2-step enzymatic digestion. Immediately after anesthesia, identical samples from neonatal rats were harvested and satellite cells were cultured *in vitro*.

Separation and culture of rat satellite cells

According to a slight modification of the methods described by Volonte [8] and Wrobel [9], under sterile condition, rat satellite cells were separated and cultured.

Index examination

Enzyme-labeled immunosorbent assay (ELISA): In strict accordance with the instruction provided by ELISA kit (Nanjing Jiancheng, China), rat skeletal muscle and serum levels of hepatocyte growth factor were determined using ELISA method and the absorbance values were read on a microplate Elx800 (BIO-TEX Instruments Inc., Winooski, VT, USA).

Transmission electron microscopy observation of skeletal muscle ultrastructure: Rat rectus femoris muscle was fixed in 2.5% glutaral for over 2 hours, washed with phosphate buffer, fixed with 1% osmic acid for 1 hour, and then ultrathin microscope sections were made. Electron microscope specimens of four rats were randomly selected from control and exhaustive exercise (E0, E24 and E48) groups. Under a transmission electron microscope, the morphological changes of the organelles, such as

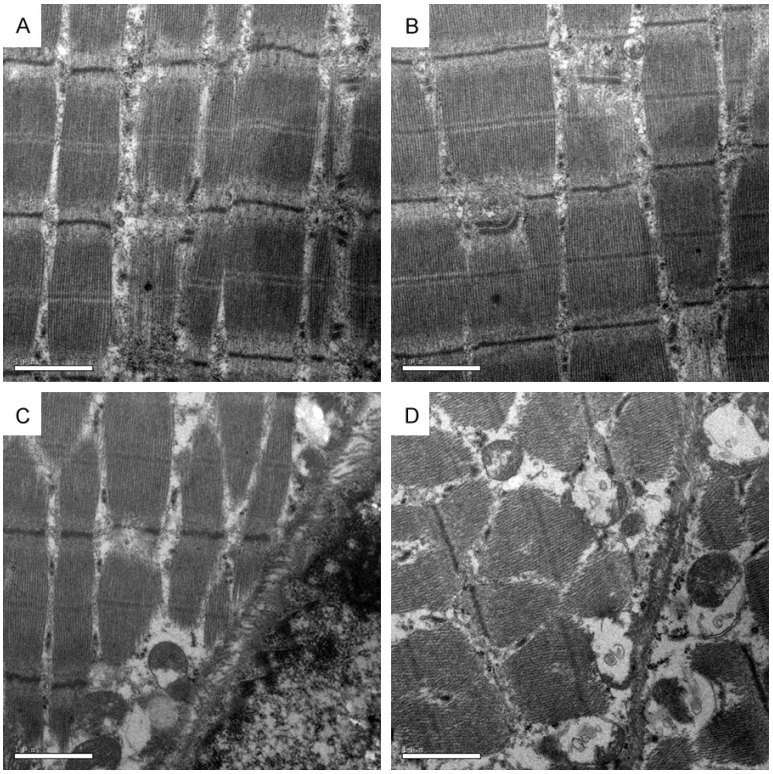

Figure 1. Changes in rat skeletal muscle ultrastructure after exhaustive exercise and during the recovery period. A: Control group (×18500). B: Immediately after exhaustive exercise group (E0 group; ×18500). C: 24 hours after exhaustive exercise group (E24 group; ×18500). D: 48 hours after exhaustive exercise group (E24 group; ×18500).

nucleus and mitochondrion and the degree of muscle fiber ultrastructure damage were qualitatively determined.

Statistical analysis

All data are expressed as the mean ± SD and were statistically processed using SPSS 17.0 software. Independent sample t-test was performed. A level of $P < 0.05$ was considered significant and a level of $P < 0.01$ highly significant.

Results

Rat physical signs after exhaustive exercise and during the recovery period

During the experimental period, rats in the control group had good appetite, lustrous fur, sparkling eyes and increase in body weight. However, rats in the exhaustive exercise groups looked tired and their four limbs were swollen at different degrees.

Changes in rat skeletal muscle ultrastructure after exhaustive exercise and during the recovery period

Electromicrographs of rat rectus femoris muscle in different groups (**Figure 1**) showed different changes as follows. Control group: rat skeletal muscle exhibited normal ultrastructure with clear endoplasmic reticulum and mitochondria, intact nucleus structure, well arranged myofibrils, and clear boundaries among H band, I band, M line and Z line. E0 group: rat skeletal muscle structure was greatly destroyed, intercellular substance was swollen, spatium intermusculare was widened, nucleus and nuclear membrane were good, endoplasmic reticulum and mitochondria were slightly deformed, myofibrils were sparse and thinned, the boundaries of H and I bands were unclear, and M and Z lines were unclear. E24 group: rat skeletal muscle structure was destroyed, intercellular substance was obviously swollen, endoplasmic reticulum and mitochondria were slightly deformed and twisted, myofibrils were sparse, thinned and even disappeared, and Z line was twisted. E48 group: rat muscle fiber injury was further aggravated, inflammatory cells were infiltrated, sarcolemma was unclear, myofibrils were arranged disorderedly, A and I bands were hardly distinguished. Sarcomere and myofilaments were poorly arranged, Z line was twisted and even disappeared, mitochondria were differently sized, swollen or pyknotic, unevenly distributed, and unclear. Vacuoles formed in the muscle.

Rat skeletal muscle and serum levels of hepatocyte growth factor after exhaustive exercise and during the recovery period

After one-time exhaustive exercise, rat skeletal muscle and serum levels of hepatocyte growth factor in each exhaustive exercise group were

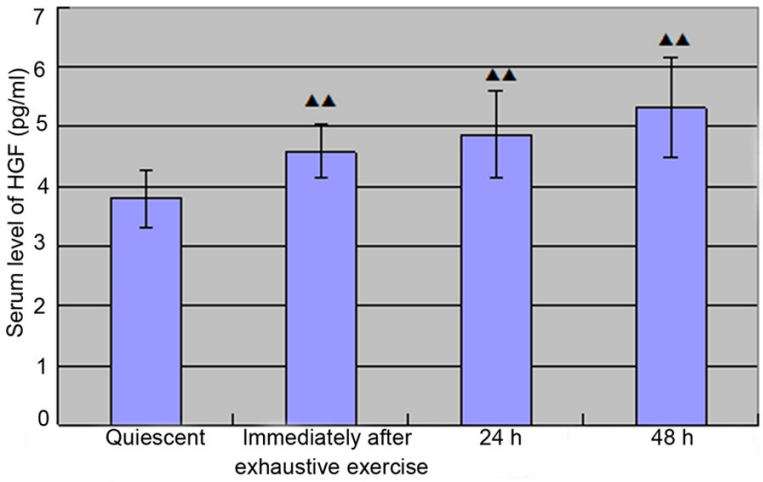

Figure 2. Hepatocyte growth factor level of skeletal muscle in rats after exhaustive exercise and during the recovery period. *P < 0.05, **P < 0.01, vs. control group.

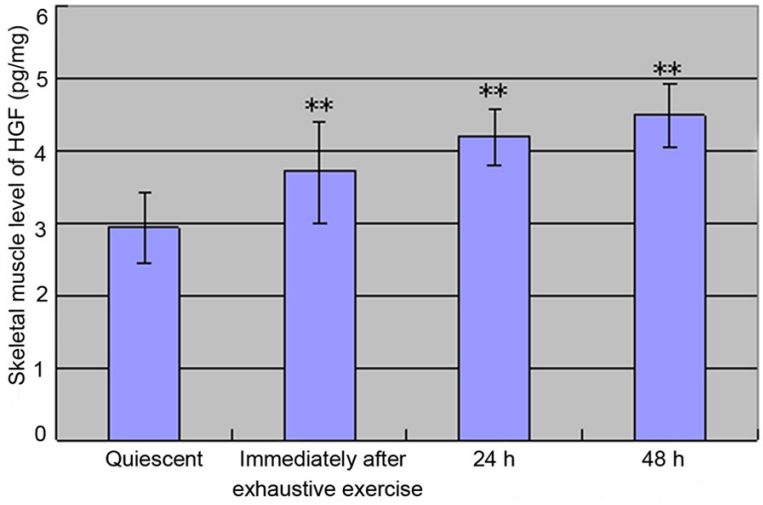

Figure 3. Rat serum level of hepatocyte growth factor after exhaustive exercise and during the recovery period. ▲P < 0.05, ▲▲P < 0.01, vs. control group.

significantly greater than in the control group (P < 0.01) (**Figures 2** and **3**).

Satellite cells cultured in vitro at different time points after one-time exhaustive exercise

Primary satellite cells were initially rounded, and most of them were suspended in the culture fluid. One day later, they gradually adhered to culture flask, were very small-sized, and were evenly distributed. Then they gradually expanded in volume and began to divide. Electron microscopy showed that 2 or 3 or more cells arranged in a row. After 3-4 days later, cells began to enter the logarithmic phase, rounded cells accounted for a larger proportion, and cells formed clusters in various regions.

The satellite cells released by 2-step enzymatic digestion were of high purity. The *ex vivo* cells were scattered on the bottom of culture flask and they were rounded and had strong refraction. Twenty-four hours after seeding, the majority of cells adhered to culture flask. With adherence time, cells changed from rounded to shuttle- or spindle-shaped, and finally monocytes fused with each other to form multicore myotubes. Twenty-four hours later, the majority of cells adhered to culture flask, they were rounded and had strong refraction, and rare cells exhibited small processes. Forty-eight hours later, almost all cells adhered to cell culture flask. A small number of dead cells suspended in clusters. Shuttle-shaped cells increased in number and some cells began to proliferate. Seventy-two hours later, cells adhered to culture flask completely, most of them were spindle-shaped and connected with each other, an increased number of proliferating cells was observed and refraction was reduced. Ninety-six hours later, cells proliferated obviously and connected with each other. Some cells differentiated and formed myotubes.

Separation, purification and culture of satellite cells were performed according to abovementioned method. Forty-eight hours later, culture fluid was refreshed. In the control group, only a few satellite cells were observed (**Figure 4A**). In the E0, E24 and E48 groups (**Figure 4B-D**), the number of satellite cells in the exhaustive exer-

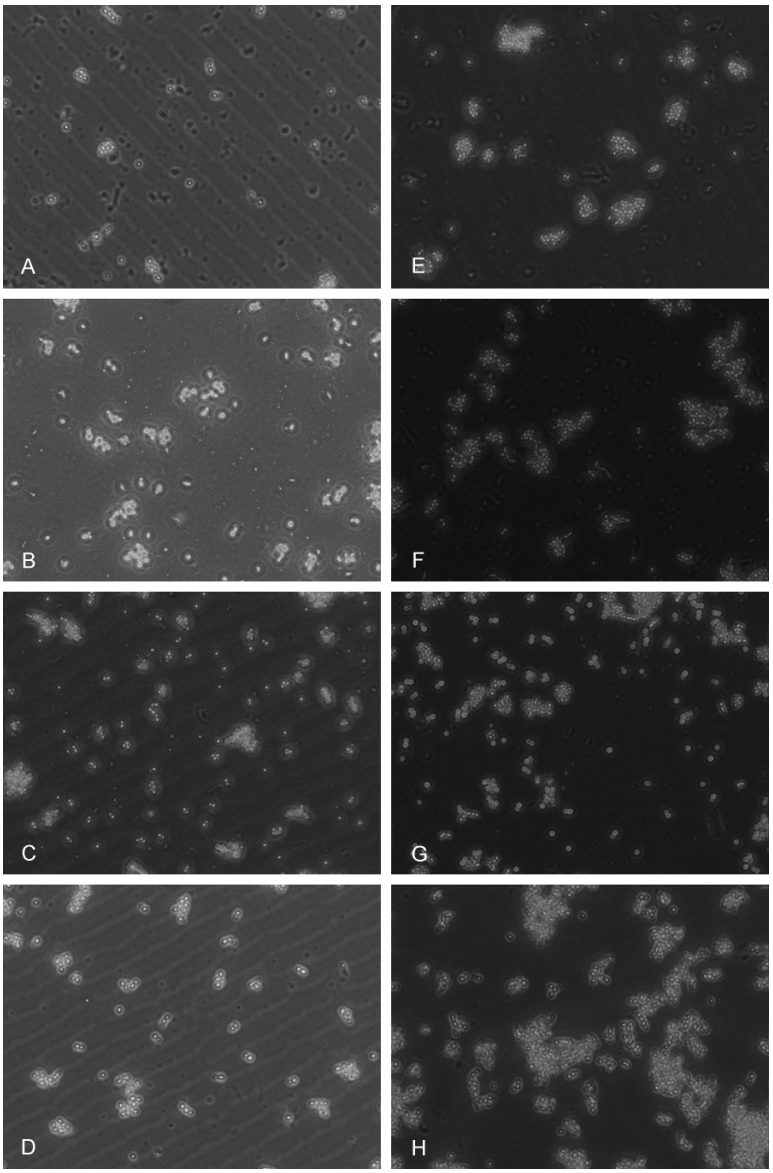

Figure 4. Satellite cells cultured *in vitro* at different time points after one-time exhaustive exercise. A: Control group (48 hours) (40× magnification). B: Immediately after exhaustive exercise group (48 hours) (40× magnification). C: 24 hours after exhaustive exercise group (48 hours) (40× magnification). D: 48 hours after exhaustive exercise group (48 hours) (40×10 magnification). E: 96 hours after exhaustive exercise group (96 hours) (40× magnification). F: Immediately after exhaustive exercise group (96 hours) (40× magnification). G: 24 hours after exhaustive exercise group (96 hours) (40× magnification). H: 48 hours after exhaustive exercise group (96 hours) (40×10 magnification).

cise groups was significantly greater than in the control group. With time after exhaustive exercise, the number of satellite cells was gradually increased.

Ninety-six hours later, satellite cells in different groups proliferated to different degrees (**Figure 4E-H**). Satellite cells in the exhaustive exercise groups proliferated more obviously than in the control group. Satellite cells in each group began to expand and simultaneously divide. Two or three or more cells arranged in a row. With time after exhaustive exercise, the number of satellite cells gradually increased.

Comparison of in vitro cultured satellite cells of adult versus neonatal rats after exhaustive exercise

Separation, purification and culture of satellite cells of neonatal rats were performed according to abovementioned method. Forty-eight hours later, culture fluid was refreshed and cells were photographed (**Figure 5A**). Immediately after exhaustive exercise, satellite cells of neonatal rats were slightly smaller than those of adult rats. The number of satellite cells per unit of skeletal muscle in neonatal rats was greater than that in adult rats. Ninety-six hours later, satellite cells of neonatal rats proliferated rapidly and soma expanded gradually and began to divide. Two or three or more cells arranged in a row (**Figure 5B**).

Discussion

Accumulative evidence exists that violent and unaccustomed load exercise, especially eccentric exercise, can cause skeletal muscle ultrastructural changes. Optical and electron microscopy findings of injured muscle fiber structure can be used as the direct evidence of sports-related skeletal muscle injury [10].

Microphotographs of rectus femoris muscle of rats in different groups showed different changes. Control group: Rat skeletal muscle exhibit-

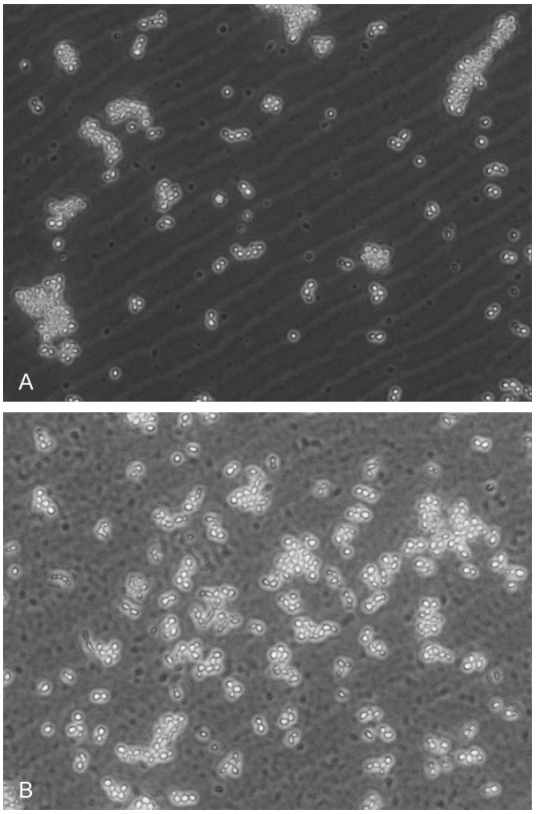

Figure 5. Comparison of in vitro cultured satellite cells of adult *versus* neonatal rats after exhaustive exercise. A: Neonatal rats (48 hours) (40×10 magnification). B: Neonatal rats (96 hours) (40×10 magnification).

ed normal ultrastructure with clear endoplasmic reticulum and mitochondria, which were evenly distributed in the muscle plasma, intact nucleus structure, well arranged myofibrils, and clear boundaries among H band, I band, M line and Z line.

Exhaustive exercise group: Rat skeletal muscle structure was greatly destroyed. Precisely, (1) Myofibrils were sparse and thinned, the boundaries of H and I bands were unclear, and M and Z lines were unclear. Z line was twisted even disappeared. (2) Intercellular substance was swollen and spatium intermusculare was widened. (3) Endoplasmic reticulum was clear but it was slightly deformed, and nucleus and nuclear membrane were good. (4) Mitochondria were obviously deformed and twisted, and even swollen and disrupted. With time after exhaustive exercise, damage to myocytes in the exhaustive exercise groups became more and more severe. Therefore, rat models established in this study meet experimental needs. Our electron microscopy results showed that compared with previously established models of sports-related skeletal muscle injury, rat models of skeletal muscle injury caused by one-time exhaustive exercise were of more suddenness.

Tatsumi *et al* [11] found that soon after skeletal muscle injury, satellite cells were activated and hepatocyte growth factor level and c-Met expression increased. Miller *et al* [12] found that after skeletal muscle injury, not only satellite cells but also normal skeletal muscle secretes hepatocyte growth factor. After muscle injury, local injection of hepatocyte growth factor can activate satellite cells, increase the number of myoblasts, and promote recovery of muscle injury. Exercise can increase the expression of hepatocyte growth factor in skeletal muscle [13, 14].

In the present study, hepatocyte growth factor expression was not observed in the control group. Hepatocyte growth factor expression in rat skeletal muscle in the E0, E24 and E48 groups was significantly greater than in the control group ($P < 0.01$). These results suggest that after exhaustive exercise, injured organism makes hepatocyte growth factor expression in rat skeletal muscle significantly increased through autocrine and paracrine. Rat serum level of hepatocyte growth factor in the E0, E24 and E48 groups was significantly greater than in the control group ($P < 0.01$). These results suggest that after exhaustive exercise, some hepatocyte growth factors may be involved in the repair of tissue injury. Therefore, hepatocyte growth factor in the serum can be considered one of factors that accelerate tissue and microvessel recovery and promote conversion of satellite cells from quiescence to activation. These findings also confirm that one-time exhaustive exercise can increase serum level of hepatocyte growth factor.

We *in vitro* cultured satellite cells after exhaustive exercise and during the recovery period and found that the number of satellite cells per unit rat skeletal muscle in the E0, E24 and E48 groups was significantly greater than in the control group and with time after exhaustive exercise, the number of satellite cells showed a gradually increased tendency. Satellite cells in the E0, E24 and E48 groups showed stronger

proliferative ability than in the control group. Inflammatory reaction occurred within 24 hours after exhaustive exercise and satellite cells were activated and proliferated within 48 hours. These results are highly similar to previous studies [15, 16]. All these results confirm that exhaustive exercise can activate quiescent satellite cells.

Satellite cell content is different under different functions, physical activity levels and ages [17, 18]. The precise mechanism is currently uncertain and needs further investigation. This suggests that the mechanism underlying satellite cell expression is complex and is influenced by various factors. The time periods, 24 and 48 hours after inoculation of neonatal rats, are cell adaptation periods. The cell growth curves till 72 and 168 hours are basically linear because these two periods are cellular logarithmic phases. Our results showed that in neonatal rats, absolute satellite cell number in muscle was lower, but the number of satellite cells per unit of skeletal muscle was higher, and satellite cells had stronger proliferative ability than in adult rats.

In conclusion, our results demonstrate that high-intensity eccentric exercise can increase hepatocyte growth factor expression, thereby activating quiescent satellite cells. We hope the present innovative and exploratory study will provide reference information for future related studies.

Acknowledgements

This study was supported by The Key Research and Development Program of Jiangsu Province (Social Development Program, Gait characteristics in older adults who fall and the early warning protection technology, No. BE2017766).

Disclosure of conflict of interest

None.

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