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Effect of Exercise and Massage Therapy on Injured Muscular Structure and C-Reactive Protein Expression

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ABSTRACT

The treatment and rehabilitation after acute skeletal muscle injuries remain major challenges of sports medicine practice and further studies could provide more information for effective treatment of this pathological condition. In this study, adult rats were divided into four groups namely natural healing (control), exercise and massage, massage only and exercise only and received acute skeletal contusion to the right tibialis anterior, followed by corresponding rehabilitation methods. The injured muscular tissues and blood samples were collected pre-injury and post-injury on day 1, 2, 4, 6, 9, 12 and 15. The exercise and massage group showed the fastest recovery reflected by muscular histological structure and C-reactive protein levels, followed by the other three groups. C-reactive protein decrease in exercise and massage, massage only or exercise only groups was significantly different comparing to control group ($p<0.05$). Combined treatment of exercise and massage therapy showed the best effect in inflammation suppression, skeletal muscle regeneration, control of skeletal muscle fibrosis and muscular tissue re-construction. The skeletal muscular structure and C-reactive protein level could reflect the recovery process after skeletal muscles injury with high sensitivity in groups with the four different rehabilitation methods.

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PL, QL and XC designed the study and wrote the manuscript. PL performed experiments and analyzed the data.

Key words
Skeletal muscular injury, Sports medicine, Massage therapy, Muscle tissue structure, C-reactive protein

INTRODUCTION

Exercises and massage therapy are two rehabilitation methods which are easy to perform, are low-cost and easy to popularize (Barnes *et al*., 2008; Tiidus, 2015). These methods could help relieve the pain, recover the function of skeletal muscles and the kinetic functions of joints, so as to improve the life quality of patients. They are widely applied in sports medicine and rehabilitation treatments (Aboodarda *et al*., 2015). It has been reported that mechanism of muscle injury followed by message therapy involves collapse of injured myofibers, muscle stem cell followed by activation of regeneration of myofibers and replacement of injured myofibers with new myofibers (Blau *et al*., 2015; Nedergaard *et al*., 2013). The recovery could be the result of whole body or partial biological effects (Weerapong *et al*., 2005), including blocking of the pain signal, improving the blood and lymphatic perfusion, suppressing local inflammations, etc. Besides this, the signaling pathways could be induced in skeletal myofibers by physical traction in certain directions, and further regulate the protein synthesis, glucose uptake and immune intervention (Thomson *et al*., 2015), as well as influence the regenerative capability of muscle stem cells and the reconstruction of injured muscle tissues (Gilbert *et al*., 2010).

Currently, little is known about how exercises and massage therapy would induce the dynamic changes at serology and histology levels during the muscular pathological and recovery processes. This scarcity of knowledge largely limits the possibility of further improvement of efficacy and safety of exercises and massage therapy. This study used the sports medicine and massage therapy as treatment of injured skeletal muscles and monitored the reconstitution level of skeletal muscle recovery by serological and histological biomarkers, to help understand the optimal methods of functional exercises and rehabilitation methods treating common sports injuries.

MATERIALS AND METHODS

Experimental animals

Healthy male adult Sprague-Dawley rats (n=48, body weight=360+22.7g) were purchased from Anhui Double-

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Crane Pharmaceuticals, Ltd., with permit # SYXK-2016-003, with level of 'clean animals'(CL) based on experimental animal classification. The experiments were approved by Institutional Animal Care and Use Committee of Anhui Normal University (approval number 2016027). The animal experiments were carried out at Wannan Medical College. ARRIVE guidelines and appropriate guide for the care and use of Laboratory animals were followed during the animal experiments. The temperature of vivarium, animal massage room and animal exercise room were maintained at 23-25°C, and the humidity at 50-60%. Vivarium was maintained with active ventilation and air circulation, and the illumination followed natural 24-hour cycle (12h on, 12h off).

Skeletal muscle contusion model

After the animals were weighed, 2.5% pentobarbital sodium (0.2ml/100g body weight) was administered through intraperitoneal (i.p.) anesthesia. Then each animal was made to lying with abdomen down on sterile platform, with right hind limb fully extended, and with 90°C dorsiflexion of ankle. A metal mass (cone-shape balance weight, 220g) was allowed to fall through a guide PVC tube (65cm of height and 3cm of diameter) twice with a three-second interval, causing the injury at the same position. The injury was at inner side of right hind leg, over the mid-belly region of the gastrocnemius muscle, and was around 1.5cm away from heel bone. The injured surface area was ~0.674cm^2, with kinetic energy of 1.372J. This model resulted in moderate closed lesion of tibialis anterior, and follow-up histological analysis confirmed a 100% success rate of the muscle contusion resulted from such injuries.

Rehabilitation methods

After the injury was introduced, the 48 animals were randomly separated into four groups (natural healing (control), exercise and massage, massage only and exercise only), each with twelve rats. For natural healing group, no rehabilitation method was applied to the animals, and they maintained their regular life cycles with regular diets. For exercise and massage group, each animal was made to do uphill running on treadmill for 3 min (+10 degree of slope, 12m/min), followed by downhill running for 3 min(-10 degree of slope, 14m/min), with no rest in between. Then the animals received massage therapy at injured leg, back and the uninjured leg, with a pressing frequency of 70-75/min for 4 min and with moderate force. The animal received both methods once per day, between 4pm-5pm. For massage only group, the animals received massage therapy at injured leg, back and the uninjured leg (starting 48 h post injury), with a pressing frequency of 70-75/min for 4 min and with moderate force. The animal received massage therapy once per day, between 5pm-6pm. For exercise only group, each animal did uphill running on treadmill for 3 min (+10 degree of slope, 12m/min), followed by downhill running for 3 min (-8 degree of slope, 14m/min), with no rest in between. The animal received exercise therapy once per day, between 4pm-5pm. The animal treadmill (Catalog#BCPT-2008, Hangzhou LiTai Technology Company, Ltd., Hangzhou, China) was provided by The Kinesiology Lab at Anhui Normal University.

Serum sample collection and analysis

Animals were fasted for 9 h every morning. Blood samples were collected once five days before injury, and seven times within 15 days after injury (first post-injury collection occurred after 12 hours of injury) at 6:30am in the morning. The catheter was inserted into the lateral tail vein at a shallow angle approximately 5 cm (around 1/3 length of the tail) from the tip of the tail. When the vein was penetrated, blood flew into the catheter and the plunger of the syringe were withdrawn slowly to collect 1.5ml at a steady rate (~20 µl per sec). To avoid any health issues due to frequent blood collection, rats in each group were randomly separated into two subgroups (#1 and #2), and blood samples were collected from animals in subgroup#1 at 1-, 4-, 9- and 15-day post injury, while samples for subgroup#2 were collected at 2-, 6- and 12-day post injury. Blood samples were transferred to 1.5ml microcentrifuge tubes, incubated in 37°C water bath until they reached 37°C, and spun at 2,000 x g. Serum were collected and stored at -20°C for further analysis.

ELISA

C-reactive protein (CRP) level in rat serum was detected by rat-CRP ELISA kit (Shanghai Jiemen Biotechnology Inc.), and the data were analyzed by microplate reader (DNM-9602, Madell Technology Corporation).

Histological analysis

Two random rats were anesthetized by diethyl ether inhalation and muscle tissues of 1cm^2 were collected from the injured area on corresponding limb as well as from the healthy limb (normal control). The surgery was performed on sterile platform. The animals were euthanized afterwards. The muscle tissues were fixed in 4% paraformaldehyde solution for 4 hours, treated with gradient dehydration by ethanol (70%-100%), xylene substitution, and embedded in paraffin wax. The paraffin blocks were sectioned, and H&E staining were performed on 7-µm sections. Each stained section was imaged

under 100X bright field microscope at 5 different fields. Histological analysis of skeletal muscles was performed for samples collected at different time points.

Statistical analysis

The data were analyzed by SPSS for Windows version 18.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA analysis and Newman–Keuls test were used for CRP level expression analysis among different rehabilitation groups ($p< 0.05$ was considered significant), and data were shown as Mean±SD.

RESULTS

Pre- and post-injury of tibialis anterior serum CRP expressions in different rehabilitation groups

As shown in Table I and Fig.1, the contusion resulted in sharply increased serum CRP expression, with an average 34.7-fold increase on day 1 post injury comparing to pre-injury levels and reached the peak value on day 2 (with a 36.08-fold increase comparing to pre-injury levels). From day 4 post injury, the CRP levels in all four groups started to drop. On day 6, the Exercise and massage group (E&M) showed the fastest decrease, with an obviously lower level comparting to that of the other three groups. On day 9 post injury, E&M group showed a CRP level that is similar to pre-injury expression, which is significantly different from the other groups ($p<0.05$). In massage only group, serum CRP expression displayed a quick drop between day 6 and day 9, and it returned to pre-injury level on day 12, which is significantly different from natural healing group and exercise only group ($p<0.05$). In exercise only group, serum CRP expression did not display an obvious change in the first 6 days, but showed a dramatic decrease from day 9 to day 12, and returned to pre-injury level on day 15. In natural healing group, serum CRP expression showed the slowest recovery rate, with no obvious change in the first 6 days, and followed by a moderate decrease from day 9 to day 12. This group showed higher CRP expression comparing to pre-injury level even on day 15.

The acute injury induced 37-fold increase of serum CRP level (compared to pre-injury level), and the rats displayed severe inflammatory reactions afterwards. On day 15 post injury, the control group still showed a 3-fold increase of serum CRP, while serum CRP in exercise and massage group dropped to pre-injury level on day 9. In massage only group, serum CRP level returned to pre-injury level on day 12 post injury, and in exercise only group it happened on day 15. CRP decrease in exercise and massage, massage only or exercise only groups was significantly different comparing to control group ($p<0.05$).

In Figure 1, serum CRP expression abnormally increased after the skeletal muscle injury in rat. The CRP increases are positively correlated with injury levels in four rehabilitation groups, while the CRP decreases are positively correlated with recovery levels in those groups, indicating that CRP could reflect the healing condition with high sensitivity, as well as the final recovery status of the injury.

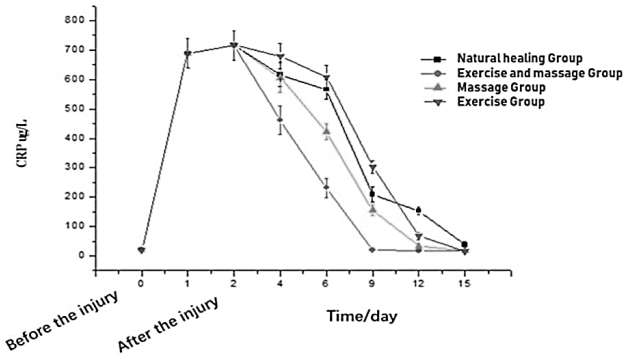

Fig. 1. Serum CRP expressions pre- and post-injury of tibialis anterior.

Ultrastructure of tibialis anterior

In natural healing group, pre-injury myofibers were complete and well organized, with even thickness (Fig. 2A).

Table I.- Serum CRP expressions pre- and post-injury of tibialis anterior (Mean±SD, mg/l).

Group	Sample number	Pre-injury	Post-injury						
			1 d	2 d	4 d	6 d	9 d	12 d	15 d
NH	12	19.86 ±6.77	689.29 ±51.32	716.61 ±50.19	617.04±41.28	566.87±33.39	209.21±25.07	153.09±12.73	38.23±10.84
E&M	12				460.88±48.82*	231.77±32.94*	20.07±12.26*	18.03±5.62*	17.88±2.37
Massage	12				606.54±50.07	422.39±26.46	156.01±19.23	32.89±10.08*	18.29±3.11
Exercise	12				679.38±43.76	608.65±40.02	302.87±20.70	67.42±12.96*	16.45±3.04

*significant differences were observed by comparing Exercises and massage group (E&M), Massage only group and Exercises only group to Natural healing group (NH), $p < 0.05$.
♦E&M group showed significant difference comparing to other three groups.

On day 2 post injury (Fig. 2B), edema and hyperemia were observed among myofibers, and part of the myofibers were disintegrated, with increased space in between and were poorly organized. On day 5 post injury (Fig. 2C), myofibers were filled with some connective tissues, yet still poorly organized, with a tendency of necrosis. On day 8 post injury (Fig. 2D), the filling among myofibers by connective tissues became obvious/dominant, and myofibers were atrophic, with obvious necrosis, and the area with degenerated myofibers were increased. On day 12 and 16 post injury (Fig. 2E and F), myofibers were still separated with large amount of crosslinked connective tissues filled in between, and were still atrophic, necrotic and poorly organized.

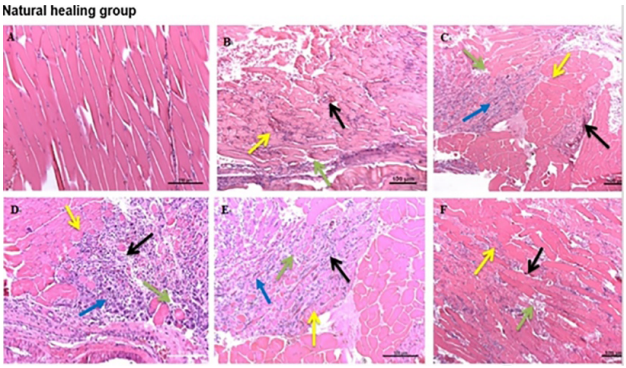

Fig. 2. Microstructure of tibialis anterior in Natural healing group (H&E staining). A, normal tissue (pre-injury); B-F, the injured tissues at day 2(B), day 5(C), day 8 (D), day 12 (E) and day 16 (F). Yellow arrows, myofiber arrays; Black arrows, myocytes; Geen arrows, connective tissues; Blue arrows, satellite cells.

In exercise and massage group, on day 2 post injury (Fig. 3B), the muscular structures were destroyed with partial myofibers disintegrated, and hyperemia was observed among myofibers, together with infiltrating inflammatory cells. On day 5 post injury (Fig. 3C), neighboring tissues were adhesive to each other, and the broken myofibers were surrounded by connective tissues, poorly organized, with some necrotic cells. On day 8 post injury (Fig. 3D), the myofibers began to align in order and tightly, with a few connective tissues filled in between. On day 12 and 16 (Fig. 3E, F), the myofibers were tightly aligned without adhesions in muscles and the nuclei of myocytes were displayed evenly. Connective tissues could still be observed (although at very low amount), and the histological structure was complete and intact.

In massage only group, on day 2 post injury (Fig. 4B), hyperemia was observed among myofibers, myofibers were disintegrated and poorly aligned and myocytes were necrotic. On day 5 (Fig. 4C), large amount of connective tissues filled up the space among myofibers, with tissue hyperplasia, poorly organized arrays and infiltrating inflammatory cells and a little necrosis. On day 8 (Fig. 4D), The myofibers were still poorly organized, with decreased infiltrating inflammatory cells and less connective tissue hyperplasia (comparing to day 5), and partially atrophic. On day 12 and 16 (Fig. 4E, F), although a few spots of stale hyperemia could still be observed, the myofibers were not adhesive to each other and had organized arrays. The nuclei of myocytes were displayed evenly, and the histological structure was complete and intact.

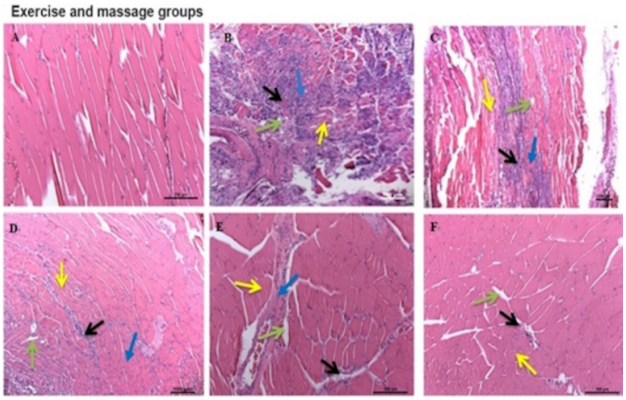

Fig. 3. Microstructure of tibialis anterior in Exercise and massage therapy group (H&E staining). A, normal tissue (pre-injury); B-F, the injured tissues at day 2(B), day 5(C), day 8 (D), day 12 (E) and day 16 (F). Yellow arrows, myofiber arrays; Black arrows, myocytes; Geen arrows, connective tissues; Blue arrows, satellite cells.

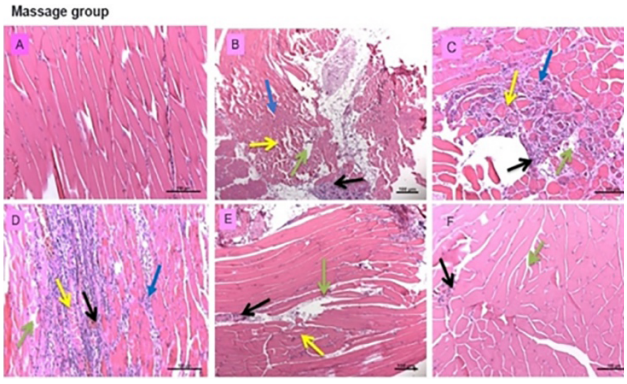

Fig. 4. Microstructure of tibialis anterior in massage only group (H&E staining) A, normal tissue (pre-injury); B-F, the injured tissues at day 2(B), day 5(C), day 8 (D), day 12 (E) and day 16 (F). Yellow arrows, myofiber arrays; Black arrows, myocytes; Geen arrows, connective tissues; Blue arrows, satellite cells.

In exercise only group, on day 2 post injury (Fig. 5B), myofibers were broken and disintegrated, with poorly organized arrays and strong inflammatory reactions. On day 5 (Fig. 5C), the myofiber arrays were disorganized, and the myocytes were atrophic and necrotic.On day 8 (Fig. 5D), the rupture of myofibers were reconnected, although the myofibers were still twisted, with cross-linked connective tissues, and relieved inflammatory reactions. On day 12 and 16 (Fig. 5E, F), myocytes were full and well-organized, and the histological structure was complete and intact.

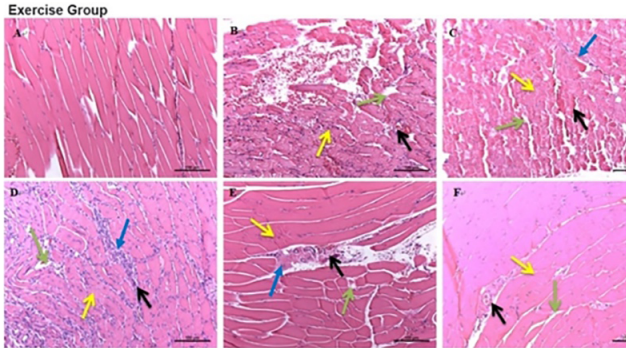

Fig. 5. Microstructure of tibialis anterior in exercise only group (H&E staining). A, normal tissue (pre-injury); B-F, the injured tissues at day 2(B), day 5(C), day 8 (D), day 12 (E) and day 16 (F). Yellow arrows, myofiber arrays; Black arrows, myocytes; Geen arrows, connective tissues; Blue arrows, satellite cells.

DISCUSSION

CRP has been a non-specific biomarker commonly used in detection of various types of acute and chronic infections, including myocardial infarction, post-surgery infection, radiation damage, gynecologic inflammation and general infection of other organs and tissues (Chen and Zhe, 2006; Liao, 2012). CRP is a classic acute-phase serum protein, with a fairly low expression in healthy individuals. However, when the tissue was injured or infected by bacteria, the inflammatory factors neutrophils, monocytes and activated macrophages release Interleukin-1(IL-1) to induce accelerated protein synthesis by hepatocytes and epithelial cells, resulting in a dramatic increase of CRP expression in serum. This induced expression could usually be detected within 6-12 hours. Comparing to other inflammatory biomarkers, CRP is faster and more sensitive to reflect the reaction during the short time course and is preferred as an important indicator of tissue injury and bacterial infection (Hou *et al.*, 2008; Liu, 2010). Liu *et al.* (2008, 2009) found that serum CRP expression increased abnormally in athletes who had muscular injuries and found that the increase of CRP is positively correlated with the injury level, and negatively correlated with recovery status. This indicates that CRP may be able to sensitively reflect the different stages during treatment and the final recovery condition of the injury.

Massage therapy could effectively regulate serum CRP expression due to skeletal muscle injuries through the suppression of inflammation. The regeneration of skeletal muscles after injury is a continuous pathological process covering the myofiber structural destruction, myocytes atrophy and necrosis, inflammatory cell infiltration, satellite cells activation and myoblast development into myofibers (Carlson and Faulkner, 1983; Kurek *et al.*, 1997). In this study, by histomorphological analysis, we observed accumulation of neutrophils at the injured locations after one hour of the acute muscular injury, which was maintained at high level for five days (data not shown). Comparing to other groups, the massage only group displayed larger number of neutrophils, with higher proliferation rate (data not shown This might be a result of induced release of inflammatory lymphokine from liver, which is triggered by the massage therapy and accompanied pressure stimulation. The monocytes were activated and released interleukin-1, while neutrophil released a series of proteolytic enzymes to degrade disrupted extracellular matrix, activate blood coagulation, and promote macrophages to move towards injured myofibers and tissue gaps to engulf cell debris, remove algogenic substances in the serum, improve the microenvironment of injured tissues, suppress the over-expression of inflammatory factor CRP, so as to suppress local inflammation. Massage therapy may serve a critical role during the process of macrophage engulfment and activation of satellite cells for skeletal muscle regeneration.

In exercise only group, the quick decrease of serum CRP expression happened around a week after muscular injuries, during which the muscles were at the end of inflammatory reaction stage and the beginning of recovery stage, which covers the process of satellite cell activation and development of myoblasts into myofibers (Jin *et al.*, 2010). On day 12 post injury, CRP level was resumed to the pre-injury level. The effective influence at later stage is closely associated with the forms of muscle exercises. The uphill running enforces concentric contractions of tibialis anterior, which leads the muscular terminals to move towards the center of the muscles. Such contractions protect the injured myofibers from getting dragged and allow the regeneration of cell membrane and reconstruction of the muscles, effectively suppressing the CRP expression. The down-hill running enforces eccentric contractions, pushing the muscular terminals towards two ends. Histological observation confirmed that eccentric contraction at early

stage of muscular injuries could result in repeated dragging of myocyte membrane and surrounding connective tissues, followed by collagen degradation and recurred damage of cell membrane (Liao, 2010). It was reported that the mechanical force from eccentric contraction could activate phospholipase A2 (PLA2), Ca^{2+}-activated proteases and lysosomal proteases (Li *et al*., 2002). The concurrent increase of intracellular could disrupt the normal functions of mitochondria, and further affect the injured structure of local tissues, represented by the damage to mitochondria and sarcoplasmic reticulum, disruption of continuous sarcolemma, and obstruction of new vessel vascularization (Li *et al*., 2002). It is worth noting that blood circulation is essential for the development from myoblasts to myofibers during the pathological process, and new vessels are the foundation of infiltration by neutrophils, macrophages and other blood-derived cells. The eccentric muscular exercises may hinder the new vessel vascularization at injured locations and delayed the engulfment and removal of necrotic cell debris by macrophages, which caused the elongated serum CRP expression.

Electron microscopy showed that in massage only group, satellite cells appeared within 24 h post injury and started division and proliferation, with collagen secretion (Fig. 4B). The capillary vessels then intrude in with growth of granulation tissues. Although the myofibers were filled with a few connective tissues, the force generated by massage pushed the fibrillar growing tissues towards well-aligned arrays along the direction of pre-existing fibers. Massage therapy obviously decreased the adhesion between tibialis anterior and skin or neighboring muscles, suppress the hyperplasia of connective tissues, myocyte atrophy and necrosis. Massage therapy also balanced the intra- and extra-cellular osmotic pressures, as well as the cell-tissue osmotic pressures, so as to maintain the stable intra- and extra-cellular levels of Ca^{2+} and recover cellular metabolism and mitochondrial transportation. Hou and Manran (2011) reported similar data in rabbit model of acute injuries, in which they introduced injuries to quadriceps femoris muscle in New Zealand rabbit, treated the animals with massage therapy, and observed the final morphology and recovery of quadriceps femoris muscle and motor endplate. Their data confirmed that massage therapy obviously improved the recovery of injured quadriceps femoris muscle and motor endplate, with higher acetyl coenzyme A activity, and effectively restrained denervation atrophy of myofibers and the regression of motor endplate. Histomorphology also indicated that the tibialis anterior scarring in massage only group was significantly decreased comparing to other groups, possibly due to that massage therapy is beneficial to new vessel vascularization at injured tissues and blood circulation speed around injured tissues, which accelerated the removal of dead cells, congestion and edema among injured tissues.

Exercise therapy exerts positive effects on the morphology, structure and motor functions of injured skeletal muscles. In the exercise only group, we performed concentric and eccentric exercises alternatively for the rehabilitation of injured skeletal muscle in rat model and observed the histology of injury recovery at different stages using various of exercise methods. Such method design is to mimic the human exercises, which are often composed of concentric and eccentric movements. The concentric movement of skeletal muscles could effective protect the injured parts from getting torn, which allow the recovery of cell membrane and reconstruction of muscle structures, which is suitable to be performed at the early stage post injury. The eccentric movement of skeletal muscle, on the other hand, could stretch the myocyte membrane and surrounding connective tissues and destroy the microstructure of muscles. Thus, it is better to carry out eccentric movement after one-week post injury. Current studies confirmed that eccentric movement causes the stretch of muscles, and pulls the muscle terminals towards two ends, which could interrupt and destroy recovery from injury. Eccentric movements with load and/or frequency that are higher than normal exercises could induce the breakage of collagen linkages, recurrent injury of cell membrane and disturbance of muscular homeostasis (including damage of myofiber membrane, twisted myofibril contraction, breakage of newly-formed myotubules, abnormal extracellular matrix and damage of cytoskeleton, suppressed the collagen fiber generation at two ends of the injured site, suppressed relinkage and synthesis of myoglobulins, etc.) (Crane *et al*., 2012; Luo and Jian, 2011; Song *et al*., 2018). However, on day 6 post injury, the activation and proliferation levels of satellite cells in exercise only group were obviously stronger than the other three groups (Figs. 2-5). Exercises result in better-organized regenerated myofibers, with intact and complete structures. This is probably due to the adapted specialization of muscles after shortening and lengthening contractions (Almada and Wagers, 2016).

CONCLUSION

CRP and histomorphological markers could sensitively and objectively represent the recovery of injured skeletal muscles during regeneration using four different rehabilitation methods. Exercises and massage therapy showed better effects in inflammation removal, skeletal muscle regeneration, skeletal muscle fibrosis control and muscle tissue reconstruction comparing to the

other three groups. Exercise only therapy showed better effects in muscular strength and balance comparing to the other groups. Eccentric exercises may cause recurrence of acute muscular injury and would be recommended to be performed one-week post injury. The alternative methods of concentric and eccentric exercises need to be used with caution, too.

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Statement of conflict of interest

We declare no conflicts of interest in this study.

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