



Effect of Massage Before or After Exercise on Delayed-Onset Muscle Soreness in a Rat Model

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Research Article

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Abstract

Background: Delayed-onset muscle soreness (DOMS), also known as tenderness of touch, refers to the pain caused by muscle mechanical stimulation, such as contraction and stretching. Chinese massage has been widely used in the treatment of sports fatigue and sports injury, but there is controversy in the efficacy. In this experiment, we established DOMS model in rats to observe the prevention and treatment effect of massage, to find the best time for intervention, and to provide scientific basis for the prevention and treatment of exercise fatigue.

Methods: 130 male SD(sprague-dawley, SD) healthy rats were randomly divided into blank group, control group and massage group. Except for blank group, the other rats received DOMS model. Professionals applied kneading and twisting methods on both lower limbs of rats. The expression of IL-2, IL-6 and IL-8 in skeletal muscle of rats were determined by western blot, PCR and ELISA, and the content of serum creatine kinase was determined by ELISA. In addition, we measured the concentration of Ca²⁺, Ca²⁺-ATPase in mitochondria of skeletal muscle. The changes of skeletal muscle structure were observed by scanning electron microscopy.

Results: After massage, the expression of IL-2, IL-6, IL-8 and CK decreased compared with control group ($P < 0.01$), the expression of IL-2, IL-6 and IL-8 in post massage group was lower than that in front massage group ($P < 0.01$), and the content of CK in front massage group was lower than that in post massage group ($P < 0.01$). The content of Ca²⁺ in front massage 24, 48 and 72h group was lower than that in post massage ($P < 0.01$), the concentration of Ca²⁺-ATPase in front massage 24h and 72h group was lower than that in post massage group ($P < 0.05$).

Conclusion: Massage can prevent the injury of muscle and reduce the inflammatory reaction of muscle after exercise. It can also improve the activity of Ca²⁺-ATPase, enhance the transport of Ca²⁺ by mitochondria and protect the skeletal muscle.

Keywords: Massage; Exercise; Delayed onset muscle soreness; Interleukin

Abbreviations: DOMS: delayed-onset muscle soreness; IL-2: Interleukin-2; IL-6: Interleukin-6; IL-8: Interleukin-8; CK: Creatine Kinase; cDNA: Complementary DNA; ELISAs: Enzyme-linked immunosorbent assays; OD: Optical density.

Introduction

Delayed onset muscle soreness (DOMS) refers to the phenomenon of muscle soreness which often occurs after the body's unadaptable exercise, especially centrifugal exercise, or sudden increase of exercise intensity [1]. It mainly manifests as local tenderness and muscle pain, and often accompany with muscle stiffness, acid swelling, limited joint movement, etc. In severe cases, it may occur acute soft tissue injury related symptoms such as muscle fiber rupture and muscle strength decrease [2]. The symptoms of DOMS are also related to the inflammatory response of sarcomere rupture [3]. At present, the physiological mechanism of DOMS is not clear. In 1902, Hough put forward the hypothesis of "mechanical injury", and thought that DOMS was related to the tear of muscle or connective tissue, as well as the obvious changes of the ultrastructure and contraction structure of skeletal muscle [4]. This is also the first time DOMS has been proposed. The hypothesis of "ischemia spasm" thought that, the pain caused by muscle ischemia after exercise will lead to muscle fiber spasm, aggravate local ischemia and form a vicious circle [5]. Theory of acute inflammatory response hold opinion that Ca^{2+} trigger a series of inflammatory reactions after mechanical injury [6], and the inflammatory mediators such as neutrophils, eosinophils and basophils are increased [7]. Inflammatory reaction and production of ROS may be the core causes of DOMS, and the interruption of excitation / contraction coupling process of muscle fibers and the disorder of muscle fibers may also be the possible causes [8]. Bradykinin and nerve growth factor also play a key role in the production of DOMS [9].

Clinically, the treatment of DOMS mainly includes hydrotherapy, ultrasound, vibration, electrical stimulation, laser therapy, anti-inflammatory drugs, acupuncture, etc. But there are different opinions on the evaluation of curative effect [10-15]. Massage is one of the important methods in treatment of DOMS in traditional Chinese medicine. It has a good effect in reducing pain and improving muscle fatigue [16]. Modern neurophysiology has found that pain is related to the disorder of neurotransmitters such as 5-hydroxytryptamine (5-HT), acetylcholine (Ach), dopamine (DA), β -endorphin (β -EP) and γ -aminobutyric acid (GABA). Research found that massage can reduce the release of 5-HT and DA in peripheral blood, and increase the release in central system [17,18], β -EP [19] and GABA [20] also increase. This plays an important role in the process of analgesia.

The dorsal horn of the spinal cord in the nervous system

is a very important integration center in the pain afferent system, and also a key part in the mechanism of massage analgesia. In 1965, Melzack and wall proposed the "gate control theory", which is the most ideal explanation for dorsal horn neurons. Type A afferent fibers excite SG cells, while type C afferent fibers inhibit. Injury cause Type C afferent tension activity, at this time, the gate is opened. Massage stimulates SG cells by stimulating Type A fibers so as to close the gate, inhibit the activity of dorsal projection neurons (T cells), reduce the transmission of harmful information to the center, and relieve the pain. This explains how massage plays an analgesic role by inhibiting pain signal transmission at the spinal cord level. In addition, massage may promote venous and lymphatic return, decompose inflammatory mediators, improve nutritional metabolism of muscle, repair muscle tissue [21]. Although massage is widely used in treatment of muscle fatigue and injury, there is controversy on efficacy of improving local circulation and assisting muscle function recovery [22-24]. At present, most of studies are about the intervention effect of massage on DOMS, while the preventive effect of massage on DOMS is less. Therefore, we established DOMS model in rats, and carried out front and post massage intervention on rats. We measured content of serum creatine kinase (CK), concentrations of mitochondrial Ca^{2+} , Ca^{2+} -ATPase and the expression of IL-2, IL-6, IL-8. We also use scanning electron microscope to observe the changes of rat skeletal muscle. Through the above study to observe the prevention and effect of massage, find the best time for intervention, and provide scientific basis for the prevention and control of sports fatigue.

Methods

Animals and groups

130 SPF (specific pathogen free, SPF) male SD healthy rats with an average body weight of 180-200g (6 weeks old). The animal is provided by Shanghai sipur bikai experimental animal Co., Ltd., license No.: SCXK (Shanghai) 2018-0006. The animals were raised in separate cages with 10 rats in each cage, free diet, natural light, room temperature (20 ± 3) °C, relative humidity of 40-60%. The rats were used in the animal laboratory for 7 days. According to the random principle, the animals were divided into blank control group, exercise control group and massage group. The massage group was divided into two groups: the experimental group before exercise and the experimental group after exercise. The massage group and the exercise control group were randomly divided into 0h, 24h, 48h and 72h groups, with 10 rats in each group. The operation process of the experiment strictly complies with the relevant provisions of the regulations of the people's Republic of China on the administration of experimental animals and the guiding opinions on treating experimental animals well.

Observation index

Content of serum CK, concentrations of mitochondrial Ca^{2+} , Ca^{2+} -ATPase and the expression of IL-2, IL-6, IL-8. We also use transmission electron microscope to observe the ultrastructural changes of rat skeletal muscle.

Main experimental instruments

The antibody was provided by Bio-World technology, Co. Ltd. The gel imaging system comes from Shanghai TianNeng Technology Co., Ltd. (model: 5300). ELISA kit is from bio swamp (China). Use -SYBR®premier ex Taq Gamma (Takara, rr420a) (Dalian Baosheng biotechnology Co., Ltd. China) carried out quantitative RT-PCR. Primers were synthesized by Shanghai Biotechnology Co., Ltd. (SANGON biotechnology, Shanghai, China). Trizol and cDNA synthesis kits were obtained. Manual strength tester comes from. The skeletal muscle structure was observed with hitachi-650 scanning electron microscope. Ca^{2+} , Ca^{2+} -ATPase test kits are provided by JianCheng company (Nanjing China. product No.: A106-1, A070-4).

Experimental protocol

DOMS protocol: Except for the blank control group, the other rats were all exercised to in exhaustion. The rats are free to drink and feed the same food. After randomization, the experiment was conducted from 7 to 10 a.m. every day. The experimental animals first swim in a glass cylinder of 100 cm * 60 cm * 70 cm with a weight-bearing capacity of 3% of the weight of the rats. Start a swimming exercise at 9 a.m. In the process of swimming, when the rat's motor coordination decreased significantly, it sank three times in a row for more than 10s, then takes animals out of swimming tank and dry it into the cage with an electric blower at room temperature. The animals in the control group are fed regularly under the same conditions and do not participate in swimming. All the rats were sacrificed by cervical dislocation to obtain the distal colon for future experiments, such as western blot, PCR, enzyme-linked immunosorbent assays (ELISA), and et al.

Massage interventions: The rats in the massage group are fixed on the fixed frame, and the kneading and twisting methods are applied to the lower limbs of the rats for 5 minutes each, and each rat is massaged for 10 minutes. Professional training for massage staff, control force is 4N. The manual strength is 0.4KG and the frequency is 120-160 times / min.

Western blotting: Fresh rat skeletal muscle tissue was added with RIPA lysate and mixture of protease inhibitor, and the specimen tissue was fully ground. The supernatant was centrifuged and quantified by BCA kit. 10µg protein samples

were separated by SDS-PAGE and transferred to PVDF membrane. After the membrane was sealed by 5% skim milk at room temperature for 2 hours, the first antibody IL-2 (BS60299, Bioworld Technology), IL-6 (MB9296, Bioworld Technology), and IL-8 (BS3479, Bioworld Technology) and GAPDH (MB001, Bioworld Technology) were incubated overnight at 4°C, and the membrane was washed with TBST for 3 times, each time for 10 minutes. Add goat anti-rabbit or mouse IgG-HRP second antibody (1:10000) and incubate at room temperature for 2h. The gel imaging system (Shanghai Tian Neng Technology Co., Ltd., model 5300) was photographed and preserved.

Quantitative real-time polymerase chain reaction(PCR): Trizol reagent was used to extract 2µg of total RNA from skeletal muscle tissue, and reverse transcription cDNA was used with revertaid first cDNA synthesis Kit (thermo), -SYBR® premier ex Taq Gamma (Takara, rr420a) (Dalian Baosheng biotechnology Co., Ltd., China, batch No.: a650t-1) was used for quantitative RT-PCR. Reaction conditions: after 30 seconds pre denaturation at 95°C, 5 seconds at 95°C, and 30 seconds at 60°C, 40 cycles were performed. After the cycle, melting curve analysis was performed to confirm the specificity of amplification. The expression level of GAPDH was used as internal control. Primers were designed by the software primer 5. The primers were synthesized by Shanghai Biotechnology Co., Ltd. and the primer sequence is shown in Table 1.

Primer	Sequence (5' to 3')	Base number of alkalis
IL-2-F	TTGCACTGACGCTTGTCCTCC TTGTCAACA	30
IL-2-R	CCATCTCCTCAGAAATTCCACCA CAGTTGC	31
β-actin-F	CTTTTGTGCCTTGATAGTTC	20
β-actin-R	GAGTCCTTCTGACCCATAC	19
IL-6-F	ATT-GTATGAACAGCGATGATGCAC	25
IL-6-R	CCAGGTAGAAACGGAACCTCCAGA	23
IL-8-F	CATGGATCTGTCTAGGGCT	20
IL-8-R	CTGACCAACAGACCAGGGTT	20

Table 1: PCR primer sequence

ELISA: The levels of CK, IL-2, IL-6 and IL-8 were measured by double antibody sandwich method. The purified rat antibody was coated on the microporous plate to make solid-phase antibody. The blood samples of rats and the standards of each factor were added into the microporous of the coated MCAB in turn, and then combined with the antibodies of CK, IL-2, IL-6 and IL-8 labeled by HRP to form the antibody antigen enzyme antibody complex. After thorough washing, the substrate TMB was added to develop the color. The

kit is provided by bio swamp (product No.: RA20686, RA20132, RA20607, RA20553). The absorbance (OD value) of the sample was determined by enzyme standard instrument (model: elx800) at the wavelength of 450nm. The concentrations of CK, IL-2, IL-6 and IL-8 in the sample were calculated by standard curve.

Mitochondrial sampling: Take 1g of the left gastrocnemius muscle of rat, cut them into pieces on the ice bath, add 5ml of extraction medium (normal saline), 4°C homogenate for 30s, and centrifuge for 10min at 2000r/min. The supernatant was centrifuged at 10000r/min for 15 min. The precipitate obtained by removing the supernatant is mitochondria, which are then suspended in the preservation medium (normal saline) for storage at -20°C.

Determination of mitochondrial Ca^{2+} concentration: The extracted mitochondria were suspended in 1ml normal saline, grinded by hand in ice bath for 3 minutes with glass homogenizer, and centrifuged at the speed of 2500R/min for 10min. After centrifugation, the supernatant was mixed with Mtb reagent, alkaline solution, deionized water, 2.5mmol/L calcium standard solution and protein clarifier. The supernatant was allowed to stand for 5 minutes. The OD value of absorbance at 610nm was recorded.

Calculation formula of calcium content in tissue (mmol/g prot) =

$$\frac{\text{Measuring tube absorbance} - \text{Blank tube absorbance}}{\text{Standard tube absorbance} - \text{Blank tube absorbance}} \times \frac{\text{Standard tube concentration (2.5mmol/L)} \div \text{Sample protein content (gprot/L)}}{1}$$

Determination of mitochondrial Ca^{2+} -ATPase: The extracted mitochondria were suspended in 1ml normal saline, grinded by hand in ice bath for 3 minutes in a glass homogenizer, and centrifuged at a speed of 3500R/min for 10min. Take 100 μL supernatant to determine phosphorus, and then cool it to room temperature after 20 min water bath at 45°C. The OD value of absorbance at 610nm was recorded. The amount of 1 μmol inorganic phosphorus produced by protein decomposition of ATP per milligram per hour is regarded as an ATPase activity unit, namely $\mu\text{molPi/mgprot/hour}$, Ca^{2+} -ATPase calculation formula =

*The reaction time is 10min, multiplying by 6 is 1h

$$\frac{\text{OD of measuring tube} - \text{Control tube OD}}{\text{Standard tube OD}} \times \frac{\text{Standard tube concentration (1}\mu\text{mol/ml)} \times \text{Sample dilution ratio (2.8)} \times 6 \div \text{protein content}}{1}$$

Protein content unit: mgprot

Scanning electron microscope of gastrocnemius: Two rats in each group were randomly selected. Within one minute after the rats sacrificed, the left gastrocnemius muscle tissue granules were taken immediately, the size of which was

1*1*1mm. Fixed solution (1.5-2ml): 4% glutaraldehyde, prepared with PBS (phosphoric acid buffer, pH = 7.2-7.4), stored at 4°C. Muscle tissue granules were fixed, washed with phosphoric acid buffer for 3 times, 10 minutes each time, fixed for 2 hours after 1% osmium acid, washed with phosphoric acid buffer for 3 times, 10 minutes each time, dehydrated with 50%, 70% and 90% ethanol gradients for 15 minutes respectively, dehydrated with 100% ethanol for 3 times, 30 minutes each time. Three times of acetone replacement, three minutes each time, soaking for more than 10 hours, embedding with epoxy resin, polymerizing the embedding plate at 40-60°C for 48 hours, repairing the ultra-thin section for 50-90mm, double staining with uranyl acetate and lead citrate for 5-10min, and observing the changes of myofibrils, myofilaments etc. under scanning electron microscope of Hitachi-650 SEM.

Statistical analysis

SPSS 22.0 software (IBM SPSS Inc., USA) was used for statistical analysis of the data. Results were expressed as means \pm standard error of the mean (SEM). One-way analysis of variance (ANOVA) and Dunnett's T3 test were performed for statistical analysis. $P < 0.05$ was considered the level of statistically significant.

Result

Changes of IL-2、IL-6 and IL-8 expression level

Changes of IL-2 expression level: IL-2 plays an important role in inflammatory response and is a key factor in cytokine network. Western blot showed difference in the expression of IL-2 protein between post massage 48h and 72h group ($P < 0.05$); 0h, 24h and 48h, the expression of massage group was lower than that of control group ($P < 0.01$), and the expression of front massage 72h group was higher than that of control group. The expression of post massage 48h group and 72h group were significantly lower than front massage ($P < 0.01$) (Figure 1a, 1b). PCR showed that there was no significant difference in the expression of IL-2 mRNA between post massage 24h group and 48h group, and there was significant difference in other experimental groups ($P < 0.01$); Compared with control group, the expression of front massage 0h、48h、72h group were lower than control group ($P < 0.01$), and the expression of post massage group were all lower than control group ($P < 0.01$), the most significant difference was found in 0h group; the expression of post massage 0h and 24h group was significantly lower than front massage group ($P < 0.01$) (Figure 1c). ELISA showed that the level of IL-2 in front massage 0h, 24h and 48h group were lower than control group ($P < 0.01$); compared with control group, the content of each period in post massage group was lower than control group, the most significant difference was found in the 48h group ($P < 0.01$); the content of each

period in post massage group was decreased significantly compared with front massage group in the same period ($P <$

0.01); the content of post massage group was significantly lower than front massage group ($P < 0.01$) (Figure 1d).

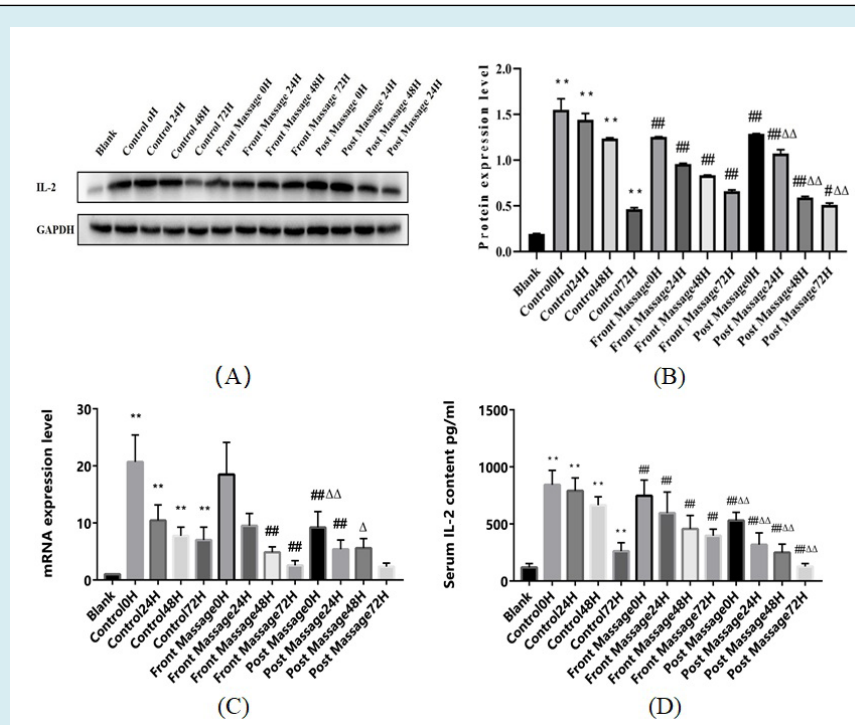


Figure 1: Changes of IL-2 expression in DOMS rats. Data were mean \pm SEM. A. Western blot analysis IL-2 protein expression in rats. ** $P < 0.01$ compared with blank group; ## $P < 0.01$ compared with the control groups in the same period; # $P < 0.05$ compared with the control group in the same period; $\Delta\Delta$ $P < 0.01$ compared with the front massage groups in the same period; B. PCR detection for IL-2 mRNA. ** $P < 0.01$ compared with blank group; ## $P < 0.01$ compared with the control group in the same period; Δ $P < 0.05$ compared with the front massage groups in the same period; $\Delta\Delta$ $P < 0.01$ compared with the front massage groups in the same period; C. ELISA showed the IL-2 content in rats. ** $P < 0.01$ compared Blank group; # $P < 0.05$ compared with the control group in the same period; ## $P < 0.01$ compared with the control group in the same period; $\Delta\Delta$ $P < 0.01$ compared with the front massage groups in the same period.

Changes of IL-6 expression level: Early studies suggested that IL-6 was only expressed in monocyte macrophages, vascular endothelial cells and fibroblasts. In recent years, it has been found that IL-6 can be expressed in most cells, including skeletal muscle cells. Western blot showed that the expression in front massage 0h、24h group was significantly different ($P < 0.01$); there was significant difference between post massage 0h and 72h group ($P < 0.01$); 0h, 24h and 48h, the expression of front massage group was decreased compared with control group ($P < 0.01$), the expression of front massage 72h group was increased compared with control group; the expression of post massage group was decreased compared with control group, and the difference was significant in 72h group ($P < 0.01$); the expression of post massage 0h, 48h and 72h groups was significantly lower than front massage group, the most significant difference was

found in 72h group ($P < 0.01$) (Figure 2a, 2b). PCR showed that there was a significant difference between control 0h and 24h group; there was no significant difference between post massage 24h and 48h group, but there was significant difference in rest period ($P < 0.01$); the expression in front massage 48h and 72h group was significantly decreased compared with control group ($P < 0.01$); the expression of post massage group was decreased significantly compared with control group ($P < 0.01$), 0h group was the most; the expression of post massage 0h and 24h groups was significantly lower than front massage group, 0h group is the most. ($P < 0.01$) (Figure 2c). ELISA showed that there was no significant difference in post massage 24h and 48h group, other periods have significant difference ($P < 0.01$); 0h, 24h and 48h, the content of front massage group was decreased compared with control group ($P < 0.01$); the content of post

massage group was decreased compared with control group, the most significant difference was found in 48h group ($P < 0.01$); the content of post massage group in each period was

significantly reduced compared with front massage group, the most significant difference was found in 48h group ($P < 0.01$) (Figure 2d).

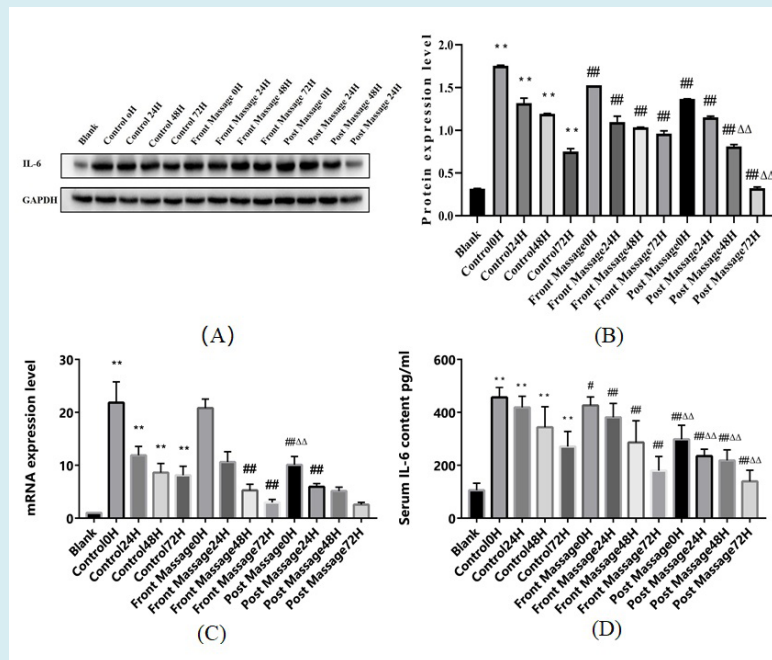


Figure 2: Changes of IL-6 expression in DOMS rats. Data were mean \pm SEM. A. Western blot analysis IL-6 protein expression in rats. ** $P < 0.01$ compared with blank group; ## $P < 0.01$ compared with the control group in the same period; ΔΔ $P < 0.01$ compared with the front massage groups in the same period; B. PCR detection for IL-6 mRNA. ** $P < 0.01$ compared with blank group; ## $P < 0.01$ compared with the control group in the same period; ΔΔ $P < 0.01$ compared with the front massage groups in the same period; C. ELISA showed the IL-6 content in rats. ** $P < 0.01$ compared with blank group; ## $P < 0.01$ compared with the control group in the same period; ΔΔ $P < 0.01$ compared with the front massage groups in the same period.

Changes of IL-8 expression level: IL-8 can promote the release of neutrophils, activate neutrophils, and promote the release of cytotoxic particles, resulting in the inflammatory response of self-tissue damage. Western blot showed that the most significant difference was found in the post massage 0h and 72h group ($P < 0.01$); the expression of front massage group was decreased compared with control group, the most significant difference in the 24h group ($P < 0.01$); the expression of post massage group was significantly decreased compared with control group ($P < 0.01$); the expression of post massage group was significantly decreased compared with front massage group ($P < 0.01$) (Figure 3a, 3b); PCR showed that there was no significant difference in post massage 48h and 72h group, and significant difference in other periods ($P < 0.01$); 0h, 48h and 72h, the expression of

front massage group was less than control group ($P < 0.01$), but was not significant in 48h group ($P < 0.05$); the expression of post massage group was less than control group, the most significant difference in 0h group ($P < 0.01$); the expression of post massage 0h, 24h and 48h groups was significantly lower than front massage group ($P < 0.01$) (Figure 3c); ELISA showed that there was no significant difference in post massage 48h and 72h group, but significant difference in other periods ($P < 0.01$); the content of front massage group was less than control group ($P < 0.01$); the content of post massage group was lower than control group ($P < 0.01$), the most significant difference was found in 48h group; the content of post massage group was significantly lower than front massage group ($P < 0.01$), with the most significant difference in 24h group (Figure 3d).

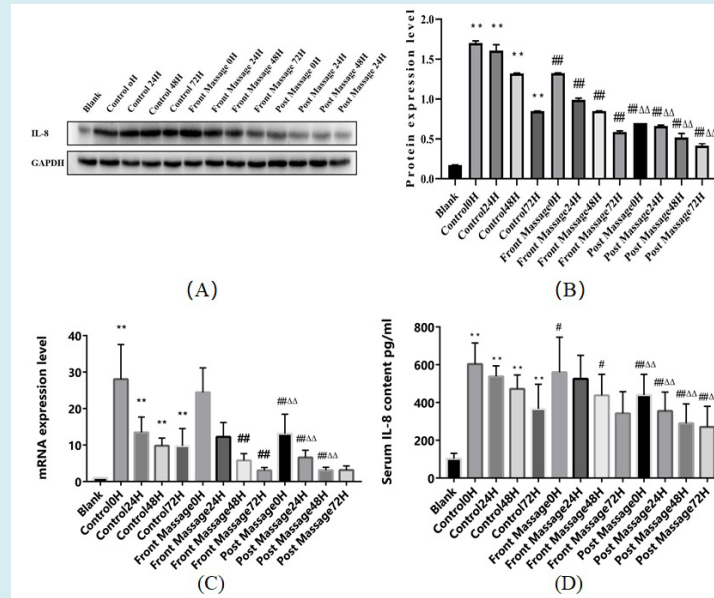


Figure 3: Changes of IL-8 expression in DOMS rats. Data were mean±SEM. A. Western blot analysis IL-8 protein expression in rats. ** $P < 0.01$ compared Blank group; ## $P < 0.01$ compared with the control group in the same period; ΔΔ $P < 0.01$ compared with the front massage groups in the same period; B. PCR detection for IL-8 mRNA. ** $P < 0.01$ compared Blank group; ## $P < 0.01$ compared with the control group in the same period; ΔΔ $P < 0.01$ compared with the front massage groups in the same period; C. ELISA showed the IL-8 content in rats. ** $P < 0.01$ compared Blank group; # $P < 0.05$ compared with the control group in the same period; ΔΔ $P < 0.01$ compared with the front massage groups in the same period.

Changes of serum CK content

CK activity in muscle tissues is higher than other tissues, so serum CK activity has become a common indicator for the diagnosis of some skeletal muscle diseases. ELISA shows that, compared with blank group, the CK content of massage group and control group was increased ($P < 0.01$); the content of massage group varied significantly at each period, and on declining curve ($P < 0.01$); the content of front massage

group was decreased significantly compared with control group, the difference is significant between 0h and 24h groups ($P < 0.01$); the content of post massage group was less than control group, most significant in 0h group ($P < 0.01$), but the content of 72h group was more than control group; the content of post massage group was significantly higher than front massage group ($P < 0.01$), 0h and 48h group were significantly different (Figure 4).

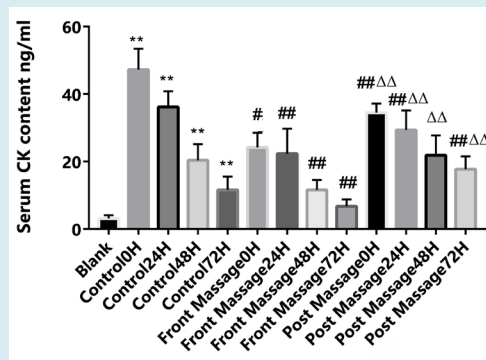


Figure 4: Changes of serum CK content in DOMS model rats. Data were mean±SEM. ** $P < 0.01$ compared with blank group; ## $P < 0.01$ compared with the control group in the same period; ΔΔ $P < 0.01$ compared with the front massage groups in the same period.

Changes of mitochondrial Ca^{2+} 、 Ca^{2+} -ATP concentration

We measured the changes of Ca^{2+} and Ca^{2+} -ATPase concentration in the mitochondria of the left gastrocnemius muscle of rats. The results showed that compared with the blank group, Ca^{2+} and Ca^{2+} -ATPase concentration in control group and massage group increased significantly ($P < 0.01$).

There was a significant difference in the concentration of massage group in each period ($P < 0.01$), the lowest value was in 0h group, the highest value was in 24h group, and the decreasing trend was in 24h, 48h and 72h group.

The concentration of Ca^{2+} and Ca^{2+} -ATPase in massage 0h group was lower than that in control group ($P < 0.01$), and the content of 24h and 48h group were higher than that in control group ($P < 0.01$). The concentration of Ca^{2+} -ATPase in massage 72h group was higher than that in control group ($P < 0.05$); the concentration of Ca^{2+} in front massage 0h group was higher than that in post massage group ($P < 0.05$), 24h, 48h and 72h group was significantly lower than that of post massage group ($P < 0.01$); the concentration of Ca^{2+} -ATPase in front massage 24h and 72h group was lower than that in post massage group ($P < 0.05$) (Figure 5a 5b)

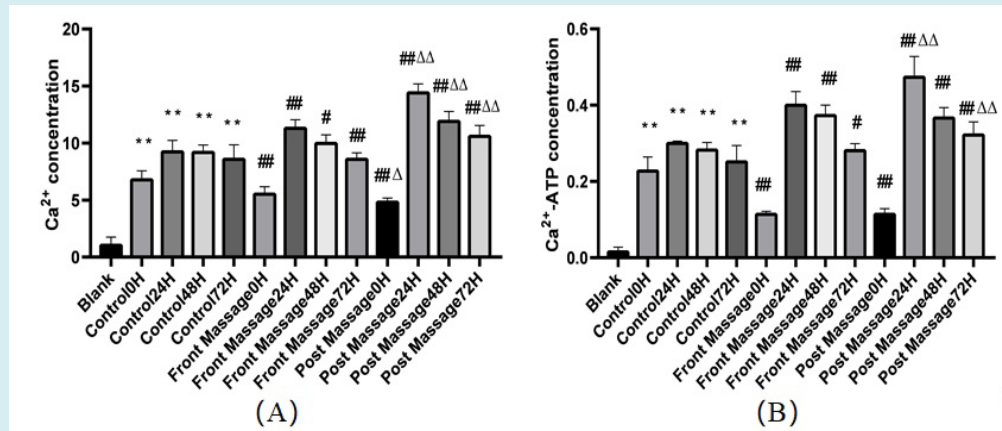


Figure 5: Changes of Ca^{2+} , Ca^{2+} -ATPase concentration in DOMS rats. A. Changes of Ca^{2+} concentration. ** $P < 0.01$ compared with blank group; # $P < 0.05$ compared with the control group in the same period; # $P < 0.05$ compared with the control group in the same period; Δ $P < 0.05$ compared with the front massage groups in the same period; ΔΔ $P < 0.01$ compared with the front massage groups in the same period. B. Changes of Ca^{2+} -ATPase concentration. ** $P < 0.01$ compared with blank group; ## $P < 0.01$ compared with the control group in the same period; ΔΔ $P < 0.01$ compared with the front massage groups in the same period.

Ultrastructural observation of skeletal muscle

We observed the changes of myofibrils and filaments of gastrocnemius in rats by scanning electron microscope. In blank group, the structure of sarcomere was intact and the morphology was normal (Figure 6a). The sarcoplasmic reticulum swelling occurred in different degrees in control group, and contraction, compression, distortion and disintegration were observed in sarcomere and myofibril

structure, and edema of myofibrils was found between fibers (Figure 6b). The swelling of sarcoplasmic reticulum, slight contraction of sarcomere, compression and distortion of some myofibrils were observed in the 0h and 24h massage group. 0h group was damaged more significantly (Figure 6c, 6d, 6g, 6h). 48h, 72h group, sarcomere structure was more complete, the morphology was basically normal; the myofibrils were closely arranged, the effect of 72h group was more significant (Figure 6e, 6f, 6i, 6j).

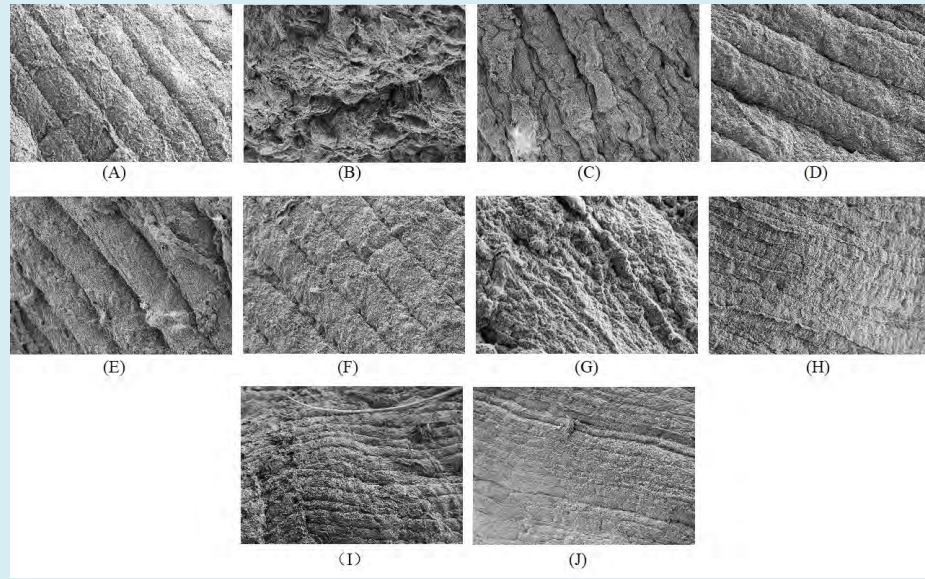


Figure 6: Changes of myofibrils and filaments of gastrocnemius in DOMS rats. a. blank group b. control group c. front massage 0h group d. front massage 24h group e. front massage 48h group f. front massage 72h group g. post massage 0h group h. post massage 24h group i. post massage 48h group j. post massage 72h group.

Discussion

Analysis of DOMS experimental model

Most of researchers take rats as the objects, and adopt the method of animal experiment to study DOMS. The main exercise methods include downhill running and swimming exhaustion. Swimming is the instinctive exercise of rats, which has a significant effect on the heart and posterior muscle groups [25]. Compared with downhill running, swimming is closer to large amount of exercise in daily life, so we build DOMS model by swimming exhausted exercise. In order to avoid the extra energy consumption caused by cold environment stimulation, we keep the water temperature of the swimming pool between 31-35°C, and static water depth of swimming pool is more than twice the length of rats, so as to prevent rats from using tail to support and rest at the bottom of pool and affect exercise load. For the judgment of swimming fatigue of rats, there are different conclusions, researchers commonly used methods is: rats can't surface again after sinking in water for a certain period (10-60s, etc.); breathe deeply, rapidly and greatly after leaving the water; eyes are not bright; lie prone; limbs are drooping when held, etc., which can be regarded as fatigue of rats. In practice, the above standards are dangerous. In order to avoid model falling off due to the death of rats, and considering the difference of swimming ability of rats, we think that when the coordination of rats' movement is significantly reduced, repeated sinking occurs, sinking for more than 10s, and sinking for three times in a row is the fatigue state of

rats. We believe that: 1) the judgment of fatigue method of rats is mainly based on the external characteristics, which has certain limitations, and may have the phenomenon of "pseudo exhaustion"; 2) there are differences in swimming ability between rats, different exercise intensity that can be borne, and weight-bearing can affect the results. The above two points need to be paid attention to in the future molding process. We need to pay attention to whether there is a more suitable weight-bearing, and judgment of fatigue state of rats.

Effect of massage on IL-2、IL-6、IL-8 in DOMS model rats

DOMS can cause muscle tissue damage, and make muscle fibers produce inflammatory reaction, thus increasing the content of inflammatory markers such as neutrophils and eosinophils [26,27]. Cytokines are mainly involved in immune response, immune cell differentiation, inflammatory response and tissue repair. Short term or long-term training can affect cytokines.

Effect of massage on IL-2 in DOMS model rats: The effect of muscle injury on IL-2 is complex, and the conclusions are different. The experimental results support that IL-2 increases, decreases and has no obvious change after exercise [28-30]. Compared with other inflammatory factors, there are few studies on IL-2. We found that the expression of IL-2 protein and mRNA increased, and the content of serum increased in control group. Massage can significantly reduce

the above content. Post massage has a greater impact on IL-2 than front massage. In general, we think 48h group has the most significant difference. The increase of β -EP after massage is an important mechanism of massage analgesia [31]. Therefore, we believe that massage can improve the level of β -EP, inhibit the release of IL-2, and inhibit mediating effect on inflammation. The pain of DOMS usually reaches the peak at 48-72 hours after exercise [32]. The results of this experiment show that changes of IL-2 in post massage 48h group are the most significant. Therefore, we speculate that post massage 48h group has most obvious inhibitory effect on release of IL-2, but more research is needed to prove this hypothesis.

Effect of massage on IL-6 in DOMS model rats: Centrifugal exercise leads to mechanical injury of muscle cells, and tissue fragments act as antigens to activate macrophages. After the injury of skeletal muscle, the permeability of cell membrane changes and the release of IL-6 into the blood can cause the increase of IL-6 expression. Researchers observed the changes of IL-6 after DOMS in traditional Chinese medicine and water bath etc., but the results were different [33,34]. We found that expression of IL-6 protein and mRNA increased, and the content of serum increased in control group. The peak time of IL-6 expression was 0h in control group, and gradually decreased with time. The expression level remained high in 24h and 48h group, which was basically consistent with the muscle morphological damage observed by our electron microscope. According to a previous review [35], massage can significantly reduce the content of IL-6, which is one of the most effective treatments for DOMS. We think that IL-6 plays an important role in sports injury and can be used as an important index of DOMS detection. The overall expression of IL-6 in model rats with massage intervention was significantly reduced, and the peak value was decreased, so we analysis that, massage can reduce the injury of skeletal muscle fiber and inflammatory response caused by exercise, inhibit the expression of IL-6, and reduce the symptoms of DOMS: front massage can reduce expression of IL-6, prevent the occurrence of DOMS; post massage can reduce the production of IL-6, thus reduce the symptoms of DOMS, and the prevention and intervention effect of 72h group is the best. The findings are also in good agreement with the results of electron microscopy.

Effect of massage on IL-8 in DOMS model rats: IL-8 has a two-way regulatory effect on inflammation. Curcumin, Rhodiola, ibuprofen and so on have some influence on IL-8, but there are few studies on this index [36-38]. We found that the changes of IL-8 protein level, mRNA expression and serum content in DOMS rats were similar to those of IL-6. Exercise can make body produce stress reaction, activate neutrophils, make skeletal muscle chemotaxis to inflammation, and increase the expression of IL-8. The expression of IL-8 in

control group increased significantly, and reached the peak at 0h. Intense stress leads to immunosuppression, so the expression of IL-8 in the whole group decreased significantly after 72h of exercise. From the point of the influence of massage before and after exercise on IL-8, we believe that massage can inhibit the activation of neutrophils, reduce muscle injury and inflammatory reaction by inhibiting the expression of IL-8, so as to have a good prevention and intervention on the production of DOMS. The intervention of massage after exercise is better than that before exercise, and the effect of 72h group is the most obvious. Based on the above analysis, massage can intervene the occurrence of DOMS through the action of cytokines.

Effect of massage on CK of DOMS model rats

After muscle injury, the leakage of CK enters the lymphatic system through the intercellular fluid, and finally into the circulatory system. Therefore, the muscle tissue injury leads to the increase of serum and muscle enzyme activity. Exercise intensity, muscle injury degree and serum CK activity are in direct proportion [39]. Short-term high-intensity exercise causes mechanical injury of muscle fiber and increases serum CK activity; long-term endurance exercise causes less mechanical injury, and the metabolic change of cell membrane causes normal function loss of muscle cells, increases cell membrane permeability and CK leakage, increase the of serum CK activity. The influence of physical therapy such as electromagnetic field and vibration on CK is controversial [39,40]. We found that through massage intervention, serum CK value of model rats in front massage group decreased significantly, which indicated that massage can prevent the muscle damage caused by exercise, reduce the leakage of CK, and increase the clearance of CK in the process of lymph blood circulation, so as to reduce the serum CK activity level [39]. The rule of change is consistent with that of gastrocnemius under electron microscope. We also found that compared with control group, the serum CK in post massage group was lower, but higher than that in front massage group. Therefore, we believe that massage therapy after intensive exercise cannot prevent the damaged cells from leaking CK in time, or even increase the activity of serum CK by accelerating the process of CK entering the blood circulation from lymph. Massage can reduce the activity level of CK, which is a marker of muscle injury, but it needs further study to determine whether massage can recover muscle injury by CK value.

Effect of massage on Ca^{2+} 、 Ca^{2+} -ATP in DOMS model rats

Ca^{2+} is the coupling factor of excitation and contraction of skeletal muscle. Its main function is to promote ATP hydrolysis and provide energy for muscle silk sliding.

Mitochondria regulate Ca^{2+} by internal and external transport, and transport Ca^{2+} in cytoplasm into mitochondria by the energy produced by Ca^{2+} -ATPase hydrolysis. The uptake of Ca^{2+} by skeletal muscle mitochondria is affected by the content of Ca^{2+} in cytoplasm. After exercise, the increase of Ca^{2+} content in cytoplasm stimulated Ca^{2+} -ATPase hydrolysis to release energy, which enabled mitochondria to take Ca^{2+} actively and reduced the increase of Ca^{2+} in cytoplasm to cause muscle damage.

However, when exercise fatigue occurs, the activity of mitochondrial Ca^{2+} -ATPase decreases, which makes the ability of Ca^{2+} transport into mitochondria decrease, and a large amount of Ca^{2+} accumulates in cytoplasm, which results in decrease of exercise ability. It is believed that permeability of plasma membrane increases and release of Ca^{2+} decreases after DOMS injury [40]. Ca^{2+} exchange through Na^+ - Ca^{2+} and Ca^{2+} -ATPase binding to cytoplasm to support the absorption of Ca^{2+} by cells [41]. We found that Ca^{2+} and Ca^{2+} -ATP of mitochondria in control group increased, peaked in 24h, and then decreased, which was in step with exercise fatigue; The activities of Ca^{2+} and Ca^{2+} -ATP in massage group were higher than those in control group. We think that massage can interfere with Ca^{2+} and Ca^{2+} -ATPase: except for 0h period, the Ca^{2+} -ATPase content of massage group is higher than that of control group. We can think that massage can enhance Ca^{2+} -ATPase activity, then enhance the transport ability of mitochondria to Ca^{2+} , so that protect skeletal muscle tissue. The effect of front massage 24h group was the most significant. In addition, DOMS leads to the increase of Ca^{2+} level and Ca^{2+} accumulation in vacuoles, which may be an effective mechanism for body to reduce Ca^{2+} content in fibro cytoplasm, so as to maintain the activity of muscle fibers [40]. Whether massage can promote the emergence of this mechanism is worthy of further study.

Effect of massage on the ultrastructure of skeletal muscle in DOMS model rats

The ultrastructural changes of gastrocnemius muscle in DOMS rats were basically consistent with those of other observation indexes. The results of electron microscopy showed that massage group was improved compared with control group, and results of front massage 72h group were similar to blank group, therefore, we analysed that the preventive effect of front massage on the structural change of skeletal muscle in DOMS rats was better than post massage.

Conclusion

From the results of each index, we find that DOMS is the result of multiple factors and multiple time periods. IL-6 can be used as an important index of DOMS detection. The effect of front massage on CK was better than post massage, and the

effect of post massage on IL-2, IL-6 and IL-8 was better than front massage. Combined with the results of Ca^{2+} , Ca^{2+} -ATPase and electron microscopy, we believe that massage can prevent muscle injury caused by large amount of exercise and long-term exercise, reduce the inflammatory reaction of muscle after exercise, and protect skeletal muscle by increasing the activity of Ca^{2+} -ATPase and increasing the transport capacity of mitochondria to Ca^{2+} . Based on comprehensive analysis of experimental results, we believe that post massage 72h has the best effect on DOMS. However, whether there is a relationship between the inflammatory indexes and whether massage has more influence on transport mechanism of Ca^{2+} needs further study.

- **Ethics approval and consent to participate**

All experiments were approved by the Laboratory Animal Ethics Committee at Nanjing University of Chinese Medicine (Number: 201908A015) and conducted under the Animal Experiments Guidelines and Animal Care of Chinese Academy of Sciences.

- **Consent for publication**

Not applicable.

- **Availability of data and materials**

The data used to support the findings of this study are available from the corresponding author upon request.

- **Competing interests**

The authors declare that they have no competing interests.

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- **Authors' contributions**

FL L and QB W designed the experiment. Y Z and ZQ S treated the animals. Q Z, JL G, and J L collected animal blood and tissues and performed laboratory measurements. Q Z and JL G analyzed and interpreted the data. Q Z and QB W were major contributors to the writing of the manuscript. All authors drafted, improved, and revised the manuscript. All authors read and approved the final manuscript.

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