Original Article Effects of intermittent pressure imitating rolling manipulation in traditional Chinese medicine on ultrastructure and metabolism in injured human skeletal muscle cells

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Abstract: Skeletal muscle injuries can cause significant change in the ultrastructure and the metabolism of the skeletal muscle cells. Observation of the ultrastructure and measurements of the metabolism biomarkers such as total superoxide dismutase (T-SOD), malondialdehyde (MDA), and creatine kinase (CK) can be used to evaluate the degree of damage in human skeletal muscle injury. Rolling manipulation is the most popular myofascial release technique in Traditional Chinese Medicine. This study aimed to investigate the effects of intermittent pressure imitating rolling manipulation (IPIRM) of Traditional Chinese Medicine on ultrastructure and metabolism in the injured HSKMCs. Methods: In vitro techniques were used to culture HSKMCs, which were injured with high doses of dexamethasone sodium phosphate. Cells were divided into four groups-control normal group (CNG), control injured group (CIG), rolling manipulation group (RMG), and sine pressure group (SPG). RMG and SPG cells were cyclically exposed to 3.0 Kg (6.6 Pounds) of maximum force at a frequency of 2.0 Hz for 10 min in the Flexcell compression system for duration of 3 days continually. The cell ultrastructure, total superoxide dismutase (T-SOD) activity, malondialdehyde (MDA) content, and creatine kinase (CK) activity of the groups were assessed. Conclusion: These results suggest that the mechanical effects of rolling manipulation in TCM could not only improve the recovery of injured skeletal muscle cells by ameliorating organelles arrangement, reducing organelle swelling, and maintaining nuclear membrane integrity, but also ameliorate the functions of cellular metabolism by increasing T-SOD activity and decreasing MDA content and CK activity in injured skeletal muscle. Then the Hippo/Yap signal pathway was detected, and the proteins in each group were detected by Western Blot. The protein expression of upstream protein p-LATS1 and downstream protein p-Yap (Ser127) in each group was observed to explore the biomechanical mechanism of the method. The relative protein expression of p-LATS1 and p-Yap in (RMG) group was significantly higher than that in injured (CIG) group (P < 0.05). It was suggested that Hippo/Yap pathway was related to the stimulation of 3D human skeletal muscle cells, and the proliferation pathway of 3D human skeletal muscle cells could be opened by stimulation of three dimensional human skeletal muscle cells. It may be one of the biological mechanisms caused by the mechanical effects of manipulations in TCM.

Keywords: Traditional Chinese medicine, rolling manipulation, Flexcell FX-5000 compression system, human skeletal muscle cells, cell ultrastructure, total superoxide dismutase, malondialdehyde, creatine kinase, Hippo/Yap pathway

Introduction

As a natural therapy, Chinese medical manipulation (also known as Tuina) is one of the most significant parts in TCM. The Tuina manipulations can activate internal biological pathways against diseases by coordinated and rhythmic stimulation on human body surfaces [1]. Such manipulations are specific and standardized techniques characterized by periodic force changes. In other words, the mechanical effects of the manipulations on human tissue are taken by periodic force changes, and then the mechanical effects are converted into the biological effects to relieve clinical symptoms. Many studies have verified that manipulation could promote blood circulation, improve mechanical characteristics of muscle tissues, increase pain threshold [2-4]. The effectiveness of manipulation therapy is more than 80% for treating cervical spondylosis and more than 90% for lumbar herniated disc [5-7].

The main indications of the manipulation therapy are chronic musculoskeletal injuries including lower back pain, cervical spondylopathy, and frozen shoulder, which can be manifested as muscle tension, stiffness, and pain [8]. Many studies have proved that the main cause to skeletal muscle injury is a homestatic imbalance of intracellular Ca²⁺ in the skeletal muscles [9, 10] causing significant changes in markers of skeletal muscle metabolism, such as superoxide dismutase (T-SOD), malondialdehyde (MDA) and creatine kinase (CK), which eventually damages the structure and function of muscle cells [11, 12].

Rolling manipulation is one of the most influential techniques among diverse traditional Chinese manipulations in the contemporary world, which is characterized by continual and rhythmic stimulation of the body surface while maintaining a certain pressure, moderate strength, and significant therapeutic effects [12]. As an important therapy the rolling manipulation is easy, simple, and safe to maneuver, widely used in TCM clinical practice, highlyacceptable by the public, and has a great effect on a variety of body dysfunction. The rolling manipulation is conducted by the operator's rhythmic forearm pronation with elbow flexion and forearm supination with elbow extension on the surface of the treated body part. The operator places force on the surface with his/ her hypothenar area during the supination with elbow extension phase. During the manipulation with the hypothenar eminence, the rolling frequency of the hypothenar on the treatment area is 120-160 times per minute [13]. The application of force is from dorsal parts of the fifth and fourth metacarpal bone to the hypothenar.

Cells are the fundamental functional unit in human body and the target of manipulation

effects. The key factor to illustrate the therapy mechanism is to figure out how cells in the body identify force changes caused by manipulation and convert unique mechanical signals from manipulation into biological signals to cause a series of biological effects. The analvsis of the mechanism of manipulation at the level of cell molecular biomechanics is an important link in the study of manipulations. while the vital element is how the cells are transformed into electrophysiological and chemical signals under the stimulus of manipulative mechanical signals to conditionally cause a series of biological effects. Therefore, the study of the biomechanical effects on cells is of great significance for understanding therapy of manipulative therapy [14].

The biological mechanism of mechanical stimulation is still unknown to the world. This study employed in vitro techniques to culture injured HSKMCs, which were loaded with the intermittent pressure imitating Rolling manipulation (IPIRM) in Traditional Chinese Medicine in Flexcell compression system. The ultrastructure and the intracellular metabolic markers (T-SOD activity, MDA content and CK activity) were measured in order to investigate the effects of manipulation-like mechanical stimulation on cell ultrastructure and metabolism in injured HSKMCs.

Materials and methods

Curve fitting of rolling manipulation in Flexcell compression system

The pressure-time curve of rolling manipulation was recorded in the Manipulation Technique Parameter Analyzer (Type II, Shanghai Research Institute of Traditional Chinese Medicine, China) when the operator was performing rolling manipulation. The data of the pressure-time curve was imported into the Flexcell compression system (Type FX-5000, Flexcell Inc, USA). The waveform editing program of Flexcell compression system was used to edit and simulate the curve of rolling manipulation. According to our previous study results, the optimal maximum force of rolling manipulation was 4.0 kg (8.8 pounds) in the human body, and the decay rate of the force was 25% at 2.0 cm under the skeletal muscle [15]. Therefore, the cells were cyclically exposed to 3.0 kg (6.6 pounds) of intermittent pressure imitating roll-



Figure 1. Pressure-time curve fitting of IPIRM and sine in Flexcell compression system. The solidline curve shows that the cells were cyclically exposed to 6.6 pounds of IPIRM at 2.0 Hz in frequency, and the dotted curve shows that the cells were loaded at cyclical pressure as the sine curve preserved in the Flexcell compression system.



Figure 2. A. Normal human skeletal muscle cells. B. Injured human skeletal muscle cells.

Company, Netherlands): 1. The shape of HSKMCs were spindle shaped. 2. No floating cells were found which indicated that the cultured cells had a good capacity of cellular adherence to wall of the culture flask. 3. The cell nuclei were oval-shaped without any sign of breaking out, dissolving, or pyknosis. 4. The culture flasks were clear without pollution. Once the HSKMCs grew up to the whole bottom of each culture flask, they were harvested and divided into two portions in order to further down (Figure 2A).

According to previously published protocols, the excessive dosage of dexamethasone could produce injury to muscle cells [15, 16]. When HSKMCs showed good adherence and covered 80%-90% of the bottom of the bottle under the microscope, fresh DMEM highglucose culture media containing dexamethasone sodium phosphate injection fluid was added to the culture flask. The final concentration of dexamethasone sodium phosphate

ing manipulation (IPIRM) at a frequency of 2.0 Hz (**Figure 1**).

Cell culture and establishment of injured cell model

The human skeletal muscle cells (HSKMCs; US Type Culture collection warehousing, San Diego, USA) from the 4-8th generation of skeletal muscle cell strain were used for this study. All cells were kept in CO_2 cell culture box (Heal Force Bio-meditech Holdings Co., Ltd, USA) at 37°C in a humidified atmosphere containing 5% CO_2 . HSKMCs were cultured in DMEM high-glucose medium (HyClone Company, USA) containing 4.5 g glucose, 100,000 U penicillin, 100 mg streptomycin, and 3.0% fetal calf serum (FCS; HyClone Company, USA) per liter. They were considered to be cultured successfully when the following four criteria were identified under an inverted microscope (PHILIPS (Suzhou No. 3 Pharmaceutical Factory Co., Ltd, China) in the culture media was 2.5 mg/ml, and the culture flask was placed in a 5% CO₂ culture incubator for 24 hours to establish the injured cell model of human skeletal muscle (**Figure 2B**).

Cell grouping and treatment of HSKMCs

The normal HSKMCs were used as control normal group (CNG), and they were cultured in 6 flasks. The injured HSKMCs were further divided respectively into 3 different groups with 6 flasks per group: control injured group (CIG), rolling manipulation group (RMG), and Sine pressure group (SPG). CNG and CIG cells were cultured in the same conditions as RMG and SPG cells except the amount of loaded pressure. RMG and SPG cells were cyclically exposed to the maximum force 3.0 Kg (6.6 Pounds) of IPIRM or sine pressure in the Flexcell



Figure 3. Effects of IPIRM on the cell ultrastructure of injured human skeletal muscles. CNG control normal group, CIG control injured group, RMG rolling manipulation group. A-C. Illustrating the electron microscopic finding (10 µm) of CNG, CIG and RMG cell. D-F. Illustrating the electron microscopic finding (2 µm) of CNG, CIG and RMG cell.

compression system at a frequency of 2.0 Hz for 10 min/day for 3 continual days. No mechanical loading was conducted for CNG and CIG cells and only observations were carried out for the control.

Cell ultrastructure study method

Cells in various groups were collected and fixed in 2.5% glutaraldehyde solution (Sinopharm Chemical Reagent Co., Ltd, China) for 4 hours. Subsequently, the sample was rinsed 3 times with 0.1 M phosphate buffer solution (Suzhou No. 3 Pharmaceutical Factory Co., Ltd, China) for 30 min at a time. Then the sample was placed in a solution of 1% osmium tetroxide (Alfa Aesar Company, UK) in phosphate buffer (Suzhou No. 3 Pharmaceutical Factory Co., Ltd, China) for 3 h, and was rinsed for 15 min. again as before. It was successively soaked in 30% and 50% ethanol (Sinopharm Chemical Reagent Co., Ltd, China) for 20 min. at 4°C, and then was remained overnight in 70% ethanol acetate dioxy uranium (Sinopharm Chemical Reagent Co., Ltd, China). The next day, the sample was successively soaked for 20 min. in 80% ethanol and 90% ethanol: 90% acetone (1:1) solution (Sinopharm Chemical Reagent Co., Ltd, China) at 4, and then was rinsed in 100% ethanol 3 times for 20 min each time at room temperature. After placed in pure acetone and resin (1:1) solution (Shanghai Resin Factory Co., Ltd, China) for 20 min. at room temperature, the sample was successively placed in pure resin in an oven for 1.5 h. two times, 12 hours at 37°C, and 48 h at 60°C. After completion of the material preparation procedure, the ultra-thin 0.5 µm sections were obtained with a diamond knife in ultramicrotome (Leica Microsystems, Germany). In the end, the histological slides were stained with lead citrate (Sinopharm Chemical Reagent Co., Ltd, China), and analyzed with the transmission electron microscope (Shanghai Zhongheng Inc., China).

Procedure of measuring T-SOD activity, MDA content, and CK activity

The medium was removed by aspiration from culture vessels of each cell group described

Table 1. Effects of IPIRM on cellular metabolism in injured HSKMCs

Group	T-SOD (U/mg)	MDA (nmol/mg)	CK (U/mg)
Numbers of cases	6	6	6
CNG	57.43±28.61	4.79±2.06	1.06±0.38
CIG	15.50±3.22*	9.29±1.05*	1.76±0.11*
SPG	22.64±6.27	8.98±0.69	1.73±0.12
RMG	56.99±16.30∆,☆	5.09±1.81∆,☆	1.22±0.26∆,☆

T-SOD, total superoxide dismutase; MDA, malondialdehyde; CK, creatinkinase; CNG, control normal group; CIG, control injured group; SPG, sine pressure group; RMG, rolling manipulation group. What comparing with CNG, *, meant P < 0.05; What comparing with SPG, \Leftrightarrow , meant P < 0.05; What comparing with SPG, \Leftrightarrow , meant P < 0.05.

above. 1 ml of 0.25% trypsin (Gibco Inc, USA) was added into culture vessels, then placed in 37°C incubator for approximately 1.5 min. The trypsin was removed by aspiration until HS-KMCs appeared rounded when they were observed using an inverted microscope. 6.0 ml of DMEM high-glucose medium was added into culture vessels to terminate the trypsinization process. HSKMCs were collected at 1000 r/ min for 10 min. and preserved in the refrigerator at -20°C. T-SOD activity, CK activity, and MDA content were quantified in the same experiment and in duplicates with the use of commercially available T-SOD, CK and MDA kits. T-SOD was tested by the hydroxylamine method, MDA was measured by the thiobarbituric acid method, and CK was detected by phosphorus determination methods (Nanjing Jiancheng Bioengineering Institute, China).

Procedure of measuring Hippo/Yap signal path detection

According to the principle of random and control, three-dimensional human skeletal muscle cells were divided into three groups: normal group (N, no treatment), (CIG) dexamethasone sodium phosphate solution intervention, no treatment), and treatment group (RMG, dexamethasone sodium phosphate solution intervention). The number of cells per sample was about 0.57 * 107. The first step is to extract and quantify the total protein, then prepare the glue and SDS-PAGE electrophoresis, last step is to Semi-quantitative analysis of the target protein. The picture scanning is saved as a computer file, and each specific strip gray value on the picture is digitized by ImageJ analysis software, and then export the software.

Statistical analysis

All data were continuous data that were reported as means \pm SD for descriptive expression. One-way Analysis of Variance (ANOVA) with post hoc multiple comparisons was conducted to analyze the differences between different groups. All the data were processed with software (Statistical Package for the Social Sciences, ver-

sion18.0). A *p* value of less than 0.05 was considered to be statistically significant.

Results

Effects of IPIRM on the cell ultrastructure of injured human skeletal muscles

CNG cells in electron microscope were generally normal with intact cellular and nuclear membrane structure, and without identifiable swelling of organelles (Figure 3A and 3D). Cell morphology of CIG was disrupted in disordered arrangement with unclear cell membrane, swelling and round organelles, and disappeared nuclear membrane (Figure 3B and 3E). RMG cell showed that the cell exposed to IP-IRM had completed morphology with relatively intact cell and nuclear and membrane, and organelles with less swelling (Figure 3C and 3F).

The electron microscope results showed the rolling intervention could improve the recovery of injured skeletal muscle cells which was mainly presented as improving organelles arrangement, reducing organelle swelling, and maintaining nuclear membrane integrity.

Effects of IPIRM on cellular metabolism in injured HSKMCs

Table 1 shows the effects of IPIRM on T-SODactivity, MDA content, and CK activity in injuredHSKMCs.

Rolling manipulation pressure-time curve effects on T-SOD activity of HSKMCs

As shown in **Table 1** and **Figure 4A**, T-SOD activity in the injured HSKMCs in CIG was signifi-



Figure 4. Rolling manipulation pressure-time curve effects on T-SOD activity (A), MDA content (B) and CK activity (C) of HSKMCs. T-SOD total superoxide dismutase; MDA, malondialdehyde; CK, creatinkinase; CNG, control normal group; CIG, control injured group; SPG, sine pressure group; RMG, rolling manipulation group. What comparing with CIG, α meant P < 0.05; What comparing with CIG, α meant P < 0.05; What comparing with CIG.

Table 2. Effects of IPIRM on Hippo/YAP pathway in injured HSKMCs

Group	P-LATS1	P-Yap (Ser127)
Numbers of cases	3	3
Ν	1373.67±628.38	396.67±50.56
CIG	714.33±268.36*	177.33±10.84*
RMG	1220.00±252.41∆	671.67±111.01∆,☆
RMG	1220.00±252.41Δ	671.67±111.01Δ,☆

What comparing with N, *, meant P < 0.05; What comparing with CIG, Δ , meant P < 0.05; What comparing with CIG, Ξ , meant P < 0.05; What comparing with N, Ξ , meant P < 0.05.



Figure 5. Expression of P-LATS1 and P-Yap (Ser127) proteins in human skeletal muscle cells.

cantly decreased as compared with that of the normal HSKMCs in CNG (P < 0.05), indicating that such a decrease might lead to the decreased resistance ability against oxidative damage. Meanwhile, the T-SOD activity in SPG was not different from that of GIG (P > 0.05), indicating that the sine pressure had no effect on T-SOD activity in the injured HSKMCs.

However, the T-SOD activity in RMG was significantly higher than that of CIG (P < 0.05), indicating that IPIRM could reverse the T-SOD decrease in the injured HSKMCs. Furthermore, the T-SOD activity in RMG was also significantly increased than that of SPG (P < 0.05), showing that the rolling manipulation curve was better than SPG in increasing resistance against oxidative damage in the injured HSKMCs. Rolling manipulation pressure-time curve effects on MDA content of HSKMCs

As shown in **Table 1** and **Figure 4B**, the MDA content in the injured HSKMCs in CIG was significantly increased as compared with that of the normal HSKMCs in CNG (P < 0.05), indicating the increas-

ing levels of oxygen free radicals after the muscle cell injury. Meanwhile, the MDA content in the injured HSKMCs in SPG was not different from that of GIG (P > 0.05), showing that the sine pressure had no effect on MDA content in the injured HSKMCs.

However, the MDA content in RMG was remarkably decreased as compared with that of CIG (P < 0.05), indicating that IPIRM could reduce MDA content in the injured HSKMCs. Furthermore, the MDA content in RMG was also significantly decreased as compared with that of SPG (P < 0.05), showing that the rolling manipulation curve was better than the sine curve in clearing oxygen free radicals in the injured HSKMCs.

Rolling manipulation pressure-time curve effects on CK activity of HSKMCs

As shown in **Figure 4C**, the CK activity in the injured HSKMCs in CIG was obviously increased as compared with that of the normal HSKMCs in CNG (P < 0.05), indicating the energy transportation was increased among cells after the muscle cell injury. Meanwhile, the CK activity of the injured HSKMCs in SPG was not different



Figure 6. Expression of P-LATS1 relative protein in 3D human skeletal muscle cells.





from that in GIG (P > 0.05), suggesting that the sine pressure had no effect on the CK activity of injured HSKMCs.

However, the CK activity in RMG was significantly lower than that of CIG (P < 0.05), indicating that IPIRM could ameliorate the CK release after the muscle cell injury in HSKMCs. Furthermore, the CK activity in RMG was also decreased as compared with that of SIG (P < 0.05), showing that the rolling manipulation curve was better than the sine curve in ameliorating intracellular energy transportation.

Rolling manipulation pressure-time curve effects on Hippo/Yap pathway of HSKMCs

Table 2 and Figures 5-7 shows the effects ofIPIRM on Hippo/Yap pathway of HSKMCs.

The results showed that the expression of P-LATS1 protein in 3D human skeletal muscle cells stimulated by high dose dexamethasone sodium phosphate solution was significantly decreased in CIG group and N group, indicating that the expression of 3D human skeletal muscle cell protein was inhibited and the proliferation of 3D human skeletal muscle cells was significantly inhibited.

Compared with group N, the expression of P-Yap (Ser127) protein in 3D human skeletal muscle cells stimulated by high dose dexamethasone sodium phosphate solution decreased significantly (P < 0.05), indicating that the expression of protein in 3D human skeletal muscle cells was inhibited and the proliferation of human skeletal muscle cells was significantly inhibited.

Compared with CIG group, the content of P-LATS1 protein in injured three-dimensional human skeletal muscle cells was significantly increased after treatment with traditional Chinese medicine stimulation, and skeletal muscle cells proliferated and self-updated.

Compared with CIG group, the content of P-Yap (Ser127) protein in injured three-dimensional human skeletal muscle cells was significantly increased (P < 0.05), and the proliferation and self-renewal of skeletal muscle cells were carried out after the injury of three-dimensional human skeletal muscle cells was stimulated by traditional Chinese medicine.

Compared with N group, the protein content of P-Yap (Ser127) in RMG group was significantly higher than that in N group (P < 0.05). The results showed that TCM stimulation was related to Hippo/Yap pathway, which might open the pathway of human skeletal muscle proliferation and regeneration.

In summary, with IPIRM for three days, the injured HSKMCs exhibited a relatively complete morphology with relatively intact cell membrane, organelles with less swelling and intact nuclear membrane, and the SOD activity was increased; whereas the MDA content and the CK activity were decreased in the injured HSKMCs. In addition, IPIRM could regulate the expression of P-LATS1 and P-Yap (ser127) proteins, which may be one of the manipulation mechanisms of the regeneration and self renewal in injured HSKMCs.

Figure 7. Expression of P-Yap (Ser127) relative protein in three-dimensional human skeletal muscle cells.

Discussion

The essence of TCM manipulation is mechanical stimulation, the operational demands of TCM manipulation recommends persistent, energy and forceful and gentle application of force on symmetrical in the treat, so that the force can permeate to deep tissues in the body [17]. Rolling technique as one of the Tuina manipulation was a typical widespread manipulation in clinical practice with great influence in China. The study edited the pressure-time curve of rolling manipulation in Flexcell compress system according to the recorded date in the Manipulation Technique Parameter Analyzer when the operator was performing rolling manipulation. As advanced loading stress equipment on cells in the world, the Flexcell compress system was composed of a computer, air compressor, pressure control module, fixed stage, gas pipes, valves, and other components. It could provide cyclic or static tension, pressure, and fluid shear stress loading to various types of cells and tissues. The computer in this system could precisely control cycle, magnitude, frequency, and duration of the loading force [18]. At the same time, the cyclic sine pressure-time curve was selected as the control group and used to load on injured HSKMCs under the same conditions as comparing with the effects of IPIRM.

Effects of IPIRM on the ultrastructure of injured HSKMCs

During manipulation research, chemical methods have been used to induce skeletal muscle injury, resulting in more uniform structure, greater differences, and fewer uncontrollable factors. Therefore, the chemical methods show many intuitive advantages on the establishment of skeletal muscle injured models.

The microscopic structure of skeletal muscles in our present study could be observed that cell morphology was disrupted in disordered arrangement with unclear cell membrane, swelling and round organelles, and disappeared nuclear membrane when they were injured. After three days of IPIRM, the injured HSKMCs exhibited a relatively complete morphology with relatively intact cell membrane, less swelling organelles, and intact nuclear membrane. It suggested that rolling manipulation intervention could ameliorate the recovery of injured skeletal muscle cells, which was mainly manifested as improving organelles arrangement, reducing organelle swelling, and maintaining nuclear membrane integrity.

Effects of IPIRM on cellular metabolism in injured HSKMCs

The muscle cells are the basic function units in the human body and the final target of the manipulation force. How the muscle cells recognize the rolling changes of the mechanical force and then convert the signals into some kind of chemical signals further causing a series of its biological effects is the key to explaining the mechanism of the rolling manipulation. In our present study, the SOD activity, the MDA content, and the CK activity were used as the biomarkers of the cultured muscle cell injury model and further examined after exposure to various experimental conditions.

As compared with that of the normal HSKMCs in CNG, the T-SOD activity was significantly decreased, while both the MDA content and the CK activity were evidently increased in the injured HSKMCs in CIG, demonstrating that the resistance ability against oxidative damage was decreased and the levels of oxygen free radicals and the energy transportation among cells were significantly increased when the skeletal muscle cells were injured. Meanwhile, the T-SOD activity, the MDA content, and the CK activity in SPG was not different from that of GIG, indicating that the sine pressure had no effect on cellular metabolism in the injured HSKMCs. However, with IPIRM for three days, the SOD activity was increased, whereas the MDA content and the CK activity were decreased in the injured HSKMCs in RMG as compared with CIG and SPG, showing that IPIRM could reverse the biomarkers of cellular metabolism in the injured HSKMCs, and proving that the rolling manipulation curve was better than the sine curve in ameliorating cellular metabolism in the injured HSKMCs.

Lipid peroxidation plays an active part in chronic skeletal muscle injuries. The accumulation of oxygen free radicals can cause an increase of the lipid peroxidation leading to the damage of the cell structure and functions. Toxic superoxide anion free radicals are converted to harmless water and oxygen under the combined effects of catalase and SOD, which maintain cellular homeostasis [19]. Known as the 'garbage collector' in the human body, SOD is an enzymatic defense mechanism in the contingency procedure to defend the lipid peroxidation, and is also one of the major antioxidant enzymes in human body, especially in musculature [20].

Our study revealed that T-SOD activity in the injured HSKMCs in CIG was remarkably decreased than that of CNG. SOD activity in SPG was not different from that of GIG indicating that the sine pressure had no effect on the SOD activity in the injured HSKMCs. Furthermore, with the treatment of intermittent pressure imitating rolling manipulation (IPIRM), the T-SOD activity was significantly increased in the RMG. This indicated that IPIRM could increase the T-SOD activity and enhance the antioxidant ability of injured HSKMCs. This result agrees with the previous report that the SOD activity was increased by the manipulation intervention like intermittent pressure [21].

MDA is formed in a series of reactions between the unsaturated fatty acids and the oxygen free radicals in the body. MDA content reflects the active status of oxygen free radicals in the injured tissue [22, 23]. Under pathological conditions, the dynamic equilibrium of lipid peroxidation is disrupted, resulting in reduced clearance of oxygen free radicals and increased production of oxidative damage products such as MDA. Lipid peroxidation caused by oxygen free radicals is closely related to the skeletal muscle injury [24, 25]. Similar to tissue SOD activity, the MDA content is another biomarker commonly used for chronic skeletal muscle injury.

In our study, the MDA content in the injured HSKMCs in CIG was significantly increased as compared with that of the normal HSKMCs in CNG, demonstrating the existence of the muscle cell injury in the CIG. However, the MDA content in the injured HSKMCs in SPG was not different from that of CIG, indicating that the sine pressure had no effect on ameliorating muscle cell injury in the injured HSKMCs. With intermittent pressure imitating rolling manipulation (IPIRM), the MDA content in RMG was remarkably decreased in the injured muscle cells as compared with that of CIG, indicating that the ameliorating effect of IPIRM in the injured HSKMCs may be achieved through an improve-

ment of the dynamic balance of lipid peroxidation in the injured HSKMCs. Similar results were reported previously where the MDA content was significantly increased in rat triceps after exhaustive downhill run, which could remarkably be decreased by the delivery of vitamin E, one product against lipid peroxidation caused by the oxygen free radical [26].

CK is present in the cytoplasm and mitochondria of skeletal muscles, heart, brain, and other tissues where the oxygen consumption is significant and the metabolism is rapid. It is an important kinase in aerobic cells which takes part in intracellular energy transport, ATP regeneration, muscle contraction, and other relevant functions in the body. The CK activity in the skeletal muscle cells is closely associated with the transmembrane flow of Ca²⁺ and excitation-contraction coupling [27-29].

In our study, the CK activity was significantly increased in the injured HSKMCs in CIG as compared with that of the normal HSKMCs in CNG, reflecting the reliability of our muscle cell injury model. Meanwhile, the CK activity in the injured HSKMCs in SPG was not different from that of GIG, indicating that the sine pressure had no ameliorating effect on the muscle cell injury in the injured HSKMCs. However, with the exposure to the intermittent pressure IPIRM, the CK activity returned to the same level as that of the normal control group in RMG. Same as our previous report, the present study gives direct evidence again that IPIRM ameliorates the muscle cell injury in our HSKMCs [1].

Hippo/Yap pathway is the key regulator of organ growth [30]. Hippo pathway is a key member of Yap protein (Yes-associated protein, Yap), is a transcriptional cofactor, which is expressed in all cells, including skeletal muscle cells [31]. Hippo protein pathway is based on protein kinase Mst1/2 (Mammalian sterile 20-like kinase1/2, Mst1/2) and auxiliary proteins like ww45 and mob1. Hippo protein pathway is the key regulator of organ growth. Hippo pathway is the key regulator of organ growth. Hippo pathway is the key member of Hippo pathway. Hippo protein is a transcription cofactor that is expressed in all cells, ww45 and mob1 interacts and plays an indispensable role in the inheritance of upstream protein Lats1/2. The kinase of LATS1/2 protein, which is phosphorylated by the residues of Warts kinase

540C series, can effectively promote the phosphorylation of downstream Yap protein. The phosphorylated Yap binds to 14-3-3 protein and retains it in the cytoplasm [32]. In addition, Vissergrieve et al. found that the expression of LATS1 relative protein in Hippo pathway increased, which could regulate actin in skeletal muscle cells and cause muscle contraction, cell proliferation and fineness [33]. Mao Xing and others also found that Hippo pathway can regulate the transcriptional activity of Yap protein downstream through upstream phosphorylation, so as to limit the excessive growth of tissues and cells, which is an important way to maintain intracellular proliferation and apoptosis homeostasis [34].

The pathological process of skeletal muscle injury mainly includes the degeneration and necrosis of skeletal muscle, the destruction of muscle fiber structure and the infiltration of inflammatory cells (mainly macrophages). Skeletal muscle cells, as a kind of cells with the ability of damage and repair, the key cells that play a role in repair are a kind of skeletal muscle satellite cells with the characteristics of stem cells. In the process of skeletal muscle repair, muscle satellite cells are activated, dividing and proliferating to form new muscle tube cells and then developing muscle fibers, and then the regenerated muscle fibers are restored to muscle function after differentiation and maturation [35]. The expression of Yap relative protein increased during skeletal muscle cell activation [36]. Yap can be widely expressed in the amplification of somatic stem cells and group cells. Yap high level expression activates skeletal muscle satellite cells mainly by activating genes related to cell cycle, ribosomal biogenic angiotensin signal transduction pathway and or inhibiting related differentiation genes to maintain the high value-added activity of skeletal muscle satellite cells [37]. Compared with the myoblasts in the proliferating phase the phosphorylation of total Yap and Yap Ser127 proteins increased significantly by 2.1-2.5 times at 24 h and 48 h after differentiation of satellite cells [38].

Yap pathway can be regulated by mechanical stimulation [39]. When mechanical stimulation passes through the body surface to the cell, the intracellular microenvironment converts the mechanical force into a mechanical signal to regulate the shape of the cell and affect the

state of the cell [40]. Ma's study found that the expression of Yap in cartilage cells was related to tensile stress. Combined with this experiment, the damaged three-dimensional human skeletal muscle cells were stimulated by stimulation [41]. The protein expression of upper protein P-LATS1 and downstream protein P-Yap (Ser127) in Hippo/Yap pathway was used as the index of activating Hippo/Yap pathway.

In our study, The results showed that when the CIG group was compared with the control group, the results showed that the expression of Hippo/Yap pathway was significantly higher than that of the control group. Compared with group N, the expression of P-Yap (Ser127) protein was significantly decreased in group N (P < 0.05), which indicated that the expression of protein in 3D human skeletal muscle cells was inhibited and the proliferation of human skeletal muscle cells was inhibited. When compared with CIG group, the protein content of P-LATS1 and P-Yap (Ser127) in RMG group was significantly higher than that in N group (P < 0.05), and the protein content in P-Yap (Ser127) group was significantly higher than that in N group (P < 0.05). The results showed that the protein content of RMG group was significantly higher than that of P-LATS1 and P-Yap (Ser127) protein is related to human skeletal muscle cells, which opens the pathway of human skeletal muscle cell regeneration and self-renewal. However, it has not been reported that how this stimulation can regulate Yap, but in the future research, it is still necessary to determine the mechanical sensor or receptor and further improve and verify the mRNA expression of the mechanical sensor or receptor and its corresponding protein.

In addition, it has been reported that excessive activation of Yap-Ser127 in non-static skeletal muscle satellite cells can lead to embryonic rhabdomyosarcoma-like tumors [42]. It has also been reported that the expression of Yap-Ser127 in muscle fibers can cause disease at the same time [43], while other types of Yap or Yap mutants can cause muscle hypertrophy [44]. The promotion of skeletal muscle cell regeneration by Yap or soft tissue tumors mainly depends on the level of expression after Yap activation [45]. In the molecular signal transduction network responsible for regulating skeletal muscle cells, overload-induced muscle hypertrophy is known. Protein synthesis is stimulated by mTORC1 signal transduction [46], while E3 ubiquitin ligase includes Atrogin-1 and MuRF-1 to regulate protein degradation in catabolism environment. Rapid to slow fiber phenotypic transformation and increased mitochondrial biogenesis can also respond to stimuli such as endurance exercise training and calmodulin-NFAT, AMPK and PGC-1-mediated signals [47] whether there is a correlation with TCM stimulation pathway will be further explored in future experiments.

These results suggest that the mechanical effects of rolling manipulation in TCM can not only improve the recovery of injured skeletal muscle cells by ameliorating organelles arrangement, reducing organelle swelling, and maintaining nuclear membrane integrity, but also can ameliorate the functions of cellular metabolism by increasing resistance against oxidative damage, clearing oxygen free radicals, ameliorate intracellular energy transportation, and promote the healing of the injured HSKMCs in order to treat and improve chronic muscle injury disease. It may be one of the biological mechanisms caused by the mechanical effects of manipulations in TCM; therefore, further studies by testing other parameters will be needed in future.

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Disclosure of conflict of interest

None.

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References

[1] Zhang H, Liu H, Lin Q, Zhang G and Mason DC. Effects of intermittent pressure imitating rolling manipulation on calcium ion homeostasis in human skeletal muscle cells. BMC Complement Altern Med 2016; 16: 314.

- [2] Ma HS, Zhang H and Shen GQ. Analysis of effector mechanisms by which Tuina rolling manipulation boosts blood circulation. Jiangsu Journal of Traditional Chinese Medicine 2007; 39: 39-41.
- [3] Hu J, Zhang XL and Yan JT. Effects of massage on satellite cells of acute contusive skeletal muscles. Journal of Acupuncture and Tuina Science 2007; 2: 6-9.
- [4] Yang X, Zhao H and Wang J. Chinese massage (Tuina) for the treatment of essential hypertension: a systematic review and meta-analysis. Complement Ther Med 2014; 22: 541-548.
- [5] Huang CH. Efficacy of Tuina therapy in 100 cases of neck, shoulder, lower back, and leg pain. Seek Medical and Ask The Medicine 2013; 11: 160-161.
- [6] Li DH, Fang M, Zhu QG and Jiang SY. Research on the effects of Tuina manipulation on degree of neck fatigue in patients with cervical spondylosis. Beijing Journal of Traditional Chinese Medicine 2012; 31: 10-12.
- [7] Wang YG, Guo XQ, Liu K and Zhu LG. Systematic evaluation of randomized controlled trials of Tuina in treatment of lumbar disc herniation. Chinese Archives of Traditional Chinese Medicine 2013; 31: 1638-1642.
- [8] Kuijer P, Van der Beek AJ and Visser B. Effect of job rotation on need for recovery, musculoskeletal complaints, and sick leave due to musculoskeletal complaints: a prospective study among refuse collectors. Am J Ind Med 2005; 47: 394-402.
- [9] Zorbas YG, Kakuris KK and Deogenov VA. Inadequacy of calcium supplements to normalize muscle calcium deficiency in healthy subjects during prolonged hypokinesia. Nutrition 2008; 24: 217-223.
- [10] Fraysse B, Liantonio A and Cetrone M. The alteration of calcium homeostasis in adult dystrophic mdx muscle fibers is worsened by a chronic exercise in vivo. Neurobiol Dis 2004; 17: 144-54.
- [11] Luo CG, Wen YQ and Zhu DL. Correlation of Ca²⁺-ATPase activity with pathological changes in cervical vertebra muscular tissue and cervical syndrome. Journal of Clinical Rehabilitative Tissue Engineering Research 2007; 11: 2745.
- [12] Chen XG and Liu Y. Changes of energy metabolism in the skeletal muscle of rats in injury induced by the dynamic load. Journal of Chengdu Sport University 2010; 9: 65-68.
- [13] ZY Xie and SX Xu. Frequence-domainalysisnto rolling manipulation machanics of traditional Chinese medical massage. Med Biomech 1996; 114: 208-211.
- [14] Zhang H, Ma HS, Men ZT, Yan JT, Tong XJ and Miao ZJ. Mechanical parameter optimization for Tuina rolling manipulation. Shanghai Jour-

nal of Traditional Chinese Medicine 2006; 09: 68-69.

- [15] Ma HS, Zhang H, Miao ZJ, Yan JT, Tong XJ and Men ZT. Experimental study on dynamic parameter optimization of massage method. Chinese Journal of Rehabilitation Medicine 2006; 21: 1116-1118.
- [16] Zhang X, Mei XY and Bian HM. Construction of a skeletal muscle cell injury model. Pharmaceutical Journal of Chinese People's Liberation Army 2003; 19: 85.
- [17] Shen G and Yan J. Illustration of Tuina Manipulations (Chinese-English Edition) Shanghai: Shanghai Scientific and Technical Publishers, 2004.
- [18] Garvin J, Qi J, Maloney M and Banes AJ. Novel system for engineering bioartificial tendons and application of mechanical load. Tissue Engineering 2003; 9: 967-979.
- [19] Men ZT. Effects of Tuina manipulation on rat skeletal muscle cell SOD, MDA, and CK. Journal of Liaoning University of Traditional Chinese Medicine 2010; 10: 35-37.
- [20] Amir Aslani B and Ghobadi S. Studies on oxidants and antioxidants with a brief glance at their relevance to the immune system. Life Sci 2016; 146: 163-173.
- [21] Liu CZ and Lei B. Effect of Tuina on oxygen free radicals metabolism in patients with chronic fatigue syndrome. Zhongguo Zhen Jiu 2010; 30: 946-8.
- [22] Che LL, Feng SG and Qian H. Difference of the effects of long-term endurance exercise on nitric oxide and GSH and MDA level between in the cardiac muscle and in the gastrocnemius muscle. Journal of Jiangsu University (Medicine Edition) 2008; 18: 507-509.
- [23] Li GZ, Wang S and Feng X. Facilitating effect of vitamin E on the plerosis of skeletal muscle after strain injury. Chinese Journal of Clinical Rehabilitation 2004; 8: 848-849.
- [24] Oredsson S, Qvarfordt P and Plate G. Polymorphonuclear leucocytes increase reperfusion injury in skeletal muscle. Int Angiol 1995; 14: 80-88.
- [25] Luo XX, Ogata H, Xu X and Ishitobi F. Protective effect of tetramethylpyrazine on ischemic neuronal damage in the gerbil hippocampus. No To Shinkei 1994; 46: 841-846.
- [26] Wang XT, Tian Y, Xu WX, Cui LH, Xiang SY and Lü SC. Protective effects of modeled superoxide dismutase coordination compound (MSO-Da) against ischemia/reperfusion injury in rat skeletal muscle. Cell Physiol Biochem 2015; 37: 465-476.
- [27] Zhou DQ, Gong L and Hu Y. Relationship between creatine kinase isoenzymes, its genes, and endurance. Chinese Journal of Sports Medicine 2006; 7: 494-496.

- [28] Mendonça LR, Joviliano EE, Ramalho FS, Ramalho LN, Evora PR and Piccinato CE. Bradykinin impairs and HOE 140 does not protect rat hindlimb skeletal muscle against tourniquetinduced reperfusion injury. J Invest Surg 2016; 29: 13-19.
- [29] Balnave CD and Thompson MW. Effect of training on eccentric exercise-induced muscle damage. J Appl Physiol (1985) 1993; 75: 1545-1551.
- [30] Zhao B, Tumaneng K and Guan KL. The Hippo pathway in organ size control, tissue regeneration and stem cell self-renewal. Nat Cell Biol 2011; 13: 877-883.
- [31] Watt KI, Goodman CA, Hornberger TA and Gregorevic P. The hippo signaling pathway in the regulation of skeletal muscle mass and function. Exerc Sport Sci Rev 2018; 46: 92-96.
- [32] Li Z. Study on the structure and function of transcription Factor complex YAP-TEAD downstream of hippo signaling pathway. Fudan University 2009 PhD Thesis.
- [33] Vissergrieve S, Zhou Z, She YM, Huang H, Cyr TD, Xu T and Yang X. LATS1 tumor suppressor is a novel actin-binding protein and negative regulator of actin polymerization. Cell Research 2011; 21: 1513-1516.
- [34] Mao X, Zhou XM, Wu XT, et al. Research Progress of Hippo Pathway regulating Cell Proliferation and apoptosis. Journal of Southeast University (Medical Science Edition) 2016; 35.
- [35] Relaix F and Zammit PS. Satellite cells are essential for skeletal muscle regenerationthe cell on the edge returns centre stage. Development 2012; 139: 2845-2856.
- [36] Judson RN, Tremblay AM, Knopp P, White RB, Urcia R, De Bari C, Zammit PS, Camargo FD and Wackerhage H. The Hippo pathway member Yap plays a keey role in influencing fate decisions in muscle satillite cells. J Cell Sci 2012; 125: 6009-19.
- [37] Mizuno T, Murakami H, Fujii M, Ishiguro F, Tanaka I, Kondo Y, Akatsuka S, Toyokuni S, Yokoi K, Osada H and Sekido Y. Yap induces malignant mesothelioma cell proliferation by upregulating transcription of cell cycle-promoting genes. Oncogene 2012; 31: 5117.
- [38] Judson RN, Tremblay AM, Knopp P, White RB, Urcia R, De Bari C, Zammit PS, Camargo FD and Wackerhage H. The Hippo pathway member Yap plays a key role in influencing fate decisions in muscle satillite cells. J Cell Sci 2012; 125: 6009-19.
- [39] Meng Z, Moroishi T and Guan KL. Mechanisms of Hippo pathway regulation. Genes Dev 2016; 30: 1-17.
- [40] Li Z, Zuo QF, Cao LY, et al. The mechanism of ECM and Yap/TAZ in determining the fate of cells. Progress in Modern Biomedicine 2016; 16: 785-788.

- [41] Ma ZX. Effects of cyclic tensile stress on proliferation and apoptosis of rat growth plate cartilage cells. Huazhong University of Science and Technology 2015 PhD Thesis.
- [42] Tremblay AM, Missiaglia E, Galli GG, Hettmer S, Urcia R, Carrara M, Judson RN, Thway K, Nadal G, Selfe JL, Murray G, Calogero RA, De Bari C, Zammit PS, Delorenzi M, Wagers AJ, Shipley J, Wackerhage H and Camargo FD. The hippo transducer Yap1 transforms activated satellite cells and is a potent effector of embryonal rhabdomyosarcoma formation. Cancer Cell 2014; 26: 273-287.
- [43] Judson RN, Gray SR, Walker C, Carroll AM, Itzstein C, Lionikas A, Zammit PS, De Bari C and Wackerhage H. Constitutive expression of yesassociated protein (Yap) in adult skeletal muscle fibres induces muscle atrophy and myopathy. PLoS One 2013; 8: e59622.
- [44] Watt KI, Turner BJ, Hagg A, Zhang X, Davey JR, Qian H, Beyer C, Winbanks CE, Harvey KF and Gregorevic P. The Hippo pathway effector Yap is a critical regulator of skeletal muscle fibre size. Nat Commun 2015; 6: 6048.

- [45] Sun C, De Mello V, Mohamed A, Ortuste Quiroga HP, Garcia-Munoz A, Al Bloshi A, Tremblay AM, von Kriegsheim A, Collie-Duguid E, Vargesson N, Matallanas D, Wackerhage H and Zammit PS. Common and distinctive functions of the hippo effectors Taz and Yap in skeletal muscle stem cell function. Stem Cells 2017; 35: 1958-1972.
- [46] Philp A, Hamilton DL and Baar K. Signals mediating skeletal muscle remodeling by resistance exercise: Pl3-kinase independent activation of mTORC1. J Appl Physiol 2011; 110: 561-8.
- [47] Lin J, Wu H, Tarr PT, Zhang CY, Wu Z, Boss O, Michael LF, Puigserver P, Isotani E, Olson EN, Lowell BB, Bassel-Duby R and Spiegelman BM. Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres. Nature 2002; 418: 797-801.