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Original Article

Effects of inverse moxibustion on the translocation of telomerase from cardiomyocyte mitochondria of growing mice following exercise

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Abstract: We aimed to investigate the influence of inverse moxibustion on the translocation of telomerase from cardiomyocyte mitochondria of growing mice following exercise. Sixty mice were divided into six groups (n = 10 each). For the control groups, mice were handled each day, but not subjected to moxibustion. For the inverse moxibustion groups, Guan yuan and Zusanli acupuncture points were moxibusted daily using 10 columns. Mice were then subjected to the appropriate swimming exercise (or lack of exercise for the no exercise groups). Increased apoptosis was observed for the moderate exercise group compared with the no exercise control group and for the exhaustive exercise group compared with the moderate exercise group. For all paired groups, the application of inverse moxibustion led to a decrease in the rate of apoptosis. Additionally, reduced expression of the mitochondrial ND1 gene was observed for the moderate exercise group compared with the no exercise control group and for the exhaustive exercise group compared with the moderate exercise group. In contrast, for all paired groups, the application of inverse moxibustion led to an increase in the expression of the mitochondrial ND1 gene. TERT expression in whole cells and mitochondria was enhanced as the exercise intensity increased. Application of inverse moxibustion in the moderate and exhaustive exercise groups increased TERT expression compared with the moderate exercise and exhaustive exercise control groups, respectively. Moxibustion effectively protected the myocardial cells from high-intensity exercise-induced injury by reducing mitochondrial damage and cell apoptosis, increasing telomerase activity, and promoting mitochondrial translocation of telomerase.

Keywords: Moxibustion, exhaustive exercise, myocardial cell, telomerase, mitochondria, exercise intensity

Introduction

In competitive sports, athletes must strive to maximize and improve personal potential in physical, psychological, and athletic abilities; this pursuit includes high-intensity training, which can lead to pathological changes in the body, particularly the cardiovascular and skeletal muscle system. Continuous and excessive movement is also one of the reasons that chronic diseases are difficult to cure [1]. The heart, which is one of the most sensitive organs to movement, is the engine of the blood circulation; excessive oxygen free radicals are produced after a series of high-strength and continuous training sessions, causing high levels of oxidative stress, which is the key pathological basis of myocardial injury [2-4]. Dysfunction of the cardiac electrophysiology and conduction system as well as damage to the myocardial structure, such as the cytoskeleton and gap junctions, can occur after overtraining and is the main factor associated with ventricular remodeling, electrical remodeling, and arrhythmia in athletes [5, 6].

Inverse moxibustion is an intervention that should be administered when the body is not ill and that seeks to make the body produce a moderate pre-stress (benign stress) state, resulting in a reduction in the degree of malignant stress. Recent studies have shown that inverse moxibustion can prevent myocardial injury caused by exhaustive exercise. Moreover, inverse moxibustion has protective effects against damage to myocardial cells from

exhaustive exercise by regulating endocrine function, scavenging free radicals, preventing oxidative damage, and modulating muscle energy metabolism [7]. Research has shown that the level of telomerase can be significantly affected by enhancing antioxidant defense [8]. Moreover, telomerase can also promote cell survival and resistance to the stress-dependent effects of non-telomere length, protecting mitochondrial function by maintaining the integrity of mitochondrial DNA and mitochondrial membrane potential [9], which is mainly achieved by telomerase translocation [10].

Although the effects of moxibustion on antioxidative stress and myocardial protection are clear, few studies have examined the cellular and molecular mechanisms through which moxibustion protects myocardial cells. Such information would be particularly useful for special populations, such as athletes, who have restricted capacity to take medications; thus, moxibustion may be an appropriate alternative therapy in these populations.

In this study, we examined the effects of inverse moxibustion on cardiomyocyte mitochondria of growing mice following exhaustive exercise, with a particular focus on mitochondrial translocation of telomerase, in order to provide theoretical experimental evidence for the control and prevention of movement-related diseases.

Materials and methods

Experimental animals

One-month-old healthy male Kunming mice weighing 18-22 g each were purchased from Nanjing University of Chinese Medicine Laboratory Animal Center (animal batch number: 11400700043392) and were screened for their ability to swim. Sixty mice were chosen for the study. All animal experiments were carried out according to protocols approved by the Animal Rights Ethics Committee of our institution (Approve ID: 11400700043392).

Animal maintenance and grouping

Mice were housed in cages in a room with controlled temperature (22°C ± 1°C) and humidity (50%) and were fed standard mouse chow. During the 1-week acclimation period, mice were subjected to two swimming training sessions, followed by an exhaustive swim on day 8. Mice were then randomized into six groups as follows (n = 10 mice per group): the no exercise control group, no exercise inverse moxibustion group, moderate exercise control group, moderate exercise inverse moxibustion group, exhaustive exercise control group, and exhaustive exercise inverse moxibustion group. The average exhaustive swimming time did not differ significantly between groups ($P > 0.05$).

Instruments and reagents

The glass swimming box measured 100 × 50 × 50 cm. The paraffin slicer was purchased from Leica (Germany), and analytical balances were purchased from Sartorius (Germany). The ultraviolet instrument (UV-2450) was purchased from Shimadtzu (Japan), the MultiGene Gradient polymerase chain reaction (PCR) cycler was purchased from Labnet (USA), and the quantitative PCR cycler (DA7600) was obtained from Zhongshan Ann (China). A nucleic acid electrophoresis instrument (Liuyi, Beijing, China) and Gel Doc XR (Bio-Rad, USA) were used. The western blotting electrophoresis instrument was purchased from Bio-Rad, and the high-speed refrigerated centrifuge (SH03014) was purchased from Jung Instrument Company (USA). Primary antibodies against β-actin were purchased from Immunoway; secondary antibodies (goat anti-mouse and goat anti-rabbit IgG-HRP) were purchased from Jackson Laboratories (USA). The First Strand cDNA Synthesis Kit was obtained from Fermentas (Lithuania), and the Real-time PCR Master Mix (SYBR Green) was purchased from TOYOBO (Japan). Agarose was purchased from Biowest (Spain), and the Fluorescence and Colorimetric TUNEL Apoptosis Assay Kit was obtained from Wuhan Boster Biological Engineering Co., Ltd.

Treatments and interventions

For mice in the control groups, technicians held the mice (pressing gently, but without moxibustion) each day for 8 min, for a total of 20 times for the study duration. For mice in the inverse moxibustion groups, moxibustion treatment was started 1 h before swimming training. The acupuncture points were as described by Yu and Xu [11] (the Guan Yuan and Zusanli points) and were moxibusted using 1 g/wick cones with 3-year 1:40 golden moxa obtained from Nanyang wolong han medicine moxa factory of Henan province (China). At the Guan Yuan point, moxibustion was performed in 10 columns daily, while at the Zusanli point, 10 columns were alternately moxibusted on the left and right daily. The moxa cone was removed when

about one-third of the moxa remained. Each column took 30-35 s, resulting in a moxibustion time of about 8 min per day for a total of 20 times.

Exhaustion swimming parameters

Ten mice per tank were subjected to swimming. The water depth in the 50-cm-deep tank was 30 cm to prevent the mice from grabbing the side of the tank to rest. The water temperature was maintained at 30 ± 2°C. Mice were determined to be exhausted from swimming when they became uncoordinated and could not resurface after being submerged in water for 3 s [12]. The exercise time in the moderate exercise group was set to half of the average exhaustive swim time for mice on each day.

Collection of tissue samples

Immediately after the final training session, mice were sacrificed by cervical dislocation. Heart tissues were removed, washed with ultrapure water, and wiped dry. Part of the tissue was then fixed in 10% neutral formaldehyde solution for embedding in paraffin and subsequent apoptosis assays, while the remaining tissue was used directly for quantitative PCR and western blotting.

Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay

Myocardial apoptosis was detected using TUNEL assays. Tissue sections were digested by proteinase K for 5 min and then rinsed with double-distilled water and Tris-hydroxymethylaminomethane-buffered saline (TBS). Sections were then incubated with the end labeling enzyme in a water bath at 37°C for 2 h. The slices were then washed with TBS and incubated for 30 min at room temperature. After rinsing, myocardial cells were stained with DAB and observed under a light microscope. Brown granules in the nucleus were representative of apoptotic cells. The apoptosis rate was calculated according to the number of brown-stained cells in nine randomly selected fields for each section.

Quantitative real-time reverse transcription PCR (RT-qPCR)

Oxidative damage of mitochondrial DNA was detected by RT-qPCR. After mitochondrial extraction of myocardial cells, mitochondrial DNA was extracted by proteinase K and 8 M NH_4Ac. First-strand cDNA was then prepared by reverse transcription, and the cDNA was amplified by PCR. The primer sequences were as follows: forward, 5'-CTCAACCTAGCAGAAACAAACC-3'; reverse, 5'-GGCCGGCTGCGTATTCTAC-3'; probe, 5'-CCCCCTTCGACCTGACAGAAGGAGA-3'. The PCR was carried out according to the following protocol: denaturation at 94°C for 40 s, annealing at 55°C for 35 s, extension at 72°C for 2 min 10 s. A final extension was performed at 72°C for 7 min. Each sample was analyzed three times, and the relative expression of the *ND1* gene was determined using the $2^{-\Delta\Delta CT}$ method.

Expression of TERT protein

TERT protein expression was detected by western blotting of whole cell lysates and mitochondrial lysates. Lysates were diluted to the some concentration using lysis buffer, and equal amounts of sample (70 μg) were heated for 5 min at 95-100°C. Electrophoresis was carried out at 80 V for 20 min followed by 80 min at 100 V. Proteins were then transferred to PVDF membranes at a constant current of 200 mA for 1 h. Membranes were blocked in 5% nonfat milk at room temperature for 1 h and then incubated with antibodies (anti-TERT, diluted 1:600; anti-β-actin, diluted 1:4000) overnight at 4°C. After washing four times for 5 min each, the membranes were then incubated with the appropriate horseradish peroxidase (HRP)-linked secondary antibodies (diluted 1:5000) at room temperature for 1-2 h. The membranes were then washed five times for 5 min each, followed by development at room temperature for 1 min.

Statistical analysis

Data were analyzed using SPASS16.0. All data were found to be normally distributed. Data are presented as mean ± standard deviation. Groups were compared by one-way analysis of variance (ANOVA). Differences with *P* values of less than 0.05 were considered significant.

Results

Effects of exercise and inverse moxibustion on myocardial cell apoptosis

The results of TUNEL staining are shown in **Figure 1**. Significantly increased apoptosis was observed for the moderate exercise group com-

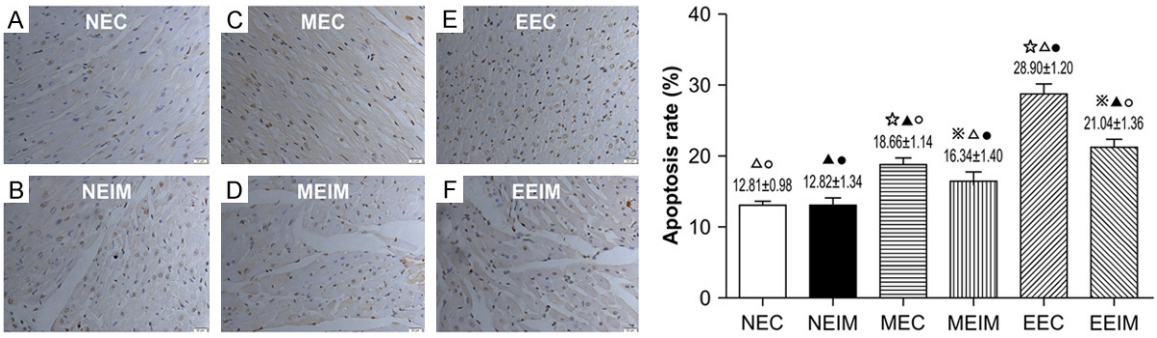

Figure 1. Effects of exercise and moxibustion on myocardial cell apoptosis. Top panels show images of TUNEL staining at 400 × magnification. Bottom panels show quantification of staining. Groups: NEC, the no exercise control group (n = 9); NEIM, no exercise inverse moxibustion group (n = 9); MEC, moderate exercise control group (n = 8); MEIM, moderate exercise inverse moxibustion group (n = 9); EEC, exhaustive exercise control group (n = 8); EEIM, exhaustive exercise inverse moxibustion group (n = 8). ☆$P < 0.05$ compared with the no exercise control group; Δ$P < 0.05$ compared with the no exercise inverse moxibustion group; ▲$P < 0.05$ compared with the moderate exercise inverse moxibustion group; ○$P < 0.05$ compared with the exhaustive exercise control group; ●$P < 0.05$ compared with the exhaustive exercise inverse moxibustion group.

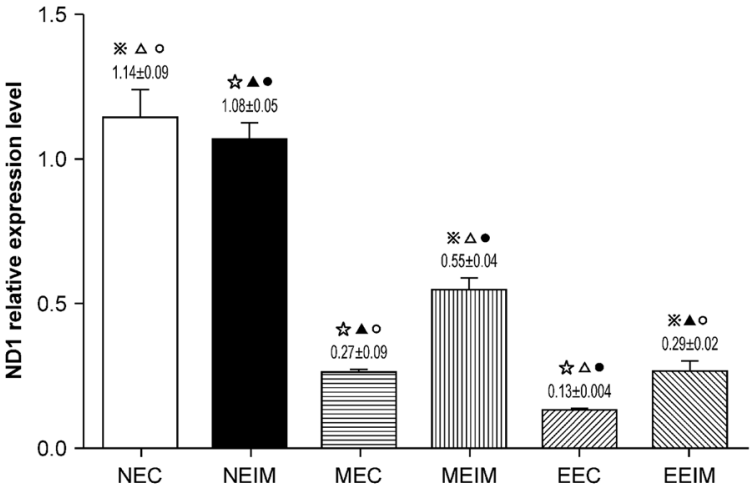

Figure 2. Effects of exercise and inverse moxibustion on the expression of the *ND1* gene. Groups: NEC, the no exercise control group (n = 9); NEIM, no exercise inverse moxibustion group (n = 9); MEC, moderate exercise control group (n = 8); MEIM, moderate exercise inverse moxibustion group (n = 9); EEC, exhaustive exercise control group (n = 8); EEIM, exhaustive exercise inverse moxibustion group (n = 8). ☆$P < 0.05$ compared with the no exercise control group; ※$P < 0.05$ compared with the no exercise inverse moxibustion group; Δ$P < 0.05$ compared with the moderate exercise control group; ▲$P < 0.05$ compared with the moderate exercise inverse moxibustion group; ○$P < 0.05$ compared with the exhaustive exercise control group; ●$P < 0.05$ compared with the exhaustive exercise inverse moxibustion group.

pared with the no exercise control group ($P < 0.01$) and for the exhaustive exercise group compared with the moderate exercise group ($P < 0.01$). In contrast, in mice subjected to moderate or exhaustive exercise, the application of inverse moxibustion led to a decrease in the rate of apoptosis ($P < 0.01$ for both).

RT-qPCR detection of mitochondrial DNA oxidative damage

Expression of the mitochondrial DNA marker *ND1* was then examined by RT-qPCR (**Figure 2**). Reduced expression of the mitochondrial *ND1* gene was observed for the moderate exercise group compared with the no exercise control group ($P < 0.01$) and for the exhaustive exercise group compared with the moderate exercise group ($P < 0.01$). In contrast, in mice subjected to moderate or exhaustive exercise, the application of inverse moxibustion led to an increase in the expression of the mitochondrial *ND1* gene ($P < 0.01$ for both).

Effects of exercise and inverse moxibustion on the expression of TERT protein in whole cells and mitochondria

Finally, we examined TERT expression in whole cell and mitochondrial lysates using western blot analysis (**Figure 3**). For both whole cells and mitochondria, TERT expression was significantly enhanced as the exercise intensity increased ($P < 0.05$ for moderate exercise versus no exercise; $P < 0.01$ for exhaustive exer-

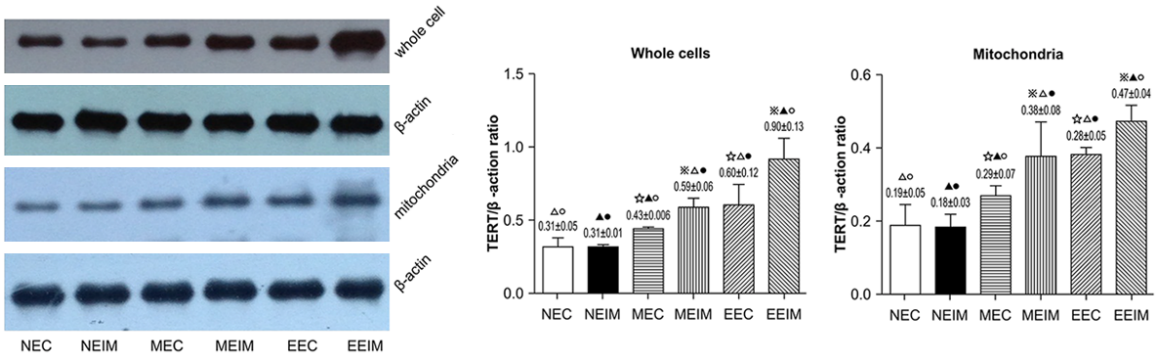

Figure 3. Effects of exercise and inverse moxibustion on the expression of TERT protein in whole cells and mitochondria. Protein expression was analyzed by western blotting and normalized to the expression of β-actin. Western blots are shown in the top panels, and quantitative results are shown in the lower panels. Groups (n = 5 each): NEC, the no exercise control group; NEIM, no exercise inverse moxibustion group; MEC, moderate exercise control group; MEIM, moderate exercise inverse moxibustion group; EEC, exhaustive exercise control group; EEIM, exhaustive exercise inverse moxibustion group. ☆$P < 0.05$ compared with the no exercise control group; ※$P < 0.05$ compared with the no exercise inverse moxibustion group; Δ$P < 0.05$ compared with the moderate exercise control group; ▲$P < 0.05$ compared with the moderate exercise inverse moxibustion group; ○$P < 0.05$ compared with the exhaustive exercise control group; ●$P < 0.05$ compared with the exhaustive exercise inverse moxibustion group.

cise versus no exercise or moderate exercise). Moreover, application of inverse moxibustion in the moderate and exhaustive exercise groups significantly increased TERT expression compared with the moderate exercise and exhaustive exercise control groups, respectively ($P < 0.01$ for both). Thus, these data suggested that increased exercise intensity enhanced TERT expression in cells and mitochondria. Similarly, inverse moxibustion increased TERT expression following exercise.

Discussion

In this study, we examined the effects of moxibustion on mitochondrial integrity and myocardiocyte survival following moderate or exhaustive exercise. We found that long-term and high-intensity exercise training induced apoptosis of myocardial cells and that moxibustion effectively protected myocardial cells from high-intensity exercise-induced injury by reducing mitochondrial damage and cell apoptosis, increasing telomerase activity, and promoting mitochondrial translocation of telomerase.

Repeated high intensity exercise can cause myocardial structural failure, ventricular remodeling, and electrical remodeling. Experimental studies have shown that long-term endurance exercise and the use of antioxidants can effectively protect myocardial cells by reducing myocardial cell injury and apoptosis in exhaustive exercise [13, 14]. However, effective treatments or preventive measured for reducing myocardial injury, particularly in athletes who undergo excessive training, have not yet been developed. While allopurinol has been shown to have some protective effects [15], the use of allopurinol is also associated with safety concerns. Additionally, in competitive sports, many drugs and hormones are banned from use. Therefore, moxibustion therapy may have applications as an alternative to traditional western medicine. Indeed, in our study, we found that application of inverse moxibustion prior to exercise had various protective effects against mitochondrial DNA damage in myocardial cells of mice. Consistent with this, clinical trials have confirmed that moxibustion can improve the physique of athletes and represents an effective, simple, feasible method for eliminating exercise-induced fatigue [16, 17]. Moxibustion has also been shown to prevent cardiovascular disease [18], protect the digestive tract mucosa [19], prevent bone marrow suppression caused by chemotherapy [20], and provide resistant to fatigue [17], among other beneficial effects. Thus, moxibustion may have applications as an alternative therapy for reducing myocardial damage during strenuous exercise in athletes.

Telomerase is a ribonucleoprotein reverse transcriptase found in eukaryotic cells. This protein has been shown to prevent cellular aging and

maintain cell immortalization [21]. The telomerase complex includes the template subunit of RNA, human telomerase RNA (hTR), protein components, human telomerase-related proteins, and human telomerase reverse transcriptase (hTERT) [21]. hTERT and telomerase act in concert to carry out the various functions of the telomerase complex [22, 23], including functions associated with cell survival and stress responses [10, 24, 25]. Interestingly, telomerase can translocate from the nucleus to the mitochondria under conditions of oxidative stress [10, 24]; in the mitochondria, telomerase protects mitochondrial DNA and enhances mitochondrial membrane potential, effectively reducing the levels of free radicals within cells, boosting mitochondrial activity, and providing protection against oxidative stress [9, 26]. After reduction of cellular oxidative stress, TERT will translocate from the mitochondria to the nucleus [27]. Thus, telomerase translocation is important for protecting cells against oxidative damage. In our study, we observed increased TERT expression following exercise and further increased expression after moxibustion therapy, suggesting that moxibustion therapy may exert its protective effects through induction of TERT expression.

Moxibustion is a type of thermal stimulation that can induce stress tolerance. Studies have confirmed that moderate heat stimulation can increase the body's responses to oxidative stress and prolong life [28]. A certain degree of thermal stimulation can induce mitochondrial synthesis and improve the adaptability of skeletal muscle during endurance training. Moreover, thermal stimulation can enhance mitochondrial enzyme activity and protein content in the respiratory chain and increase p70S6K phosphorylation, leading to dephosphorylation of acetyl CoA [29]. Moxibustion can also activate heat shock proteins [30], thereby reducing the accumulation of reactive oxygen species [31]. In the present study, we found that inverse moxibustion may promote translocation of TERT to the mitochondria following moderate-to-exhaustive exercise, thereby playing a protective role in mediating exercise-induced myocardial damage. However, additional studies are required to fully elucidate the mechanisms involved in this process.

In conclusion, long-term and high-intensity exercise training induced apoptosis of myocardial cells, with more substantial increases seen as the exercise intensity increased. Moxibustion effectively protected the myocardial cells from high-intensity exercise-induced injury by reducing mitochondrial damage and cell apoptosis, increasing telomerase activity, and promoting mitochondrial translocation of telomerase. Due to the level of telomerase in growing period of myocardial cells in mice is low, using immunohistochemical were not observed to corresponding results. We will improve the experimental methods in the later experiment.

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Disclosure of conflict of interest

None.

Authors' contribution

JLT designed the study and HRZ, YHG and YH conducted the collection, analysis and interpretation of data. JLT and HRZ drafted the manuscript. All the authors participated in writing and giving feedback on the manuscript. All authors have approved the final manuscript and made the decision to submit the article for publication.

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