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Experimental Research

Effects of electroacupuncture pretreatment on mitochondrial energy metabolism in the rats with myocardial ischemia reperfusion ☆

电针预处理对心肌缺血再灌注大鼠线粒体能量代谢的影响

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ABSTRACT

Objective: To explore the effects of electroacupuncture pretreatment on mitochondrial energy metabolism in the rats with myocardial ischemia reperfusion injury (MIRI).

Methods: A total of 60 SPF Wistar rats were randomly divided into a sham-operation group (sham group), a myocardial ischemia reperfusion injury group (MIRI group) and an electroacupuncture pretreatment group (EA group), 20 rats in each one. The rats in the sham group and the MIRI group were binded for 7 days, once a day, 20 min each time. On the 8th day, the sample was collected after the heart exposed for 50 min in thoractomy in the sham group and the sample was collected after ischemia for 20 min and reperfusion for 30 min in thoractomy in the MIRI group. In the EA group, the pretreatment intervention with electroacupuncture was applied at "Nèiguān (内关PC6)", "Guānyuán (关元CV4)" and "Zúsānlǐ (足三里ST36)" in the rats for 7 days, once a day, 20 min each time. On the 8th day, after ischemia for 20 min and reperfusion for 30 min in thoractomy, the sample was collected in the EA group. The changes in ST_{II} segment of electroacardiogram (ECG) were observed and measured. Using enzyme-linked immunosorbent assay (ELISA), the concentrations of cardiac troponin T (cTnT) and cardiac troponin I (cTnI)were detected. Using nitro blue tetrazolium chloride monohydrate (NBT) staining, the myocardial infarction weight percentage was measured. Using ELISA, the concentrations of mitochondrial adenosine monophosphate (AMP), adenosine diphosphate (ADP)and adenosine triphosphate (ATP)were detected.

Results: (1) ST_{II} changes: in 20 min of ligation, compared with the sham group, the ST_{II} segment of electrocardiograph (ECG) was elevated significantly in the MIRI group and EA group (both $P < 0.01$), but the elevation range in the EA group was lower than that of the MIRI group ($P < 0.01$). After reperfusion for 30 min, the ST_{II} segment was fallen by over 50% in the MIRI group and the EA group. Simultaneously, the ST_{II} segment in the EA group was lower than that of the MIRI group ($P < 0.01$). (2) Regarding myocardial infarction weight percentage, compared with the sham group, the infarction weight was larger in the MIRI group and the EA group (both $P < 0.05$) and the infarction weight in the EA group was lower than that of the MIRI group ($P < 0.05$). (3) Regarding the levels of serum cTnt and cTnI, compared with the sham group, the levels of serum cTnT and cTnI were higher in the MIRI group and the EA group (all $P < 0.01$) and the levels of cTnT and cTnI in the EA group were lower than that of the MIRI group (both $P < 0.01$). (4) Regarding the concentrations of AMP, ADP and ATP, compared with the sham group, ATP

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concentration was lower in the MIRI group and the EA group (both $P < 0.01$) and the concentrations of AMP and ADP were higher ($P < 0.05$, $P < 0.01$). Compared with the MIRI group, ATP concentration was higher in the EA group ($P < 0.05$) and the concentrations of AMP and ADP were lower (both $P < 0.01$).

Conclusions: Electroacupuncture pretreatment reduces the elevation of ECG ST_{II} segment, decreases the concentrations of myocardial injury markers, cTnT and cTnI and regulates the transfer among AMP, ATP and ADP. The protective effect of electroacupuncture pretreatment may result from the regulation of mitochondrial energy metabolism.

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Introduction

Cardiovascular disease is at the top of the list of the three fatal diseases, characterized as high morbidity, high mortality and high recurrence rate [1]. Regarding the treatment of ischemic cardiac diseases, restoring blood perfusion has become the basic principle of treatment [2]. However, while blood supply is restored, the reperfusion injury may occur and even the reversible damage may turn into the irreversible one, named myocardial ischemia reperfusion injury (MIRI). It is a very common pathological process in clinic [3-4]. Its occurrence is closely relevant with many mechanisms, such as calcium overload, energy metabolism, inflammatory reaction and apoptosis [5-8]. Of them, mitochondrial energy metabolic disorder is the initial link of MIRI [9]. Once myocardial ischemia and hypoxia occur, the mode of energy metabolism will change rapidly, from aerobic metabolism to anaerobic metabolism, resulting in a sharp decline of adenosine triphosphate (ATP) and mitochondrial energy metabolic disorder. Mitochondrial energy metabolic disorder may lead to the abnormality of gene structure and expression, which eventually induces apoptosis [10-11]. In this research, associated with the current research reports, taken MIRI rats as the subjects, in view of mitrochondrial energy metabolism, the anti-apoptosis effect of electroacupuncture pretreatment was explored in MIRI rats so as to further elaborate the mechanism of electroacupuncture pretreatment against MIRI.

Materials and methods

Experimental animals and group division

In this research, 60 SPF male Wistar rats [provided by Hubei Provincial Experimental Animal Center, Animal Licence Number SCXK (Hubei) 2015-0018] were adopted, aged 42 to 48 days, weighted 180 g to 220 g, all fed in SPF Animal Room, the Experimental Animal Center of Hubei University of Chinese Medicine. The experiment started 1 week after adaptive feeding. During this period, the rats were fed routinely with feed and sterile water, in at room, (25± 2)°C at room temperature, 50% to 60% in humidity. Before experiment, according to the random number table, the rats were divided into 3 groups, named a sham-operation group (sham group), a myocardial ischemia reperfusion injury group (MIRI group) and an electroacupuncture pretreatment group (EA group), 20 rats in each one.

Main reagents and instruments

The main reagents included 2,3,5-triphenyltetrazolium chloride (TTC) (manufactured by Wuhan Servicebio Co., Ltd.), ELSA kits of rat myocardial specific troponin T (cTnT) and troponin I (cTnI) (Jiangsu Boshen biotechnology Co., Ltd.), tissue mitochondrial isolation kit (Beyotime), BCA protein concentration determination kit (Beyotime), rat enyme-linked immunosorbent assay (ELISA) kit of adenosine monophosphate (AMP), adenosine diphosphate (ADP)and adenosine triphosphate (ATP) (Jiangsu Meimian Industrial Co., Ltd.).

Main instruments: HANS-200 electroacupuncture therapeutic apparatus (Nanjing Jisheng Medical Technology Co., Ltd.), ALC-V series animal ventilator (Shanghai Alcott Biotech), BL-420S biological function experimental system (Chengdu Techman Software Co., Ltd.), HMIAS 2000 high-definition color graphic analysis system (Wuhan Champath Image Technology Co., Ltd.), C16000 automatic biochemistry analyzer (Abbott Laboratories, USA), enzyme marker instrument (Shanghai Kehua Biological Engineering Co., Ltd.), 0.25 mm × 13 mm acupuncture needle (Suzhou Global Acupuncture Medical Instrument Co., Ltd.).

Model establishment

Establishment of in vivo MIRI model [12-13]: the rats were fasted of food and water one night before modeling and were anesthetized by intraperitoneal injection of 10% uratan, 10 mL/kg. When the rats had no obvious superficial reflex, the skin of the neck and chest was sterilized for subsequent operation. The rats were fixed on the operating table in supine, with the leads of electrocardiography (ECG) instrument connected. After sterilized with 75% alcohol on the midline skin of the neck, the skin was incised along the midline and the tissues of each layer were dissected bluntly to let the trachea exposed. The indwelling needle connected with the ventilator was inserted in the space between the 3rd and 4th tracheal cartilage rings directly to the trachea. Pulling out the needle core, the ventilator duct was fixed. The frequency of ventilator was set up to be 75 times/min, the respiratory ratio 1: 1, the tidal volume 11.5 mL/100 g and the oxygen flow 1 L/min at the inspired air intersection of ventilator. According to the breathing rate and depth of rats, the breathing parameters were adjusted. Sterilized the skin of the chest with 75% alcohol, the transverse incision was cut between the 3rd and 4th ribs on the left side of the sternum to dissect the tissues of each layer bluntly and expose the ribs. In the 3rd intercostal space, about 0.5 cm distant to the left border of the sternum, the intercostal muscles were dissected bluntly. With the chest opener of small animal, the ribs were opened to expose the heart. The pericardium was tore carefully. A sterile dry cotton, as a size of soybean, was put between the heart and the lung. In the place between the lower border of the left atrial appendage (LAA) and the pulmonary conus arteriosus, the main trunk of the left coronary vein was found, in which, the artery runs with it. Swiftly, with 3 × 8 suture needle and 5-0 sutures, the needle was inserted from the site, about 3 mm far from the root of the artery that is located between the lower border of LAA and pulmonary conus arteriosus. The depth of insertion was 1 mm and the width was 1.5 mm to 2.0 mm. While the silicone tube on suture line was put against the ligation site, the vessel was ligated quickly and the needle was withdrawn. When the ligation was successful, it is visible with naked eyes that the

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anterior wall of the left ventricle became pale or cyanoze and the pulsation was weakened. The skin bilateral the incision was closed with arterial clamp. 20 min later, the chest cavity was opened, the silicone tube was cut off quickly to form reperfusion and 30 min later, the modeling was established.

Interventions

Sham group: the rat was binded with the self-made rat clothes and fixed on the self-prepared rat platform, once a day, 20 min each time, for 7 days consecutively. On the 8th day, the chest was opened and the heart was exposed.

MIRI group: the rat was binded with the self-made rat clothes and fixed on the self-prepared rat platform, once a day, 20 min each time, for 7 days consecutively. On the 8th day, after the chest was opened, the myocardial ischemia for 20 min and reperfusion for 30 min were applied.

EA group: the rat was binded with the self-made rat clothes and fixed on the self-prepared rat platform. In reference to *Acupoint Altlas of Experimental Animals* developed by Hua Xingbang, et al [14], "Nèiguān (内关PC6)" "Zúsānlǐ (足三里ST36)" "Guānyuán (关元CV4)", and "*Ashi points*", 0.5 cm lateral to either side of "CV4" were selected. After sterilized with 75% alcohol, acupuncture needles, 0.25 mm × 13 mm were inserted at the above points perpendicularly, 1 mm, 7 mm, 2 mm and 2 mm in depth successively. One group of electric wire was attached on "PC6" and "ST36" on the same side and another group was on "CV4" and "*Ashi points*", 0.5 cm lateral to "CV4". The electroacupuncture parameters were continuous wave, 2 Hz in frequency and 1 mA in intensity [15]. The stimulation was given once a day, 20 min each time, for 7 days consecutively. On the 8th day, after the chest was opened, the myocardial ischemia for 20 min and reperfusion for 30 min were applied.

Sample collection

After the chest opened for 50 min in the rats in the sham group and at the end of reperfusion in the rats in the MIRI group and the EA group, blood, 2 mL was collected from the abdominal aorta and the serum was separated and stored frozen at -80 °C for standby application. The hearts were collected from 10 rats in each group by random and stored frozen at -20 °C for nitroblue tetrazolium (NBT)staining. The ischemia areas of the hearts of the rest 10 rats in each group were dissected for mitochondrial extraction.

Indicator test

(1) Changes of ST_{II} in electrocardiography (ECG)

In 20 min of ischemia, the changes in ST segment of ECG were observed. The standard of ischemia was labeled by ST_{II} segment elevation by 0.1 mV, combined with cyanosis and the outward expansion of the left ventricular anterior wall [16]. After reperfusion for 30 min, the reperfusion accomplishment was determined by ST_{II} segment fallen by over 50%.

(2) Test of the concentrations of cardiac injury markers, cardiac troponin T(cTnT) and cardiac troponin I (cTnI)

The serum was collected from 10 rats of each group by random. ELISA was used, in reference to the instructions of cTnT and cTnI kits, the concentrations of them were tested by the automatic biochemical analyzer.

(3) Determination of infarction weight percentage of myocardiac sample

NBT staining method was adopted. According to the instruction of NBT kit, 0.025% NBT buffer solution was used for staining. After staining, the sample was fixed in 4% paraformaldehyde solution for 24 h and the images were captured with digital camera. The analysis was performed with HMIAS 2000 high definition color graphic analysis system and the myocardial infarction weight percentage was calculated.

(4) Test of the concentrations of AMP, ADP, ATP

According to the instructions of mitochondrial separation kit, mitochondria was extracted from myocardial tissue and the concentration of it was tested based on the instruction of BCA kit. In reference to the instructions of AMP, ADP and ATP ELISA kits, the absorbance value of each sample was tested. Besides, the standard curve was drawn and the concentrations of ADP, ATP and AMP was calculated in each sample.

Statistical analysis

SPSS 20.0 statistical software was adopted in data management. The measurement data were expressed with mean ± standard deviation (Mean ± SD) . The *t*-test of two independent samples was used for the comparison between the two groups, the one-way analysis of variance was for the comparison among multiple samples and multiple groups and *LSD* test was for the pairwise comparison. $P < 0.05$ was considered as statistically significant.

Results

Comparison of ST_{II} changes in ECG in the rats with myocardiac ischemia reperfusion among the groups

Compared with the values before ligation, in 20 min after ligation, ST_{II} was elevated from (0.0197 ± 0.0028) mV to (0.0230 ± 0.0043) mV in the sham group and it was elevated from (0.0230 ± 0.0043) mV to (0.2913 ± 0.0303) mV in the MIRI group and from (0.0210 ± 0.0037) mV to (0.2503 ± 0.0200) mV in the EA group. In 20 min of ligation, compared with the sham group, ST_{II} was higher obviously in either the MIRI group or the EA group (both $P < 0.01$) by over 0.1 mV, which also indicated the success of modeling. In the EA group, the elevation range of ST_{II} was lower than that of the MIRI group ($P < 0.01$). After reperfusion for 30 min, ST_{II} was fell to be (0.0211 ± 0.0035) mV in the sham group, it was to be (0.1812 ± 0.0091) mV in the MIRI group and (0.1199 ± 0.0078) mV in the EA group. Compared with the MIRI group, ST_{II} was lower obviously in the EA group ($P<0.05$) and ST_{II} was fell by over 50%, which was also indicating the successful reperfusion. The success rate of modeling was 100%. See Fig. 1.

Comparison of the concentrations of serum cTnT and cTnI in the rats with myocardiac ischemia reperfusion among the groups

Compared with the sham group, the concentrations of cTnT and cTnI in serum were higher in the MIRI group (both $P < 0.01$). Compared with the MIRI group, the concentrations of cTnT and cTnI in serum were lower in the EA group (both $P < 0.01$). See Table 1.

Comparison of infarction weight percentage in the rats with myocardiac ischemia reperfusion among the groups

After NBT staining, the normal myocardial tissue was blue in color and the infarct tissue was not stained. Compared with the sham group, the infarction weight percentage was higher in either the MIRI group or the EA group (both $P < 0.01$). The infarction weight percentage in the EA group was lower than that of the MIRI group ($P < 0.01$). See Table 2.

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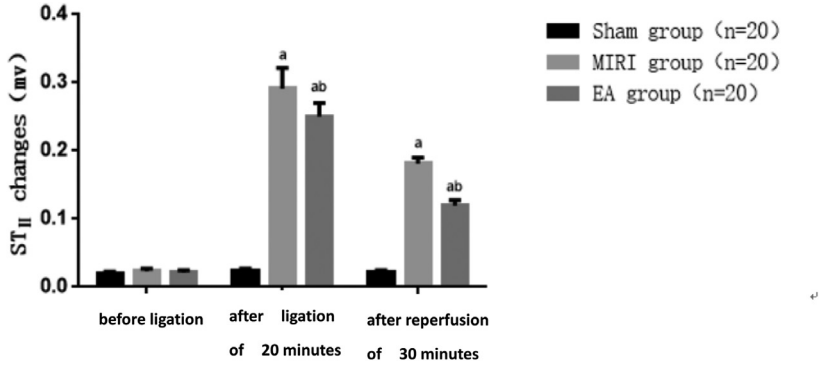

Fig. 1. Comparison of the changes in ST_{II} segment of ECG in the rats with myocardial ischemia reperfusion among the groups.
[a] Compared with the sham group in the same time point, $P < 0.01$.
[b] Compared with the MIRI group in the same time point, $P < 0.01$.

Comparison of the concentrations of ADP, AMPand ATP in the rats with myocardial ischemia reperfusion among the groups

In the sham group, the concentrations of ADP, AMP and ATP were (1150.56 ± 195.64) μg/L, (3099.40 ± 297.62) μg/L and (767.40±169.25) μg/L respectively. In the MIRI group, they were (1727.50 ± 306.31) μg/L, (3802.10 ± 382.28) μg/L and (351.80 ±107.96) μg/L respectively. In the EA group, they were (1122.50 ± 206.5) μg/L, (3210.90 ± 334.17) μg/L and (579.50 ± 67.37) μg/L respectively. Compared with the sham group, the concentrations of ADP and AMP were higher and ATP was lower in the MIRI group ($P < 0.05$, $P < 0.01$). Compared with the MIRI group, the concentrations of ADP and AMP were lower (both $P < 0.01$) and ATP was higher ($P < 0.01$) in the EA group. See Fig. 2.

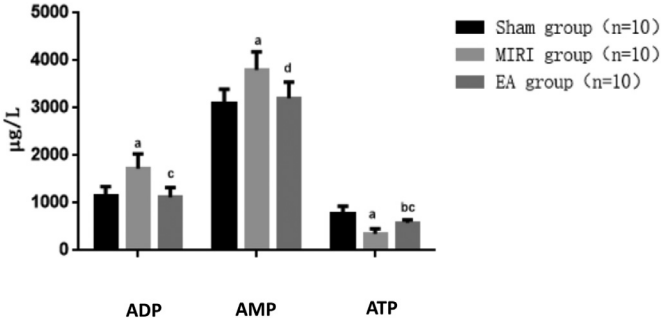

Fig. 2. Comparison of the concentrations of ADP, AMP and ATP in the rats with myocardial ischemia reperfusion among the groups.
[a] Compared with the sham group, $P < 0.05$.
[b] Compared with the sham group, $P < 0.01$.
[c] Compared with the MIRI group, $P < 0.05$.
[d] Compared with the MIRI group, $P < 0.01$.

Discussion

Myocardial ischemia is in the category of "chest *bi* syndrome and heart pain" in traditional Chinese medicine (TCM). Its basic pathogenesis is the deficiency of the root/antipathogenic *qi* (*ben*,本) and the excess of the branch/pathogens (*biao*,标) and the basic principle of treatment is consolidating the root and reducing the excessive pathogens. It is said in TCM that "the pathogens cannot invade the body when the antipathogenic *qi* is strong enough". Under the guidance of TCM in the idea of disease occurrence and the theory of disease prevention, the early prevention of disease is specially important. Acupuncture pretreatment is a kind of method of disease prevention in the field of acupuncture-moxibustion. It is the method to stimulate some acupoints of the body with acupuncture-moxibustion in advance so as to invigorate the potential vitality of the body, improve the ability of the resistance and response to disease and suppress or alleviate the subsequent damage of disease. Clinical researches [17-18] indicate that acupuncture pretreatment has satisfactory effect on myocardial ischemia and it alleviates the injury of myocardial ischemia and reperfusion. It is mentioned that "PC6 is the crucial point in treatment of the heart and chest disorders". “PC6” is the *luo*-connecting point of pericardial meridian of hand-*jueyin*. Needling at this point works on promoting three *jiao* and removing the stasis of heart meridian for the treatment of the branch/pathogens (*biao*). Needling at “CV4” and “ST36” works on consolidating the congenital and strengthening the acquire for the treatment of the root/antipathogenic *qi* (*ben*) [19-20]. In this research, it is proved again in the in vivo MIRI rat models that electroacupuncture pretreatment effectively improves in the changes of ST_{II} segment in MIRI, which is coincident with the results in our previous report [21]. In addition, the changes of relevant indicators of mitochondrial energy metabolism and serology have been observed in MIRI. It is indicated in the results of this research that electroacupuncture pretreatment effectively promotes ATP generation, improves in energy metabolic disorder of cardiomyocyte and regulates relevant serological indicators. Mitochondria is the productive organelle of cardiomyocyte. Whether its function is normal or not is the crucial factor to decide the degree of myocardial injury in MIRI [11]. In MIRI, mitochondrial energy metabolic injury is regarded as the initial link of MIRI on-

Table 1
Comparison of of the concentrations of serum cTnT and cTnI in the rats with myocardiac ischemia reperfusion among the groups (Mean±SD, μg/L)

Groups	Rats	cTnT	cTnI
Sham group	10	0.128 ± 0.015	0.057 ± 0.009
MIRI group	10	0.252 ± 0.019[a]	0.159 ± 0.026 [a]
EA group	10	0.185 ± 0.011[a,b]	0.009 ± 0.018 [a,b]

[a] Compared with the sham group, $P < 0.01$.
[b] Compared with the MIRI group, $P < 0.01$.

Table 2
Comparison of infarction weight percentage in the rats with myocardiac ischemia reperfusion among the groups (Mean±SD, %)

Groups	Rats	Infarction weight percentage
Sham group	10	0.287 ± 0.166
MIRI group	10	36.329 ± 3.793[a]
EA group	10	28.556 ± 5.533[a,b]

[a] Compared with the sham group, $P < 0.01$.
[b] Compared with the MIRI group, $P < 0.01$.

set [22]. Energy metabolic disorder must affect the normal structure and expression of genes of cardiomyocyte. The apoptosis and necrosis of cardiomyocyte are closely related to the concentration of ATP in mitochondrial energy metabolic disorder [23]. Therefore, in this research, mitochondrial ATP was taken as the main subject and the mitochondrial energy metabolism and relevant serological indicators in cardiomyocytes were observed in each group. It is very significant to give a further elaboration on the effect mechanism of acupuncture-moxibustion pretreatment for alleviating myocardial ischemia reperfusion so as to guide clinical practice.

ATP is an important energy substance in living organism and it achieves the energy transportation and transfer through the mutual transformation with ADP or AMP. The changes in the concentrations of ATP, ADP and AMP in myocardial tissue reflect the energy metabolism of the heart [24]. In MIRI, the changes of ATP, ADP and AMP are in a dynamic process. ADP and AMP are generated gradually in the process of energy release of ATP. When myocardial ischemia happens, a most of ATP will converte to ADP and AMP. During reperfusion, a most of ADP is converted back to ATP and AMP under the catalysis of adenosine kinase [25]. In this research, after ischemia and reperfusion, ATP concentration in myocardial mitochondria was reduced significantly in the rats and the concentrations of ADP and AMP generated were increased significantly. It is suggested that ischemia and reperfusion result in myocardial energy metabolic disorder. However, electroacupuncture pretreatment effectively increases myocardial ATP concentration and reduces the concentrations of ADP and AMP so as to improve the energy metabolism of heart to some extent.

Troponin (cTn) is a marker for the diagnosis of myocardial injury, with high stability and sensitivity. It is composed by three subunits, named cTnI, cTnT and cTnC. Among them, cTnT and cTnI are especially valuable in clinical diagnosis of myocardial infarction. In MIRI, cTnI enters blood vessel through the damaged cell membrane due to Ca^{2+} overload and other factors, resulting in the increase of cTn in plasma [24]. Hence, in this research, the change in the concentrations of cTnT and cTnI in myocardium was taken as one of the observation indicators to further determine whether electroacupuncture pretreatment works against MIRI in the aspect of serological study. The results of this research showed that the concentrations of cTnT and cTnI in the MIRI group were increased significantly as compared with the sham group and the concentrations in the EA group were reduced significantly as compared with the MIRI group. It is indicated that electroacupuncture pretreatment reduces the markers of myocardial injury and brings the effective protection in MIRI. Meanwhile, cTnI is the inhibitory subunit of ATP enzyme and its concentration affects the synthesis of ATP to some extent [26-27]. The results of this research prove that the concentration of cTnI in serum changes in the opposite trend to ATP. In MIRI, cTnI concentration is increased significantly, while ATP concentration is decreased significantly. Electroacupuncture pretreatment effectively reduces the concentration of cTnI and increases the concentration of ATP so as to bring them to the normal levels.

In summary, electroacupuncture pretreatment at “PC6”, “CV4” and “ST36” displays a certain protective effect in ischemia reperfusion injury, effectively improves heart function, decreases infarction weight and reduces the concentrations of myocardial injury markers as well as effectively adjust the transfer of ADP and AMP to ATP. Thus, it can be seen that the protective effect of electroacupuncture pretreatment in MIRI is possibly based on the improvements in mitrochondrial energy metabolism, which provides some evidence to the prevention and treatment of MIRI with electroacupuncture pretreatment in clinical practice. Meanwhile, the combination of “PC6”, “CV4” and “ST36” gives an reference to the acupoint combination in treatment of coronary angina pectoris with acupuncture-moxibustion in clinical application.

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