Electroacupuncture preconditioning alleviated myocardial injury via regulating mitochondrial function

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Chunai Wang
Gansu provincial hospital of traditional chinese medicine

Xi Liang
Gansu provincial hospital of traditional chinese medicine

Yan Yu
Gansu provincial hospital of traditional chinese medicine

Yulan Li
The first hospital of lanzhou university

Xiaohui Wen
Gansu university of chinese medicine

Min Liu
affiliated hospital of gansu university of chinese medicine

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Abstract

Background

In clinical research, integrated Chinese and Western medicine has been increasingly accepted due to the advantages. Clinical studies related to bupivacaine-induced myocardial injury are considered indispensable. Therefore, the purpose of this study is to verify whether acupuncture preconditioning could alleviate side effects of bupivacaine-induced.

Methods

Simulating bupivacaine-induced myocardial injury in clinical surgery, western blot, PCR, transmission electron microscope, Elisa methods were used to evaluate the bupivacaine-induced structure injury and dysfunction of mitochondria, and alleviating effect of lipid emulsion, acupoint injection, and electroacupuncture pre-treatment oxidase stress response.

Results

Bupivacaine caused structural damage, degradation, and swelling of mitochondria. Furthermore, it reduced adenosine triphosphate (ATP) synthesis and impaired energy metabolism in mitochondria. Structural and functional impairment of mitochondria were alleviated via lipid emulsion injection, acupoint injection, and electroacupuncture pre-treatment. Electroacupuncture pre-treatment yielded a greater alleviating effect than the other two approaches. Following electroacupuncture pre-treatment, the number of mitochondria increased, apoptosis was reduced, enzymatic activity of cytochrome C oxidase and superoxide dismutases, expression of uncoupling protein 2, Voltage-dependent anion channel 1, and Bcl 2 were upregulated and SLC25A6, MDA level was downregulated. Additionally, our findings indicated that electroacupuncture pre-treatment exerted an effect on mitochondria via the mitochondrial transcription factor A / nuclear respiratory factor 1 / proliferator-activated receptor-gamma coactivator -1 pathway.

Conclusion

Our study revealed that electroacupuncture pre-treatment could effectively alleviate bupivacaine-induced myocardial mitochondrial damage, thereby providing a theoretical basis for clinical studies and application of this treatment method.
Background
Local anaesthesia is often performed during surgical procedures to reduce the suffering of patients and improve success rates associated with surgery. However, local anaesthesia also leads to complications, resulting in inevitable adverse effects. Local anaesthesia affects the central nervous system and the cardiovascular system, and may lead to neurotoxicity [1], resulting in nerve injuries and pathological changes in peripheral nerves. Deaths from local anaesthesia are mainly attributed to adverse effects on the cardiovascular system, which include contractile dysfunction and cardiac arrhythmia [2], and are mainly characterized by haemodynamic changes [3].

Bupivacaine is an amide, which is a long-acting local anaesthetic commonly administered in various procedures, such as labour analgesia and total knee arthroplasty. It has the advantage of rapid onset of action [4]. However, it also results in significant adverse effects. Reportedly, bupivacaine induce muscle toxicity in skeletal muscles [4]. Bupivacaine-induced cardiotoxicity may result in cardiac arrhythmia, poor myocardial contractility and cardiac arrest due to circulatory collapse [5, 6]. Sztark et al., reported that bupivacaine may directly inhibit the mitochondrial respiratory chain complex I (MRCC-I), alter mitochondrial membrane structure, increase proton permeability in mitochondrial inner membranes (MIM), induce loss of mitochondrial calcium, and reduce mitochondrial membrane potential (MMP) [7], thereby affecting respiratory function and energy production by mitochondria, and eventually leading to mitochondrial dysfunction. Evidently, bupivacaine inhibits MRCC-I activity in a dose-dependent manner, which accounts for alterations of mitochondrial energy fluxes [8].

Bupivacaine negatively regulates fatty acid oxidation and oxidative phosphorylation, and inhibits the activity of mitochondrial carnitine transferases, thereby reducing the synthesis of ATP required for myocardial contractility [5]. Bupivacaine inhibits the aerobic respiration of mitochondria, wherein decoupling of oxidative phosphorylation alters the MMP and inhibits mitochondrial respiration, resulting in the production of reactive oxygen species (ROS) in mitochondria, which triggers the mitochondrial pathway of apoptosis.

Over the past three millennia, acupuncture has exhibited much potential in clinical studies conducted on various diseases in China and other Asian countries. Electroacupuncture is a combination of
acupuncture and electrical stimulation [9]. Because electroacupuncture allows more precise parameter tuning, it can be widely applied in clinical studies [10]. Previous studies have shown that electroacupuncture may reduce hippocampal neuronal apoptosis in mice with cerebral infarction by regulating the Notch3 signalling pathway [11]. Moreover, it can also inhibit apoptosis of cells in ischaemic regions by regulating p38, extracellular signal-regulated kinase (ERK1/2) and c-jun N-terminal kinase in rats with cerebral ischaemia-reperfusion injury [12]. Electroacupuncture is also an adjuvant therapy for various types of pain with fewer side effects, and glial cells may contribute to these analgesic effects [13]. The results of clinical studies suggested that electroacupuncture is both effective and safe for treating acute decompensated heart failure [14], and also effective against cognitive impairment in Alzheimer’s disease [15]. Besides, electroacupuncture can affect the secretion of proinflammatory cytokines and vascular endothelial growth factor, by regulating endocrine hormones [16].

Acupuncture-assisted anaesthesia like stimulation of ST36 was commonly used in clinical study to reduce the dose of bupivacaine avoiding the side effect [17]. Similarly, acupuncture of ST40 can exert a protective effect on heart structure and function through regulated the metabolism of lipid emulsions [18].

Neiguan point (Pericardium-6, PC6) is located on the medial surface of the forelimb between the tibia and the ulna. Researches have verified that PC6 play an vital role on cardiovascular diseases when stimulated. Reportedly, the protective effects of PC6 electroacupuncture are predominantly achieved via the regulation of various signals of mitochondrial energy metabolism [19]. In our research, we explored the effect of stimulation of PC6 on structure and function of mitochondria.

**Materials & Methods**

**Materials**

The main materials used in the study were as follows; Foetal bovine serum (FBS), bupivacaine, lipid emulsions, puerarin injections, pentobarbital sodium, RNeasy Mini Kit (QIAGEN), QuantiNova SYBR Green PCR Kit (QIAGEN), QuantiNova Reverse Transcription Kit (QIAGEN), Ant-UCP2 (GeneTex), Ant-NRF1 (GeneTex), Ant-SLC25A6 (GeneTex), Ant-mtTFA (GeneTex), Ant-VDAC1, Ant-PGC-1 (Abcam),
Ant-Bcl-2 (Abcam) and ATP assay kit (Abcam, ab83355).

**General animal and animal group treatment conditions**

Animal experimentation in this study was carried out in accordance with the requirements of Committee of the Animal Protection and Utilization Institute. This study was approved by animal experiment ethics of Gansu University of Traditional Chinese Medicine (No. 2018-018) and complied with the Declaration of Helsinki. Male specific-pathogen-free Wistar rats (8-week-old, weighing 300±10 g) were provided by Shanghai Experimental Animal Co., Ltd. (Shanghai, China). These animals were anaesthetised and placed in the supine position on the surgical platform, and three needle electrodes were placed under the skin to monitor the 12-lead electrocardiograms (ECGs) and other basic conditions, such as blood pressure and heart rate.

Those experimental rats were divided into 5 groups as follows: Control group rats intravenously infused with 3 ml of 0.9% physiological saline solution (kg/min) and sacrificed 30 min later; bupivacaine group rats intravenously infused with physiological saline solution for 30 min and infused with 0.5% bupivacaine to induce cardiac arrhythmia or death (standard criteria for cardiac arrhythmia); lipid emulsion group rats subject to continuous intravenous infusion with 3 ml of 20% lipid emulsions for 30 min, followed by infusion with bupivacaine to induce cardiac arrhythmia or death; puerarin group rats infused with bupivacaine after being injected with 0.1 ml of puerarin at PC6 of both forelimbs; and electroacupuncture group rats infused with bupivacaine after 30 min of electroacupuncture stimulation (Longitudinal wave: 2/10 Hz; current intensity: 2 mA; pulse width: 0.2 ms) at PC6 of both forelimbs. The rate and duration of infusion, as well as lipid emulsion administration were performed according to a study reported by Weinberg [20]. Cardiac arrhythmia was assessed by an electrocardiographer based on premature ventricular contractions (PVC) or ventricular tachycardia (VT), with the duration of QRS complex prolonged to 90 ms. At the conclusion of the experiment, the rats were euthanasia and subjected to retrograde perfusion, followed by immediate resection of their hearts for subsequent analyses.

**Isolation of mitochondria**

Myocardial mitochondria were isolated according to a previously published method with some
modifications [21]. The rats were euthanised with pentobarbital sodium, their hearts harvested, excised into small pieces and homogenised, followed by the removal of cell debris. The supernatant was centrifuged at 13,000 g for 10 min to isolate the mitochondria.

**Mitochondrial membrane potentials assay**

Mitochondrial membrane potential were measured with JC-1 kit (Beyotime, C2006) according the manufacturers’ instructions. Namely, the 1x JC-1 working solution was added into the purified mitochondria with an appropriated proportion. The results were analysed using fluorescence spectrophotometer. The excitation wavelength was 458nm and the emission wavelength was 590nm.

**Determination of calcium ion levels in mitochondria**

Calcium ion concentration of mitochondrial was measured using Fluo-3 AM according the previous study[5]. Fluo-3 AM was added in resuspended Mitochondria and incubated for 1h. Then, the Fluo-3 AM was removed. Fluorescence intensity was analyzed by flow cytometry after the mitochondria were subjected to Triton X100 and calcium chloride and EDTA, respectively.

**Reactive oxygen species measurement**

Cardiomyocytes was isolated from the SD rats. ROS levels were evaluated using DCFH-DA. The cells were incubated with DCFH-DA. After incubated for 30min, cells were subjected to washed and resuspended. Subsequently, the results were analyzed using microplate reader at an excitation wavelength of 488 nm and at an emission wavelength of 525 nm.

**ATP content**

Myocardial ATP levels were measured using a commercial assay kit (Beyotime). Briefly, tissues and cells were lysed and centrifuged at 12,000 g and 4°C for 5 min. The resulting supernatant was harvested for subsequent assays. Mitochondrial ATP content was analyzed by colorimetry using phosphomolybdic acid.

**Enzyme linked immunosorbent assay (ELISA)-**

After being homogenised in phosphate-buffered saline, tissue samples were centrifuged to obtain the supernatant for subsequent measurements. All experimental procedures were carried out in accordance with manufacturers’ instructions provided with the commercial kits. Standard samples
and samples to be tested were placed in wells intended for blank, standard, and samples to be tested, respectively. After being incubated at 37°C for 30 min, each well was washed and incubated with the enzyme-labelling reagent, following which, the wells were washed again and subjected to colour development. After the reaction was terminated, the absorbance value of each well was measured to estimate sample concentration. The experiment was repeated three times.

**qPCR**

Cardiac muscle tissues were rapidly harvested and immersed in liquid nitrogen to extract RNA for subsequent analyses. Experimental procedures were carried out in accordance with instructions provided with the commercial kits. The purity and quality of the resulting RNA samples were determined in addition to the removal of DNA, followed by a reverse transcription PCR (RT-PCR) assay. Primer sequences for target genes are listed (Table 1).

**Western blot**

After the tissue samples were homogenised, the supernatant was obtained for the purpose of isolating total proteins using a protein extraction kit. After measuring the protein concentration, total proteins were loaded in equal amounts and separated via sodium dodecylsulphate polyacrylamide gel electrophoresis. Subsequently, the proteins were transferred onto a polyvinylidene fluoride membrane, which was then and incubated with special primary antibodies, followed with secondary antibodies 1 h at 37°C. Western blot images were obtained via the enhanced chemiluminescence method. Grayscale analysis was performed on target protein bands using Image J software, and the results were analysed statistically.

**Transmission electron microscopy**

Myocardial tissues were sequentially fixed with 2.5% glutaraldehyde and 1% citric acid, followed by dehydration in an acetone gradient and resin embedding. Then the embedded tissues were dried rapidly prior to sectioning. Next, tissue sections were stained and imaged under an electron microscope. Mitochondrial injuries were assessed using Flameng score [22], whereby 5 microscopic fields were randomly selected to obtain the mean Flameng score for each group. The mitochondria were graded according to the following criteria: Grade 0 (score 0) - mitochondria with normal
ultramicrostructure and intact granules; Grade I (score 1) - mitochondria with basically normal ultramicrostructure and partial loss of granules; Grade II (score 2) - swollen mitochondria with transparent matrix; Grade III (score 3) - mitochondria with transparent matrix and fragmented cristae or formation of flocculent densities in the mitochondrial matrix; and Grade IV (score 4) - mitochondria lacking matrix with fragmented cristae and disrupted outer membrane.

Statistical analysis
Statistical analyses in this study were performed using SPSS 20.0 software, and the data were expressed as means of triplicate experiments. Multiple comparisons between groups were carried out using one-way analysis of variance. P-values of <0.05 were considered to indicate a statistically significant result.

Results
Protective effect of different treatments on bupivacaine-induced myocardial injury
Previous studies have demonstrated that electroacupuncture pre-treatment exerts an alleviating effect on bupivacaine-induced toxicity. As shown in Fig. 1, significant differences in the lethal dose of bupivacaine were observed between different groups, among which, the electroacupuncture pre-treatment of PC6 corresponded to the highest lethal dose of bupivacaine. This suggested that electroacupuncture had the greatest alleviating effect against myocardial toxicity. Rats injected at PC6 with puerarin corresponded to the second highest lethal dose of bupivacaine, while intravenous injection with lipid emulsions yielded a relatively poor alleviating effect, but still outperformed the bupivacaine group.

The effect of electroacupuncture pre-treatment on mitochondrial structure
Structural and functional damage to mitochondria is the main mechanism underlying myocardial injury. Our results revealed that the control group rats exhibited densely-packed myocardial fibres with clearly visible sarcomeres and abundant mitochondria. Mitochondrial structure was intact with a typical oblong shape and continuous cristae, as well as intact membranes and granules (Fig 2A). Rats of the bupivacaine group displayed mitochondrial degradation with swollen, fragmented and even missing cristae. The lipid emulsion group, the puerarin group, and the electroacupuncture pre-
treatment group increasingly alleviated mitochondrial injuries, in ascending order with a gradual increase in mitochondrial density. Flameng scores showed that bupivacaine was associated with the least damage to mitochondria in electroacupuncture pre-treatment group rats. Besides, there were significant differences in the Flameng scores between different groups. Mitochondrial abundance varied among different treatment groups (Fig. 2B). All treatment groups showed lower mitochondrial abundance compared with that of the control group, and the bupivacaine group rats had the lowest mitochondrial abundance, amounting to half of that of the control group. The lipid emulsion group, puerarin group and electroacupuncture pre-treatment group showed gradually increasing mitochondrial abundances, and the ATP synthesis ability was enhanced along with the changes of mitochondrial abundances suggesting mitochondrial function was recovered gradually (Fig 2C).

Functional changes in mitochondria

The result shown in Fig. 3A, the activity of Monoamine oxidases (MAO) was upregulated by bupivacaine, while the Cytochrome C oxidase (COX) activity was downregulated, mitochondrial membrane potential was declined, indicating that bupivacaine induct mitochondrial autophagy and dysfunction (Fig. 3A, 3B). The therapy of lipid emulsion, puerarin and electroacupuncture pre-treatment promoted the expression of Bcl2, UCP2, VDAC1, decreased the expression of ANT1, SLC25A6. After the treatments, the mitochondrial dysfunction was relieved and apoptosis reduced (Fig. 3C).

Bupivacaine induced the oxidative stress response

The dysfunction of mitochondria is closely related to the level of ROS. In results of ROS levels, we found that ROS production was induced by bupivacaine. The treatment of three methods increased the activity of SOD, decreased the content of MDA, playing a key role of mitochondrial protection (Fig. 4).

Effects of bupivacaine on myocardial mitochondrial biogenesis

Studies have revealed that increased intracellular Ca^{2+} levels, which significantly upregulate the expression of PGC-1, NRF-1 and mtTFA, is one of the signals mediating mitochondrial biogenesis [23],
which is consistent with the findings of our studies. Our results indicated the concentration of Ca\(^{2+}\) levels was the lowest in bupivacaine group. However lipid emulsion group, puerarin group, and electroacupuncture pre-treatment group showed higher expression of PGC-1, mtTFA and NRF than bupivacaine-induced group with statistically significant differences between groups (Fig. 5).

**Discussion**

Numerous studies have confirmed that acupuncture can prevent myocardial injury. Patients who underwent cardiac surgery showed significantly improved heart rates, blood pressure, as well as accelerated rates of recovery after receiving electroacupuncture stimulation[24]. Huang et al., found that electroacupuncture pre-treatment improved the survival rate of rats with myocardial ischaemia-reperfusion injury with decreased serum concentrations of lactate dehydrogenase and creatine kinase, as well as reduced plasma troponin levels [25]. Our findings indicated that the expression of Bcl-2 was increased, ROS production was significantly reduced, following electroacupuncture pre-treatment. Electroacupuncture pre-treatment inhibits apoptosis via the mitochondria-dependent pathway [26]. The acupuncture of PC6 can alleviate cardiac injury by reducing cardiac arrhythmia, apoptosis, and myocardial enzymes. Besides, electroacupuncture can effectively promote angiogenesis and protect myocardial tissue from damage [27].

Mitochondria, which are a central source of metabolism and energy production, play an important role in cellular energy metabolism. Mitochondria-targeting treatment, considered to be an appealing strategy for the control and management of mitochondrial injury [28], has become the focus of many important research studies [29]. Previously-published data suggest that injection of isolated viable respiration-competent myocardial mitochondria into ischaemic regions prior to reperfusion may reverse post-ischaemic functional deterioration and apoptosis, thereby limiting the infarct area [30]. Rahman et al., demonstrated that glycogen synthase kinase 3 phosphorylation-mediated PI3K/Akt extracellular signal-regulated kinase (ERK) pathway may be the mechanism underlying myocardial protection by lipid emulsions [31].

Mitochondrial injury may lead to cardiotoxicity [32]. Uncoupling of mitochondrial oxidative phosphorylation and/or inhibition of the electron transport chain leads to metabolic dysregulation in
mitochondria. The cumulative release of ROS, a decline in ATP synthesis, and leakage of mitochondrial Ca\(^{2+}\) may result in inflammatory responses, mitochondrial injury, which aggravates apoptosis and the death of cells surrounding the injured areas. In this research, electroacupuncture treatment alleviated the mitochondrial injury induced by bupivacaine, and promoted the function recovery of mitochondria. The concentration increasing of Ca\(^{2+}\) promoted the mitochondrial biogenesis.

Conclusions
Electroacupuncture pre-treatment of PC6 improved the mitochondrial damage caused by myocardial toxicity, enhanced the mitochondrial membrane potential, and altered the concentration of Ca\(^{2+}\), all of which may contribute to the prevention of bupivacaine-induced cardiac arrhythmia and the improvement of myocardial metabolism.

Declarations

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Authors’ contribution
Conceptualization: Chun-ai Wang. Data curation: Xi Liang. Formal analysis: Yan Yu. Funding acquisition: Chun-ai Wang. Investigation: Yu-lan Lee. Methodology: Min Liu. Project administration: Chun-ai Wang. Resources: Chun-ai Wang. Software: Xiao-hui Wen. Validation: Chun-ai Wang. Writing—original draft: Chun-ai Wang. Writing—review & editing: Chun-ai Wang. Approval of final manuscript: all authors

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Availability of data and material
The data is available from the corresponding author on reasonable request.

Ethic approval and consent to participate
This study was approved by Committee of the Animal Protection and Utilization Institute. Animal experimentation in this study was carried out in accordance with the requirements of Committee of
the Animal Protection and Utilization Institute. This study was approved by animal experiment ethics of Gansu University of Traditional Chinese Medicine. The carcasses were delivered to the animal center of Gansu Traditional Chinese Medicine for innocuous treatment at the hazardous waste disposal center of Gansu province (No. 2018-018).

Consent for publication

Not applicable.

Competing interest

The authors have no potential conflicts of interest to disclose.

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Table

| Genes  | Forward                  | Reverse                  |
|--------|--------------------------|--------------------------|
| GAPDH  | AATGGTGAAGGTCGGTGTGAAC   | AGGTCAATGAAGGGGTCGTTG   |
| Bcl-2  | ATGATAACCGGAGATCGTG      | GACGGTAGCGAGAGAGAAG      |
| VDAC1  | CACCAAGTGAAAGGCGAGTC    | TGCTCCCTCTTGACCCCTGT    |
| UCP2   | GCAACACCTCATGACAGACGA    | AGGAAGGCTGAACCCCTTTG    |
| PGC-1α | CATGTGAGCCCAAGACTCTG     | GTGAGGACCCTAGCAAGTT     |
| NRF1   | GCTATGGGCCAGATATGGAGT    | CGTAAGCTGCTGCTGTTGT     |
| mTFA   | GGAATCAAGAGCTGTGCCTGC   | AGAAACTGCAATGGCTCTGC    |
| ANT1   | GCTAACCACACCACCTGCTCCT  | ATGCCACCGCTAACAGACAT    |

Figures

A

![Diagram showing lethal doses of bupivacaine](image)

B

![Diagram showing arrhythmia and lethal effects](image)

Figure 1

Protective effect of electroacupuncture pre-treatment on myocardial mitochondria (A) Lethal doses of bupivacaine in different experimental groups. (B) Effect of bupivacaine on heart rate.
Figure 2

Effects of electroacupuncture pre-treatment on mitochondrial structure (A)
Ultramicrostructure and damage score of mitochondria. (B) Mitochondrial concentration. (C)
The content of ATP
Figure 3

Effects of electroacupuncture pre-treatment on mitochondrial functions (A) Bupivacaine declined mitochondrial membrane potential. (B) The content of COX and MAO. (C) The expression of Bcl2, UCP2, VDAC1, SLC25A6 and ANT1.

Figure 4

Changes of oxidase stress response of mitochondria. (A) The level of ROS. (B) The activity of SOD and level of MDA.
Figure 5

Effect of different treatment on myocardial mitochondrial biogenesis (A) Electroacupuncture pre-treatment increased the concentration of Ca2+. (B) mRNA expression of PGC, mTFA, NRF. (C) Western blot for PGC, mTFA, NRF