Electroacupuncture preconditioning alleviated myocardial injury via regulation mitochondrial function

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Abstract

Background. Electroacupuncture is well known for its advantageous neuroanalgesic and therapeutic effects on myocardial ischemia-reperfusion injury. The purpose of the present research was to verify whether electroacupuncture can alleviate bupivacaine-induced myocardial injury.

Methods. Specific-pathogen-free Wistar rats were used to establish the bupivacaine-induced myocardial injury model. Western blot, PCR, transmission electron microscope and enzyme-linked immunosorbent (ELISA) methods were used to evaluate bupivacaine-induced structure injury and dysfunction of the mitochondria as well as the alleviating effects of lipid emulsion, acupoint injection, and electroacupuncture pre-treatment of the 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 the mitochondria. Structural and functional impairment of the mitochondria was 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 (COX) and superoxide dismutase and expression of uncoupling protein 2, voltage-dependent anion channel 1, and Bcl 2 were upregulated and SLC25A6, MDA levels were downregulated. Additionally, our findings indicated that electroacupuncture pre-treatment exerted an effect on the mitochondria via the mitochondrial-transcription-factor-A / nuclear-respiratory-factor-1 / proliferator-activated-receptor-gamma-coactivator -1 pathway.

Conclusion The present study revealed that electroacupuncture pre-treatment could effectively alleviate bupivacaine-induced myocardial mitochondrial damage, thereby providing a theoretical basis for clinical studies and applications of this treatment method.

Background

Local anesthesia is often performed during surgical procedures to reduce the suffering of patients and improve surgery success rates [1]. However, local anesthesia can also lead to complications, resulting in inevitable adverse effects. Local anesthesia affects the central nervous system and the cardiovascular system and may therefore lead to neurotoxicity [2], resulting in nerve injuries and pathological changes in the peripheral nerves. Deaths from local anesthesia are mainly attributed to adverse effects on the cardiovascular system, which include contractile dysfunction and cardiac arrhythmia [3], and are mainly characterized by hemodynamic changes [4].

Bupivacaine is an amide, which is a long-acting local anesthetic commonly administered in various procedures, such as labor analgesia and total knee arthroplasty. Bupivacaine has the advantage of rapid onset of action and is relatively safe at the recommended dosage [5]. However, overdose or accidental intravasal application of bupivacaine can result in significant adverse effects. For every 1,000 peripheral

nerve blocks, 0.04 to 1.8 cases of myocardial toxicity are likely to occur, leading to severe symptoms [6]. Bupivacaine reportedly induces muscle toxicity in skeletal muscles [5]. Bupivacaine-induced cardiotoxicity may result in cardiac arrhythmia, poor myocardial contractility and cardiac arrest due to circulatory collapse [7, 8]. Sztark et al. reported that bupivacaine may directly inhibit mitochondrial respiratory chain complex I (MRCC-I), alter mitochondrial membrane structure, increase proton permeability in the mitochondrial inner membranes (MIMs), induce loss of mitochondrial calcium, and reduce mitochondrial membrane potential (MMP) [9], thereby affecting the respiratory function and energy production of the mitochondria and eventually leading to mitochondrial dysfunction.

Evidently, bupivacaine inhibits MRCC-I activity in a dose-dependent manner, accounting for alterations in mitochondrial energy fluxes [10]. Bupivacaine negatively regulates fatty acid oxidation and oxidative phosphorylation and inhibits the activity of mitochondrial carnitine transferases, thereby reducing the synthesis of adenosine triphosphate (ATP) required for myocardial contractility [7]. Bupivacaine also inhibits the aerobic respiration of the mitochondria; specifically, decoupling of oxidative phosphorylation alters MMP and inhibits mitochondrial respiration, resulting in the production of reactive oxygen species (ROS) in the mitochondria and triggering the mitochondrial pathway of apoptosis[11]. Lipid emulsion is widely used to alleviate bupivacaine-induced myocardial toxicity because this technique can enhance mitochondrial metabolism and improve myocardial contractility [12, 13]. Similarly, puerarin isolated from the traditional Chinese medicine radix puerariae has demonstrated a protective effect on myocardial infarction by targeting the mitochondria [14].

Over the past three millennia, acupuncture has exhibited significant potential in clinical studies of various diseases in China and other Asian countries. Electroacupuncture (EA) is a combination of acupuncture and electrical stimulation [15]. Because EA allows for more precise parameter tuning, it has been widely applied in clinical studies [16]. Previous studies have shown that EA can regulate mitochondria autophagy, inhibit NLRP3 inflammasome activation, and alleviate myocardial injury [17, 18]. EA can also serve as an adjuvant therapy for various types of pain with fewer side effects; glial cells may contribute to these analgesic effects [19]. Besides, EA can also affect the secretion of proinflammatory cytokines and vascular endothelial growth factor by regulating endocrine hormones [20].

Acupuncture-assisted anesthesia, such as ST36 stimulation is commonly used in clinical studies to reduce the required dose of bupivacaine and thus avoid its the side effects [21]. Similarly, acupuncture of ST40 can exert a protective effect on heart structure and function by regulating the metabolism of lipid emulsions [22]. The Neiguan point (Pericardium-6, PC6) is located on the medial surface of the forelimb between the tibia and the ulna. Researchers have verified that PC6 plays a vital role in cardiovascular diseases when stimulated. Reportedly, the protective effects of PC6 EA are predominantly achieved via the regulation of various signals of mitochondrial energy metabolism [23]. In present study, we explored the effects of PC6 stimulation on structure and function of mitochondria.

Materials & Methods

Materials

The primary materials used in the present study were as follows: fetal bovine serum (FBS), bupivacaine, lipid emulsions, puerarin injections, pentobarbital sodium, the RNeasy Mini Kit (QIAGEN), the QuantiNova SYBR Green PCR Kit (QIAGEN), the QuantiNova Reverse Transcription Kit (QIAGEN), Anti-UCP2 (GeneTex), Anti-NRF1 (GeneTex), Anti-SLC25A6 (GeneTex), Anti-mtTFA (GeneTex), Anti-VDAC1, Anti-PGC-1 (Abcam), Anti-Bcl-2 (Abcam) and and ATP assay kit (Abcam, ab83355). JC-1 kit (Beyotime, C2006), Fluo-3 AM (Abcam), DCFH-DA (Solarbio), Rat COX ELISA kit (CUSABIO), MDA ELISA Kit (Elabscience).

General animal and animal group treatment conditions

Animal experimentation was carried out in the present study 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). Each of these animals was anaesthetized and placed in the supine position on a surgical platform, and three needle electrodes were placed under the skin to produce a 12-lead electrocardiogram (ECG) and monitor other basic conditions, such as blood pressure and heart rate.

The experimental rats were divided into five groups as follows: control group (n=8) rats intravenously infused with 3 ml of 0.9% physiological saline solution (kg/min) and sacrificed after 30 min; bupivacaine group (n=16) rats intravenously infused with physiological saline solution for 30 min and then infused with 0.5% bupivacaine to induce cardiac arrhythmia or death (standard criteria for cardiac arrhythmia); lipid emulsion group (n=16) rats subject to continuous intravenous infusion with 3 ml of 20% lipid emulsion for 30 min, followed by infusion with bupivacaine to induce cardiac arrhythmia or death; puerarin group (n=16) rats infused with bupivacaine after being injected with 0.1 ml of puerarin at PC6 on both forelimbs; and EA group (n=16) rats infused with bupivacaine after 30 min of EA stimulation (longitudinal wave: 2/10 Hz; current intensity: 2 mA; pulse width: 0.2 ms) at PC6 on both forelimbs. The rate and duration of infusion and lipid emulsion administration were determined according to a study reported by Weinberg [24]. Cardiac arrhythmia was assessed by an electrocardiographer on the basis of premature ventricular contractions (PVC) or ventricular tachycardia (VT) with the duration of the QRS complex prolonged. At the conclusion of the experiment, the rats were euthanized and subjected to retrograde perfusion followed by immediate resection of their hearts for subsequent analyses.

Isolation of mitochondria

Myocardial mitochondria were isolated using a previously published method with some modifications [25]. The rats were euthanized with pentobarbital sodium, and their hearts were harvested, excised into small pieces, and homogenized. This process was followed by the removal of cell debris. Then, the supernatant was centrifuged at 13,000 g for 10 min to isolate the mitochondria.

Mitochondrial membrane potentials (MMP) assay

MMP was measured with a JC-1 kit (Beyotime, C2006) according to the manufacturers' instructions. The 1x JC-1 working solution was added to the purified mitochondria with an appropriate proportion and the results were analyzed using a fluorescence spectrophotometer. The excitation wavelength was 458nm, and the emission wavelength was 590nm.

Determination of calcium ion levels in mitochondria

Mitochondrial calcium ion concentrations were measured using Fluo-3 AM in accordance with previous study[7]. Fluo-3 AM was added to 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, calcium chloride and EDTA, respectively.

Reactive oxygen species measurement

Cardiomyocytes were isolated from the SD rats, and their ROS levels were evaluated by incubating the cells in DCFH-DA. After 30min incubation, the cells were subjected to washed, and resuspended. Subsequently, the results were analyzed using a microplate reader at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

ATP content

Myocardial ATP levels were measured using a commercial assay kit. Briefly, tissues 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 homogenized in phosphate-buffered saline, the tissue samples were centrifuged to obtain the supernatant for subsequent measurements. All experimental procedures were carried out in accordance with the manufacturers' instructions provided with the commercial kits. Standard samples and test samples were placed in wells intended for blank, standard, and test samples, respectively. After being incubated at 37°C for 30 min, each well was washed and incubated with the enzyme-labelling reagent. Then, the wells were washed again and subjected to color development. After the reaction was terminated, the absorbance value of each well was measured to estimate the sample concentration. This 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 the instructions provided with the commercial kits. The purity and quality of the resulting RNA samples were determined

alongside the removal of DNA, followed by a reverse transcription PCR (RT-PCR) assay. Primer sequences for the target genes are listed in **Table 1**.

Western blot

After the tissue samples were homogenized, the supernatant was obtained for the purpose of isolating total proteins using a protein extraction kit. After the protein concentration was measured, the 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 incubated with special primary antibodies followed by secondary antibodies for 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 analyzed statistically.

Transmission electron microscopy

Myocardial tissues were sequentially fixed with 2.5% glutaraldehyde and 1% citric acid then dehydrated in an acetone gradient and embedded in resin. 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 the Flameng score [26]. Five microscopic fields were randomly selected to obtain the mean Flameng score for each group and 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 matrices; Grade III (score 3), mitochondria with transparent matrices and fragmented cristae or formation of flocculent densities in their mitochondrial matrices; and Grade IV (score 4), mitochondria lacking matrix with fragmented cristae and disrupted outer membranes.

Statistical analysis

The statistical analyses of the present study were performed using SPSS 20.0 software, and the data were expressed as means of the scores yielded by triplicate experiments. Multiple comparisons between groups were carried out using one-way analysis of variance (ANOVA). P-values <0.05 were considered to indicate statistically significant results.

Results

Protective effect of different treatments on bupivacaine-induced myocardial injury

Previous studies have demonstrated that EA pre-treatment exerts an alleviating effect on bupivacaine-induced toxicity [8]. As shown in **Fig. 1A**, significant differences in the lethal dose of bupivacaine were observed between different groups, among which the EA pre-treatment of PC6 corresponded to the highest lethal dose of bupivacaine. This finding suggested that EA had the greatest alleviating effect

against myocardial toxicity. Rats injected with puerarin at PC6 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 results showed that bupivacaine significantly prolonged the duration of the QRS complex. The duration of QRS complex was 26ms, 32ms, 260ms in control, arrhythmia, and lethal groups respectively. The difference between the control and arrhythmia groups was significant ($P<0.05$). The difference between the QRS complex durations of the lethal and control groups was extremely significant ($p<0.01$; **Fig. 1B**).

The effect of EA pre-treatment on mitochondrial structure

Structural and functional damage to the mitochondria is the main mechanism underlying myocardial injury. The control group rats in the present study exhibited densely-packed myocardial fibers with clearly visible sarcomeres and abundant mitochondria. Their mitochondrial structure was intact with a typical oblong shape and continuous cristae, as well as intact membranes and granules (**Fig 2A**). The rats in the bupivacaine group displayed mitochondrial degradation with swollen, fragmented, and even missing cristae. The lipid emulsion group, the puerarin group, and the EA pre-treatment group exhibited increasingly alleviated mitochondrial injuries, in ascending order, along with a gradual increase in mitochondrial density. The Flameng scores showed that bupivacaine was associated with the least mitochondrial damage in the EA pre-treatment group rats. In addition, there were significant differences in the Flameng scores between different groups ($P<0.01$). Mitochondrial abundance also varied between the different treatment groups (**Fig. 2B**). All the treatment groups showed lower mitochondrial abundance than the control group, and the bupivacaine group exhibited the lowest mitochondrial abundance, amounting to half of that of the control group. The lipid emulsion group, the puerarin group, and EA pre-treatment group exhibited gradually increasing mitochondrial abundances, and the ATP synthesis abilities were enhanced along with these changes in mitochondrial abundance, suggesting that their mitochondrial function was gradually recovered (**Fig 2C**).

Functional changes in mitochondria

As shown in **Fig. 3A**, bupivacaine upregulated monoamine oxidases (MAO) activity, downregulated COX activity, and reduced MMP. This finding indicated that bupivacaine induces mitochondrial autophagy and dysfunction (**Fig. 3A, 3B**). Therapy involving lipid emulsion, puerarin and EA pre-treatment promoted the expression of Bcl2, UCP2, and VDAC1 and decreased the expression of ANT1 and SLC25A6. After these treatments, mitochondrial dysfunction was relieved and apoptosis was reduced (**Fig. 3C**).

Bupivacaine induced the oxidative stress response

Dysfunction of the mitochondria is closely related to ROS levels. The present results indicated that ROS production was induced by bupivacaine. All three methods of treatment increased SOD activity and decreased MDA content , playing a key role in mitochondrial protection (**Fig. 4**).

Effects of bupivacaine on myocardial mitochondrial biogenesis

Mitochondrial calcium uptake significantly improved depleted cells and regulated cytosolic calcium signal [27, 28]. Our results indicated that increased Ca^{2+} levels of mitochondrial significantly upregulate the expression of PGC-1, NRF-1, and mtTFA, is one of the signals mediating mitochondrial biogenesis. The concentrations of Ca^{2+} were lowest in bupivacaine group. However, the lipid emulsion, puerarin, and EA pre-treatment groups exhibited significantly higher expression of PGC-1, mtTFA, and NRF than the bupivacaine-induced group (**Fig. 5**).

Discussion

Numerous studies have confirmed that acupuncture can prevent myocardial injury. Patients who have undergone cardiac surgery have shown significantly improved heart rates, blood pressure, and rates of recovery after receiving EA stimulation[29]. Recently, researchers found that EA pre-treatment improved the survival rate of rats with myocardial ischemia-reperfusion injury with reduced apoptosis and expressions of Cyt c and cleaved caspase 3[30, 31]. EA regulated the activation of NLRP3, polarization of macrophages, declined arrhythmia scores, improving cardiac function [18, 31]. EA played a role of anti-arrhythmia through reducing ventricular tachycardia and ventricular, and related to concentration of calcium in the cytoplasm [32, 33]. EA pre-treatment inhibits apoptosis via the mitochondria-dependent pathway [34]. In addition, EA can effectively promote angiogenesis and protect myocardial tissue from damage [35].

The mitochondria, which are comprise a central source of metabolism and energy production, play an important role in cellular energy metabolism. Mitochondrial injury may lead to cardiotoxicity [36]. The uncoupling of mitochondrial oxidative phosphorylation and/or inhibition of the electron transport chain leads to metabolic dysregulation in mitochondria. The cumulative release of ROS, decline in ATP synthesis, and leakage of mitochondrial Ca^{2+} may result in an inflammatory respond, mitochondrial injury, which may aggravate apoptosis and lead to the death of cells surrounding the injured areas. Mitochondria-targeting treatment, considered to be an appealing strategy for the control and management of mitochondrial injury [37], has become the focus of many important research studies [38]. Previously-published data have suggested that the injection of isolated viable respiration-competent myocardial mitochondria into ischemic regions prior to reperfusion may reverse post-ischemic functional deterioration and apoptosis, thereby limiting the infarct area [39].

The present findings indicated that Bcl-2 expression increased and ROS production was significantly reduced following EA pre-treatment. Similarly, calcium ions concentration of mitochondria was downregulated by bupivacaine. This is consistent with previous research[7]. We thought the leakage of mitochondrial calcium ions aggravated the overloaded of cytoplasmic calcium ions in arrhythmias. While, EA promoted uptake of mitochondrial calcium ions and reduced calcium overload in the cytoplasm. Further, the increased concentration of mitochondrial calcium promoted mitochondria biogenesis, achieving the therapeutic effect. Moreover, this conjecture needs further verification.

Conclusions

EA pre-treatment of PC6 alleviated the mitochondrial damage caused by myocardial toxicity, enhanced the MMP, and altered concentration of Ca^{2+}. All of these effects 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.

Abbreviations

ATP	adenosine triphosphate
UCP2	uncoupling protein 2
VDAC1	Voltage-dependent anion channel 1
SOD	superoxide dismutases
COX	cytochrome C oxidase
MDA	malondialdehyde
mtTFA	mitochondrial transcription factor A
NRF 1	nuclear respiratory factor 1
PGC-1	proliferator-activated receptor-gamma coactivator -1
SLC25A6	ADP/ATP translocase 3, solute carrier family 25, member 6
MRCC-I	mitochondrial respiratory chain complex I
MIM	mitochondrial inner membranes
MMP	mitochondrial membrane potential
ROS	reactive oxygen species
ERK	extracellular signal-regulated kinase
PVC	premature ventricular contractions
VT	ventricular tachycardia
ELISA	Enzyme linked immunosorbent assay
MAO	Monoamine oxidases
ANT 1	adenine nucleotide translocator 1

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Table

Table 1 primer sequences

Genes	Forward	Reverse
GAPDH	AATGGTGAAGGTCGGTGTGAAC	AGGTCAATGAAGGGGTCGTTG
Bcl-2	ATGATAACCGGAGATCGTG	GACGGTAGCGACGAGAGAAG
VDAC1	CACCAAAGTGAACGGCAGTC	TGCTCCCTCTTGTACCCTGT
UCP2	GCCAACCTCATGACAGACGA	AGGAAGGCATGAACCCCTTG
PGC-1α	CATGTGCAGCCAAGACTCTG	GTGAGGACCGCTAGCAAGTT
NRF1	GCTAATGGCCCAGATATGGAGT	CGTAAGCTCTGCCTGGTTGT
mtTFA	GGAATCAAGACTGTGCGTGC	AGAAACTGCAATGGCTCTGC
ANT1	GCTAACCAACCCACTGTCCT	ATGCCACCGCTAACAAGACAT

Figures

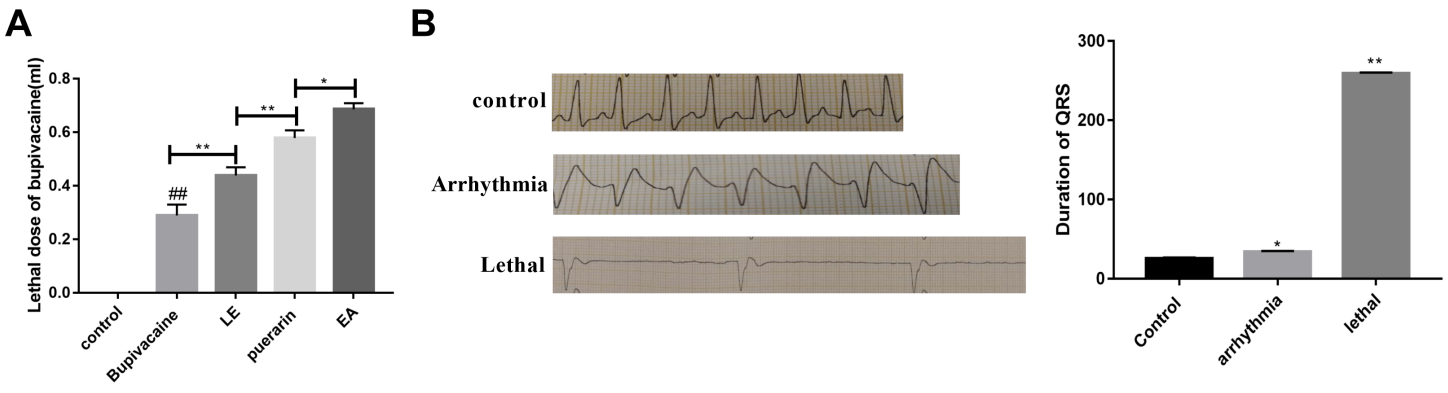

Figure 1

Protective effect of EA pre-treatment on myocardial mitochondria (A) Lethal doses of bupivacaine in different experimental groups. (B) Changes of QRS duration.

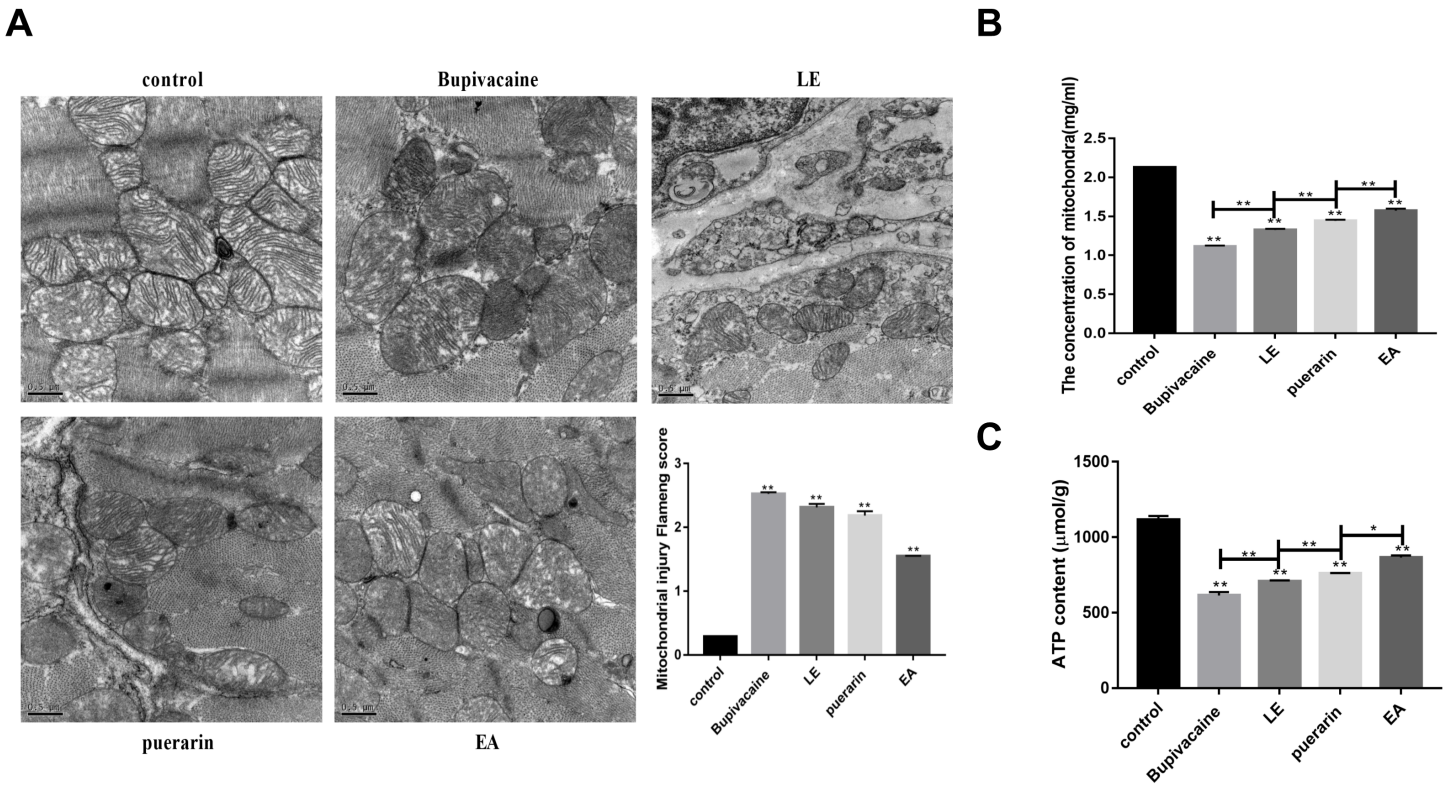

Figure 2

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

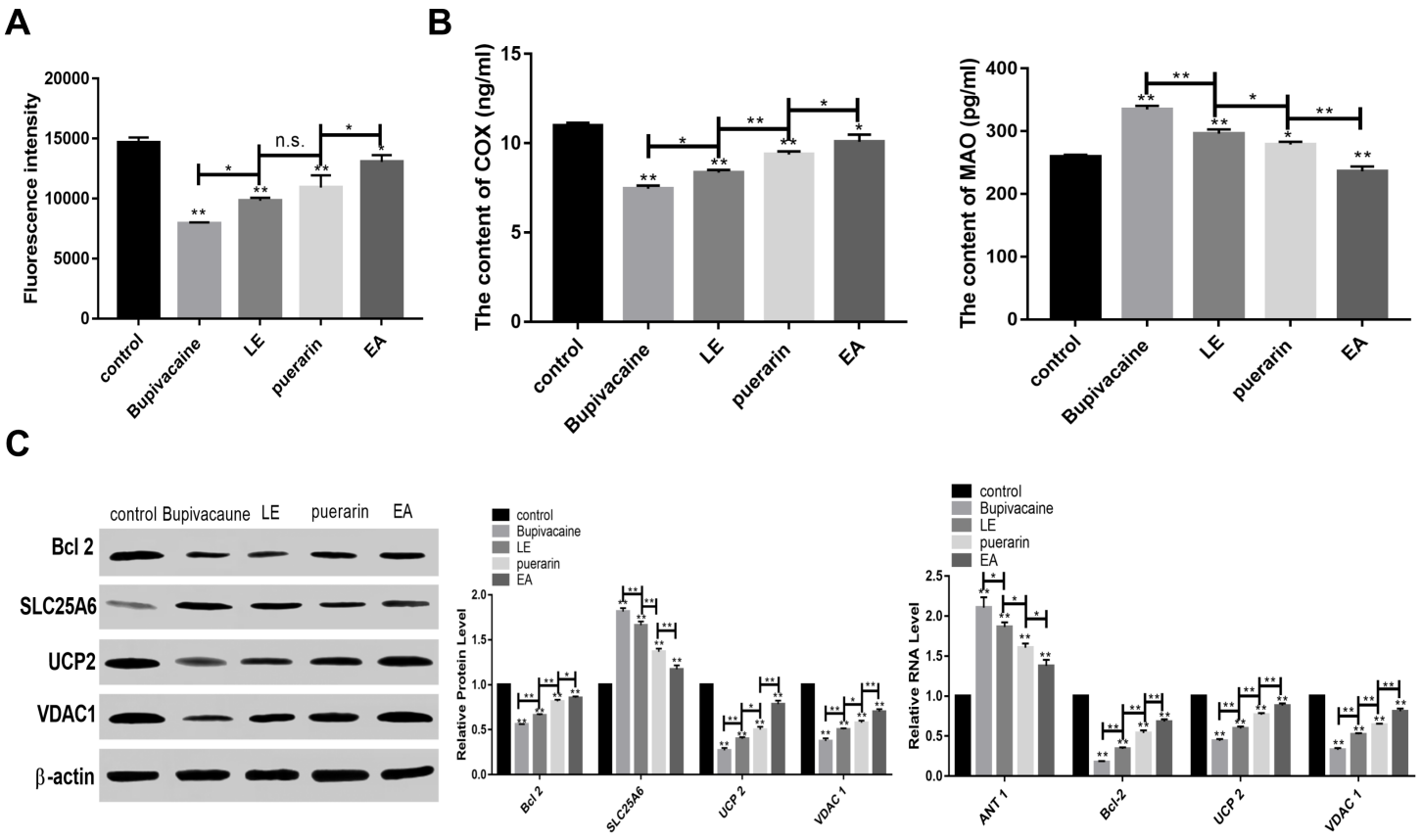

Figure 3

Effects of EA 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.

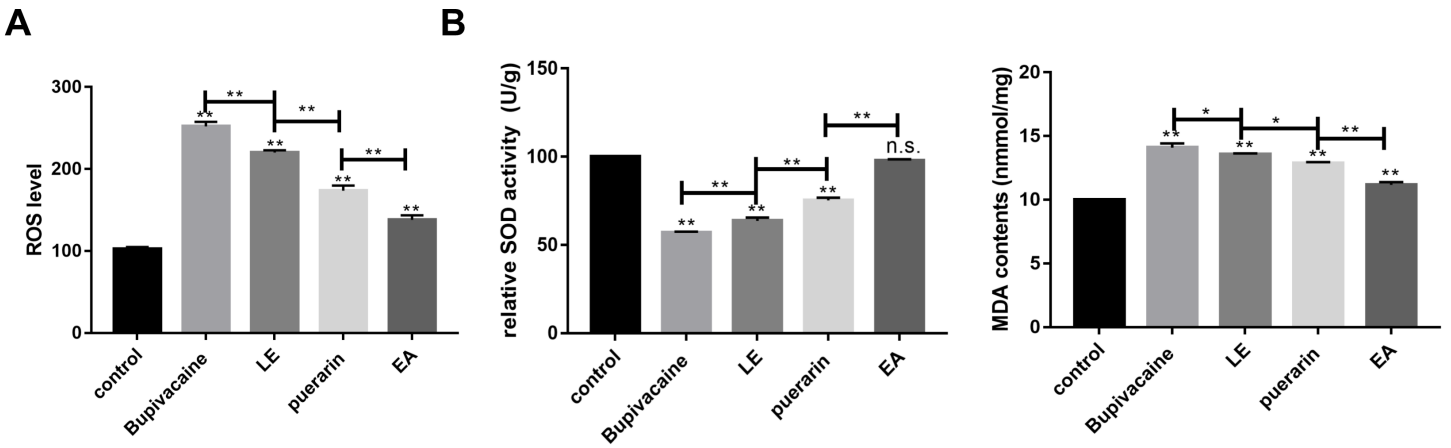

Figure 4

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

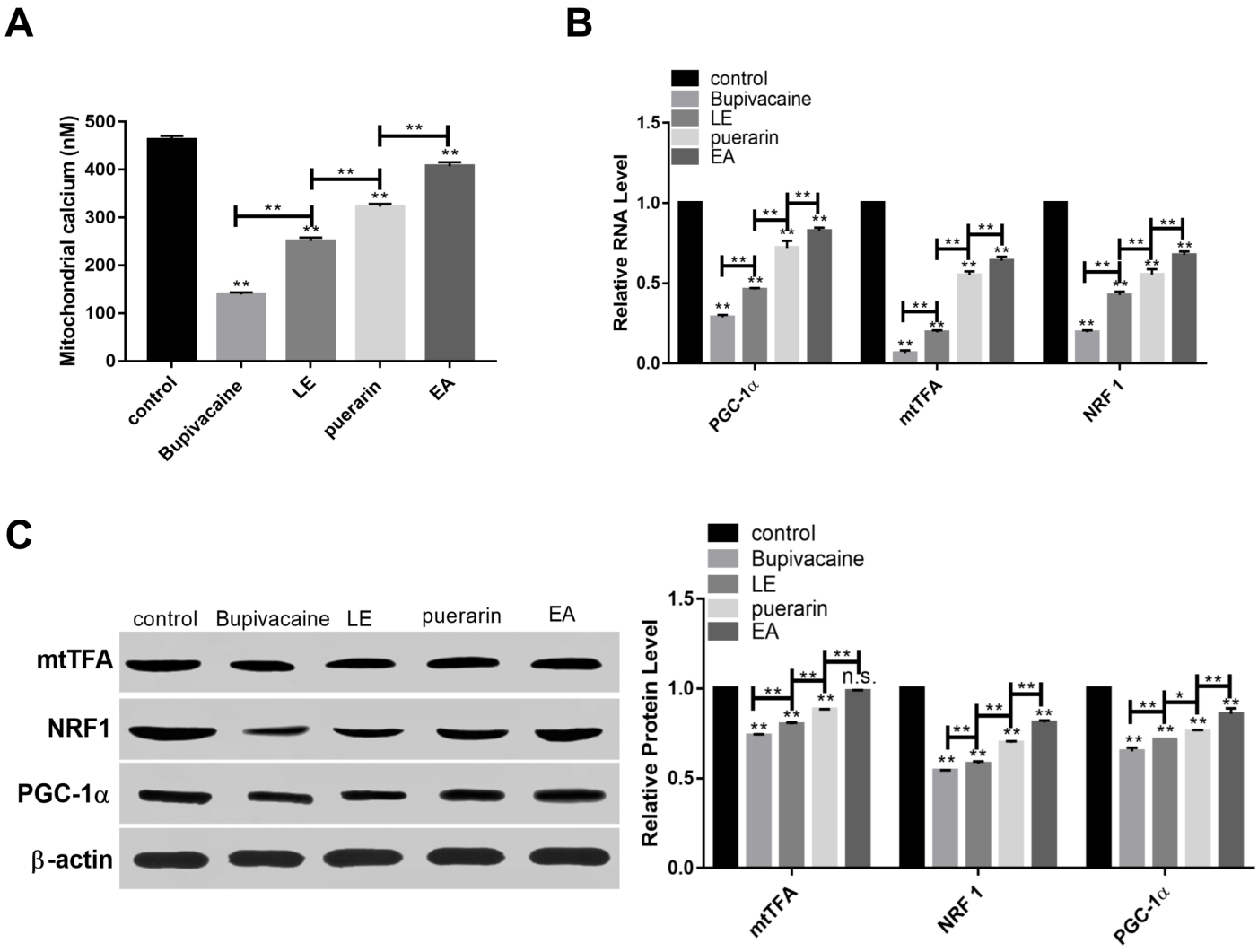

Figure 5

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