Protective effect of electroacupuncture at the Neiguan point in a rabbit model of myocardial ischemia-reperfusion injury

Hongxing Zhang PhD, Lingguang Liu MM, Guofu Huang PhD, Li Zhou MM,
Wenli Wu BM, Tangfa Zhang, Hao Huang PhD

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OBJECTIVES: To investigate possible cardioprotective mechanisms of electroacupuncture (EA) at the Neiguan point and at the Lieque point in the presence of myocardial ischemia-reperfusion injury (MIRI).

METHODS: The changes in ventricular tissue Bax and Bcl-2 protein expression, malondialdehyde (MDA) content and glutathione peroxidase (GSH-PX) activity were examined, as well as beta-endorphin (β -EP) content in a rabbit model of MIRI. Four randomized groups were studied: sham, untreated MIRI, MIRI followed by EA at the Neiguan point, and MIRI followed by EA at the Lieque point. The MIRI model involved ligating the left anterior descending coronary artery for 30 min followed by a 60 min postischemia reperfusion period.

RESULTS: EA at the Neiguan point dramatically decreased the number of apoptotic cells and the content of β -EP and MDA, and inhibited Bax protein expression while enhancing Bcl-2 expression and GSH-PX activity. Furthermore, EA enhanced Bcl-2 expression and GSH-PX activity. Lesser effects were elicited by EA at the Lieque point.

CONCLUSIONS: The cardioprotective effects of applying EA at the Neiguan point on MIRI include reducing apoptosis, regulating apoptosis-controlling genes, and decreasing myocardial MDA and β -EP while enhancing GSH-PX activity.

Key Words: *Electroacupuncture therapy; Myocardial ischemia-reperfusion injury; Neiguan point*

Effet protecteur de l'électro-acupuncture appliquée au point Neiguan dans un modèle d'ischémie-reperfusion myocardique chez le lapin

OBJECTIF : Examiner les mécanismes cardioprotecteurs possibles de l'électro-acupuncture (ÉA) appliquée au point Neiguan et au point Lieque en présence d'une lésion myocardique d'ischémie-reperfusion (LMIR).

MÉTHODE : Les auteurs ont analysé les changements affectant l'expression des protéines Bax et Bcl-2 dans le tissu ventriculaire, le contenu en malondialdéhyde (MDA) et l'activité de la glutathion peroxydase (GSH-PX), de même que le contenu en bêta-endorphine (β -EP) dans un modèle de LMIR chez le lapin. Quatre groupes randomisés ont été étudiés : factice, LMIR non traité, LMIR suivi d'ÉA au point Neiguan et LMIR suivi d'ÉA au point Lieque. Le modèle de LMIR supposait la ligature de la descendante antérieure gauche pendant 30 minutes, suivie d'une période de reperfusion post-ischémique de 60 minutes.

RÉSULTATS : L'ÉA au niveau du point Neiguan a considérablement réduit le nombre de cellules apoptotiques et la teneur en β -EP et en MDA et a inhibé l'expression de la protéine Bax, tout en rehaussant l'expression de la Bcl-2 et l'activité de la GSH-PX. En outre, l'ÉA a rehaussé l'expression de la protéine Bcl-2 et l'activité de la GSH-PX. Appliquée au point Lieque, l'ÉA a produit des effets de moindre envergure.

CONCLUSION : Les effets cardioprotecteurs de l'application de l'ÉA au point Neiguan sur la LMIR incluent la réduction de l'apoptose, la régulation des gènes contrôlant l'apoptose et la diminution du MDA et de la β -EP myocardiques et une hausse de l'activité de la GSH-PX.

METHODS

Animal preparation

Thirty-six adult Japanese male and female white rabbits, provided by the Experimental Animal Center of the Tongji Medical College of Huazhong University of Science and Technology (Wuhan, Hubei, China), were studied. The rabbits weighed between 2.0 kg and 2.5 kg. Animals were raised in a quiet, well-ventilated and well-lit environment (temperature ranged from 15°C to 25°C) before entering the study and they all had normal electrocardiograms.

Induction of regional myocardial ischemia

Following Simpson's method (2), rabbits were anesthetized with 4 mg/kg of 20% urethane administered via the marginal ear vein. Aseptic surgical methods were used and a left thoracotomy was performed without involving the left internal mammary artery or inducing pneumothorax. Following cardiac exposure, the left anterior descending (LAD) coronary artery was identified and a thread was placed under it at the juncture of its upper one-third and middle one-third. The two ends were threaded through a silicone tube that was 3 mm in diameter and

Coronary artery disease is a common cardiovascular condition that endangers the health of middle-aged and elderly individuals. Animal experiments and clinical observations have repeatedly demonstrated that myocardial ischemic injury can be aggravated by reperfusion, namely myocardial ischemia-reperfusion injury (MIRI). Clinical experience has suggested that myocardial ischemia can be effectively treated via acupuncture at the single Neiguan point. The Neiguan point is the Luo point of the pericardium meridian of hand jueyin, and is one of the eight confluence points. Performing acupuncture at the Neiguan point has proven effective clinically with respect to angina pectoris (1). To investigate the mechanisms involved in acupuncture therapy, a MIRI rabbit model was used to document potential reductions in apoptosis of cardiac myocytes and related gene alterations. The influence of electroacupuncture (EA) at the Neiguan point on myocardial cell injury and its possible protective effects were explored. This was accomplished by studying malondialdehyde (MDA) content and glutathione peroxidase (GSH-PX) activity in myocardial tissue and beta-endorphin (β -EP) activity in the medulla oblongata. In addition, the effects of EA at the Lieque point were compared with those at the Neiguan point.

Department of Acupuncture and Moxibustion, Wuhan Chinese and Western Medicine Hospital, Wuhan, Hubei, China

Correspondence: Dr Hongxing Zhang, Department of Acupuncture and Moxibustion, Wuhan Chinese and Western Medicine Hospital, 215 Zhongshan

Boulevard 430022, Wuhan, Hubei, China. Telephone 86-27-63725337, fax 86-27-84332295, e-mail zhxxj99@yahoo.com.cn

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5 mm long. Regional myocardial ischemia was induced by constricting the LAD artery to obstruct blood flow for 30 min; the constriction was then released and the artery was reperfused for 60 min. Evidence for a successful intervention was a cyanotic left anterior ventricular wall and local wall distension, as well as elevated ST segments and peak T waves on the electrocardiogram.

Exclusion criteria

Animals were excluded from the final data analysis if they died during the procedure or if ventricular fibrillation occurred more than twice, whether converted or unconverted.

Treatment groups

The rabbits were randomly divided into the following four groups: a sham group (thoracotomy only); an untreated MIRI group; a Neiguan point group (MIRI and EA treatment at the Neiguan point); and a Lieque point group (MIRI and EA treatment at the Lieque point).

Sham group: The operation was terminated when the LAD coronary artery was isolated; tissue specimens were removed 90 min later.

Untreated MIRI group: Tissue specimens were removed after successful intervention.

EA at the Neiguan point group: The procedure was the same as the untreated MIRI group. Thirty minutes after LAD ligation, acupuncture was applied bilaterally at the Neiguan points (located in the medial forelimbs at a site that is one-sixth the length of the line from the wrist to the elbow, between the radius and the ulna), soon after which a G6850 II Electro-Acupuncture Apparatus (Shanghai Medical Electric Apparatus Co Ltd, China) was applied, selecting 'disperse-dense' waves (ie, alternating frequencies of 2 Hz and 15 Hz at an intensity level of 1 mA to 2 mA). Such EA is considered to be similar to the 'twirling-rotating' acupuncture manipulation that enhances the stimulating effects of acupuncture. The rationale for using different alternating frequencies is to avoid nervous stimulation tolerance (3). Current intensity was adjusted to maintain a slight twitching of the limb. EA continued for 60 min, at which time, tissues were removed.

EA at the Lieque point group: The procedure was identical to the untreated MIRI group. Thirty minutes after LAD ligation, acupuncture was applied bilaterally at the Lieque points. These points were located using the anthropomorphic contrast method at the introcession beside the styloid process of radius. Immediately after acupuncture was performed, a G6850 II Electro-Acupuncture Apparatus was used for 60 min, with an intensity that kept the limb twitching (the 'disperse-dense' wave was turned off). Tissue specimens were subsequently removed.

Determination of apoptotic index, Bcl-2 and Bax expression, MDA content and GSH-PX activity

Preparation of sections from injured hearts: The injured myocardium beyond the ligation was excised, flushed with normal saline until no blood could be seen, then wiped with filter paper and fixed in 4% paraformaldehyde. Subsequent analyses were performed after dehydration, paraffin embedding and staining.

Histological assessment of myocardial tissue and evaluation of myocardial apoptotic index: The stained sections obtained from the hearts of each of the four treatment groups were examined by light microscopy (magnification $\times 100$). Five separate visual fields were selected in the positively stained areas, and five photographs were taken at different exposures, from bright to faint. The number of positive and negative myocardial apoptotic cells was counted. The number of positive apoptotic cells divided by the total number of myocardial cells resulted in a percentage that was expressed as the apoptotic index (AI).

Quantitation of an apoptosis-related gene – Bcl-2 and Bax protein: The streptavidin-biotin-enzyme complex was used to detect the Bcl-2 gene and Bax protein content. Kits for immunohistochemical staining of Bcl-2 and Bax were provided by Wuhan Boster Bioengineering Co Ltd (China).

For Bcl-2 protein determination, 4 μ m thick sections were dewaxed and rehydrated with freshly distilled water. Samples underwent inactivation of endogenous peroxidase at room temperature for 10 min and were then washed three times with distilled water for 2 min. Then, they were immersed in 0.1 mol/L citrate buffer solution (pH=6.0) and heated in a microwave oven until boiling (twice, with a 5 min interval). After refrigerated flushing with phosphate buffered saline (PBS) for 2 min (three times), antigen-retrieval buffers were added for 10 min and tissues were then flushed with PBS for 2 min (three times). Samples were nonspecifically blocked with normal goat serum for 20 min at room temperature. After removal of the goat serum, rabbit anti-Bcl-2 gene antigen antibody was added (incubated for 60 min at 37°C). Biotinylated goat anti-rabbit antibody immunoglobulin G was applied for 20 min and then flushed with PBS for 2 min (three times). Treated tissues were placed in a streptavidin-biotin-enzyme complex reagent, incubated at 37°C for 20 min and, after flushing with PBS for 5 min (four times), samples were stained with diaminobenzidine for 25 min. Samples were then washed with tap water for 3 min. In the final step, samples were stained with hematoxylin, and were dehydrated and mounted for microscopic examination.

For Bax protein determination, the reagents used and procedures employed were the same as those used to detect Bcl-2 protein, except for the application of rabbit anti-Bax protein antibody instead of rabbit anti-Bcl-2 antigen antibody. Images of the sections were obtained (magnification $\times 100$) using the HPIAS-1000 High Resolution Color Pathological Image Analysis System (Tongji Medical College Qianping Imaging Engineering Co Ltd, China). Surface density (positive area divided by total area) was determined by immunohistochemistry (semiquantitative analysis system).

In addition, sections were observed under light microscopy after immunohistochemical staining; cytomembrane and cytoplasm stained brown in Bax- and Bcl-2-positive cells.

Evaluation of MDA and GSH-PX: Myocardial tissue (0.2 g) was sampled with an analytical balance and placed into a 5 mL beaker that was safe for use with a Bunsen burner containing normal saline (1.8 mL). Tissue was cut quickly with ophthalmic scissors and put into a homogenizer. The tissue homogenate was then placed in a centrifuge tube and spun at 3500 rpm at room temperature for 15 min. The supernatant was extracted for determination of MDA and GSH-PX. Reagent kits for measuring MDA and GSH-PX activity were provided by Nanjing Jiancheng Bioengineering Institute (Nanjing City, China), using a 722 spectrophotometer for colorimetric determination. A wavelength of 532 nm was used for colorimetric MDA and thiobarbituric acid determinations. GSH-PX activity was assessed by dinitrobenzoic acid colorimetry and read at a wavelength of 412 nm.

Detection of β -EP in the medulla oblongata

A 0.2 g sample of tissue from the medulla oblongata was removed and heated in boiling normal saline for 5 min. After being homogenized in 1 mL hydrochloric acid, the specimen was transferred to a cuvette at a temperature of 25°C and incubated for 100 min. Then, 1 mL of sodium hydroxide was added to the specimens and, after being centrifuged for 10 min at 1000 rpm, the supernatant was transferred to Eppendorf microtubes (Eppendorf-Netheler-Hinz GmbH, Germany) at -20°C for determination of β -EP.

Radioimmunoassay kits for β -EP were provided by the Department of Neurobiology, Second Military Medical University. An SN-682 Gamma Radioimmunoassay Counter (Shanghai Hefu Photoelectric Instrument Co Ltd, China) was used. The manufacturer's instructions were followed throughout.

Statistical analysis

All data are expressed as the arithmetic mean $\pm$ SEM. SPSS software version 10.0 (SPSS Inc, USA) was used for application of the Student's *t* test. $P < 0.05$ was considered to be statistically significant.

Figure 1) Comparison of cardiac myocyte apoptosis among the groups ($\times 400$ magnification). **A** Untreated myocardial ischemia-reperfusion injury group; **B** Sham group; **C** Electroacupuncture (EA) at the Neiguan point group; **D** EA at the Lieque point group

TABLE 1
Comparison of apoptotic index (AI) among the four groups

Group	n	AI
Sham	8	0.029 $\pm$ 0.009*
Untreated MIRI	8	0.304 $\pm$ 0.064
EA at the Neiguan point	8	0.095 $\pm$ 0.016*†
EA at the Lieque point	8	0.218 $\pm$ 0.031†

Data presented as mean $\pm$ SEM. * $P < 0.01$ for comparison of sham with electroacupuncture (EA) at the Neiguan point; † $P < 0.01$ for comparison of EA at the Neiguan point with sham; ‡ $P < 0.05$ for comparison of sham with EA at the Lieque point. MIRI Myocardial ischemia-reperfusion injury

RESULTS

Effect of EA on myocardial apoptosis

Through terminal 2'-deoxyuridine 5'-triphosphate nick end labeling staining, cells with brown particles in their nuclei were regarded as positive apoptotic cells. Very few apoptotic cells were identified in the sham group. Myocardial cell degeneration was identified in the other groups. There were significantly fewer apoptotic cells in the Neiguan point group, while many apoptotic cells were identified in the Lieque point group (Figure 1).

The AI of the untreated MIRI group was clearly higher than that of the sham group (Table 1). The AI of the Neiguan point group was significantly lower than the AI of the Lieque point and untreated MIRI groups, which was also significantly different from the sham group.

Effect of EA on apoptotic gene Bcl-2 and Bax protein expression

More Bax and less Bcl-2 positive expression was detected in the myocardial tissue of rabbits in the untreated MIRI group (Figures 2A and 2B). Less Bax and Bcl-2 positive expression was seen in the sham group (Figures 2C and 2D). Less Bax and more Bcl-2 positive expression were observed in the Neiguan point group (Figures 2E and 2F). More Bax and less Bcl-2 positive expression was detected in the Lieque-point group (Figures 2G and 2H).

High expression of Bax was seen in the untreated MIRI group compared with the sham group ($P < 0.05$). Expression of Bax clearly decreased in the Neiguan point group, compared with the untreated MIRI group ($P < 0.01$), while there was an apparent increase of Bcl-2 expressed in the Neiguan point group, compared with the untreated MIRI group ($P < 0.01$) and the Lieque point group ($P < 0.05$) (Table 2).

Figure 2) Comparison of Bax and Bcl-2 protein expression in the myocardial tissue of rabbits (immunohistochemistry, $\times 400$ magnification). **A** Bax expression in the untreated myocardial ischemia-reperfusion injury group; **B** Bcl-2 expression in the myocardial ischemia-reperfusion injury group; **C** Bax expression in the sham group; **D** Bcl-2 expression in the sham group; **E** Bax expression in the electroacupuncture (EA) at the Neiguan point group; **F** Bcl-2 expression in the EA at the Neiguan point group; **G** Bax expression in the EA at the Lieque point group; **H** Bcl-2 expression in the EA at the Lieque point group

TABLE 2
Expression of Bax and Bcl-2 in myocardial tissue (surface density)

Group	n	Bax, cells/mm ²	Bcl-2, cells/mm ²
Sham	8	0.103 $\pm$ 0.012	0.183 $\pm$ 0.024
Untreated MIRI	8	0.256 $\pm$ 0.018*	0.187 $\pm$ 0.027
EA at the Neiguan point	8	0.174 $\pm$ 0.022†	0.290 $\pm$ 0.038‡§
EA at the Lieque point	8	0.206 $\pm$ 0.021	0.153 $\pm$ 0.029

Data expressed as mean $\pm$ SEM. * $P < 0.05$ compared with sham; † $P < 0.01$ compared with untreated myocardial ischemia-reperfusion injury (MIRI); ‡ $P < 0.01$ compared with untreated MIRI; § $P < 0.05$ compared with electroacupuncture (EA) at the Lieque point

Effects of EA on MDA concentration

The content of MDA in the myocardial tissue of rabbits in each group is shown in Table 3. Greater MDA content was identified in the untreated MIRI group than in the sham group ($P < 0.05$) and lower MDA content was identified in the Neiguan point group than in the Lieque point group ($P < 0.05$).

TABLE 3
Comparison of malondialdehyde (MDA) content

Group	n	MDA, nmol/mL, mean $\pm$ SEM
Sham	8	15.5 $\pm$ 2.8
Untreated MIRI	8	27.0 $\pm$ 3.5*
EA at the Neiguan point	8	16.4 $\pm$ 2.8†‡
EA at the Lieque point	8	26.2 $\pm$ 3.3

* $P < 0.05$ compared with sham; † $P < 0.05$ compared with untreated myocardial ischemia-reperfusion injury (MIRI); ‡ $P < 0.05$ compared with untreated MIRI and electroacupuncture (EA) at the Lieque point

TABLE 4
Comparison of glutathione peroxidase (GSH-PX) activity

Group	n	GSH-PX, nU/mL, mean $\pm$ SEM
Sham	8	37.2 $\pm$ 2.4
Untreated MIRI	8	28.2 $\pm$ 1.5*
EA at the Neiguan point	8	36.1 $\pm$ 2.3†‡
EA at the Lieque point	8	28.7 $\pm$ 1.5

* $P < 0.01$ compared with sham; † $P < 0.01$ compared with untreated myocardial ischemia-reperfusion injury (MIRI); ‡ $P < 0.05$ compared with untreated MIRI and electroacupuncture (EA) at the Lieque point

Effects of EA on GSH-PX activity

Changes in GSH-PX activity in the myocardial tissue of each group were identified (Table 4). The activity of GSH-PX in the untreated MIRI group was obviously lower than that of the sham group ($P < 0.01$) and GSH-PX activity in the Neiguan point group was greater than that of the untreated MIRI group or the Lieque point group ($P < 0.01$ and $P < 0.05$, respectively).

Higher myocardial MDA content was identified in the untreated MIRI group compared with the sham group ($P < 0.05$). Lower GSH-PX activity was also identified ($P < 0.01$). Comparing the Neiguan point group and the untreated MIRI group, MDA content decreased ($P < 0.05$), while GSH-PX activity increased ($P < 0.01$). Comparison between the Neiguan point group and Lieque point groups indicated significant decreases in MDA content ($P < 0.05$) and increases in GSH-PX activity ($P < 0.05$).

Effect of EA on β -EP content in the medulla oblongata

As demonstrated in Table 5, there was a prominent elevation of medulla oblongata β -EP content in the untreated MIRI group compared with the sham group ($P < 0.01$), as well as an obvious decrease in the Neiguan point group compared with the untreated MIRI group and the Lieque point group ($P < 0.01$ and $P < 0.05$, respectively).

DISCUSSION

The Neiguan point – the Luo point of the pericardium meridian of hand jueyin, which exists in a close physiological and pathological relationship with the heart – is one of the eight confluences that regulate the Qi in the thorax. Thus, acupuncture at the Neiguan point is the primary choice for treating cardiac and thoracic diseases.

In the present study, the medulla oblongata β -EP content, AI, Bax and Bcl-2 expression, MDA content and GSH-PX activity were determined to identify the cardioprotective effects of EA at the Neiguan point during MIRI. EA was also applied at the Lieque point as a comparison.

In myocardial tissue that undergoes ischemia and hypoxia, free radicals increase, whereas free-radical scavengers decrease. Lipid peroxidation occurs due to the reactions of strong, active oxygen-derived free radicals and unsaturated fatty acid. Their product, malondialdehyde, can severely damage membranes and cause protein denaturation and enzyme deactivation. The breakage of DNA chains in this process could induce apoptosis (4-6). Simultaneously, free radicals produced by endothelial cells are transferred through the vessel wall to damage

TABLE 5
Comparison of beta-endorphin (β -EP) content in the medulla oblongata

Group	n	β -EP content, ng/L, mean $\pm$ SEM
Sham	8	15.8 $\pm$ 1.8
MIRI without intervention	8	23.6 $\pm$ 1.6*
EA at the Neiguan point	8	17.1 $\pm$ 1.7†‡
EA at the Lieque point	8	23.0 $\pm$ 1.5

* $P < 0.01$ compared with sham; † $P < 0.01$ compared with myocardial ischemia-reperfusion injury (MIRI) without intervention; ‡ $P < 0.05$ compared with electroacupuncture (EA) at the Lieque point

the myocardium (7-9). GSH-PX, one of the main endogenous antioxidant enzymes, specifically and catalytically reduces glutathione and hydrogen peroxide, in addition to reducing lipid hydrogen peroxidation. Together, this results in biomembranous lipid peroxidation that converts them into harmless alcohols, thus protecting membrane structure and functional integrity.

Previous experimental studies have demonstrated the induction of cardiomyocyte apoptosis during hypoxia (10) and ischemia reperfusion (11). Biochemical features of apoptosis have been identified in the infarcted human myocardium (12). Fliss and Gattinger (13) demonstrated typical morphological changes and DNA 'ladders' in MIRI rat models, confirming that both ischemic and reperfused rat myocardium can undergo apoptotic cell death. The research of Ma et al (14) showed obvious myocardial cell apoptosis in infarcted regions in aged, acute myocardial infarction patients.

The mechanisms by which cell apoptosis in MIRI is regulated remain unclear. Recent evidence indicates that apoptosis is triggered by a cascade of gene expression. Many genes are involved in this process, among which *Bcl-2* and *Bax* play important roles. *Bcl-2*, a type of proto-oncogene that maintains cell survival, is a strong antiapoptotic factor (5). *Bax* is a member of the *Bcl-2* family, but with a reverse function to *Bcl-2* of inducing cell apoptosis. The ratio of *Bax* protein to *Bcl-2* protein determines whether cell apoptosis or survival is induced (ie, cell apoptosis occurs when *Bax* protein predominates and vice versa) (15). Misao et al (16) studied autopsied human hearts following acute myocardial infarction, using old myocardial infarction and normal hearts as controls. These authors identified *Bcl-2* protein expression in salvaged myocytes during the acute stage of infarction. Because *Bax* protein was overexpressed in older infarcts, the authors attributed an important pathophysiological role to the expression of *Bcl-2* and overexpression of *Bax* regarding the protection against apoptosis in human myocytes after ischemia/reperfusion. This is in accordance with the fact that we detected *Bcl-2* and *Bax* expression after EA at the Neiguan point.

Opioid peptides and receptors are located in a number of brain areas that are involved in the regulation of the cardiovascular system (17). β -EP is one of the most potent endogenous opioid peptides. Previous studies indicated participation of β -EP neurons originating from the nucleus of the solitary tract of the medullary bulb, which is known as 'biosis central' in the regulation of cardiovascular activities (18). In normal subjects, the administration of opioid peptides or their antagonists under basal conditions has little or no effect on heart rate or blood pressure (19,20). However, when the sympathetic nervous system is in an activated state – for instance, stressful situations cause an increase in β -EPs, which may exceed the physiological limit and inhibit the activity of the L, N, P and/or Q calcium channels, resulting in a calcium deficiency in the cells and tissues, changing endocrine functions, because intracellular calcium deficiency suppresses calcium-mediated and calcium-dependent functions (21) – opiates can exert quite potent control (mainly inhibitory) on cardiovascular function (22), which leads to bradyarrhythmia, cardiac insufficiency, hypotension, etc (23). Also, it is reported that β -EP is one of the pathological mediators of myocardial damage after acute myocardial infarction and might be used to determine the extent of cardiovascular damage (24).

In our experiments, the results obtained following EA at the Neiguan point indicate attenuation of myocardial injury, as evidenced by a reduced apoptotic index. This was consistent with an inhibition of MIRI-induced apoptosis and decreased β -EP and MDA content. In addition, increases of Bcl-2 expression and GSH-PX activity were observed, which could act to protect cardiac myocytes from undergoing apoptosis and membrane peroxidation.

Taken together, these data indicate that acupuncture applied to the Neiguan point attenuates the extent of MIRI via reduction of Bax expression and promotion of Bcl-2 expression. Together, these may act to inhibit the triggering of apoptosis concomitant with a reduction in ischemic reperfusion-induced superoxide injury and reduced free radical production. Modification of β -EP content in medulla oblongata neurons may be one of the central mechanisms whereby EA affects MIRI.

CONCLUSION

Cardioprotective effects against MIRI can be elicited by EA at the Neiguan point. In addition, comparison with EA at the Lieque point demonstrated a relative specificity of EA at the Neiguan point.

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