Original Research

Role of HO-1 in protective effect of electro-acupuncture against endotoxin shock-induced acute lung injury in rabbits

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Abstract

Heme oxygenase (HO)-1 has been reported to play a great role in attenuating lung injury during endotoxic shock in our previous research. Although electro-acupuncture has been explored to reduce oxidative stress and decrease inflammatory reaction in animals with endotoxic shock, the mechanism of this effect is still unclear. The aim of this study was to determine whether HO-1 is involved in the effect of electro-acupuncture on the injured lung during endotoxic shock in rabbits. Sixty New England white rabbits were randomly divided into groups C, Z, ES, EA, AP, and EAZ. Before inducing endotoxic shock, group ES received no electro-acupuncture, while group EA received electro-acupuncture at ST36 (zusanli) and BL13 (feishu) acupoints on both sides for five days and group AP received electro-acupuncture (EA) stimulation at a non-acupoint. Groups ES, AP, EA, and EAZ received LPS to replicate the experimental model of injured lung induced by endotoxic shock, and electro-acupuncture was performed throughout the procedure with the same parameter. Groups EAZ and Z received the HO-1 inhibitor, ZnPP-IX, intraperitoneally. The animals were sacrificed by blood-letting at 6 h after LPS administration. The blood samples were collected for serum examination, and the lungs were removed for pathology examination, detection of alveolaer epithelial cell apoptosis by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay (TUNEL assay), determination of wet to dry ratio, measurement of Evans blue (EB) contents, and determination of HO-1protein and mRNA expression. According to the results, EA at ST36 and BL13 could increase the expression of HO-1. At the same time, index of quantitative assessment (IQA) score and the number of TUNEL-positive cells decreased, while electro-acupuncture at the other points did not exert this effect, and pretreatment with ZnPP-IX in group EAZ suppressed the efficacy of electro-acupuncture preconditioning. In summary, electro-acupuncture stimulation at ST36 and BL13, while not the non-acupoint, could attenuate the lung injury during the endotoxic shock, and this effect was due to increased expression of HO-1.

Keywords: Electro-acupuncture, HO-1, acute lung injury, endotoxic shock

***Experimental Biology and Medicine* 2013; 238: 705–712. DOI: 10.1177/1535370213489487**

Introduction

Although many studies have been performed to investigate the mechanism of the acute lung injury (ALI), the mortality rate remains high in patients with the ALI or acute respiratory distress syndrome (ARDS).[1] ALI has been documented clinically following several pathological states, and endotoxic shock induced by LPS is one of the major factors that induce ALI.[2] It has been thought that apoptosis, inflammatory mediators, and reactive oxidative species play a great role in the formation of ALI or ARDS induced by endotoxin.[3] The product of oxidative stress such as malondialdehyde (MDA) significantly increased and the antioxidant enzyme superoxide dismutase (SOD) dramatically decreased during endotoxic shock.[4] In addition, it is found that the number of apotosis cells was significantly and time-dependently increased after LPS administration in mice.[5]

HO-1 is the inducible isoform of the first and rate-limiting enzyme of heme degradation, and it works as a strong

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negative regulator in the development of oxidative stress and lung inflammatory responses during endotoxin-induced ALI.[6] The cytoprotective functions of HO-1 may be attributed to heme turnover, as well as to beneficial properties of its enzymatic reaction products: biliverdin-IXα, iron, and carbon monoxide (CO).[7] It is found that when the mice were treated with a lethal dose of LPS and subsequently exposed to CO, the survival rate significantly improved and serum IL-6 and IL-1 levels also decreased.[8] When the HO-1 expression was up-regulated in the septic shock mice, we found that the CO level was increased, while MDA content decreased in plasma.[9]

Acupuncture has been used to treat different disorders in East Asian countries. Hsu *et al.*[10] reported that a patient with endotoxic shock undergoing acupuncture for 80 min maintained the vital signs stable until he has been successfully transferred to the hospital. It also was proven that acupuncture stimulation of ST36 significantly attenuated the degree of lung injury in LPS-stimulated rats.[11] In addition, electro-acupuncture treatment increased the antioxidant enzyme activities and decreased the extent of lipid peroxidation in ischemic-reperfused rat brain.[12] At the same time, electro-acupuncture also exerted the immunoregulatory effect in rats with surgical trauma.[13] According to the theory of traditional Chinese medicine, BL13 is closely related to the lung disease, and it is found that electro-acupuncture on BL-13 could protect against hypoxia-induced pulmonary hypertension in rats.[14]

However, whether electro-acupuncture treatment could cause protective effects on injured lung by up-regulation of the HO-1 protein during endotoxic shock has not been confirmed systematically. Therefore, this study was designed to clarify the effect of electro-acupuncture treatment on heme oxygenase-1 in injured lung in rabbits with endotoxic shock.

Materials and methods

Experimental animals

Two-month-old male New England white rabbits were purchased from Laboratory Animal Center of Nankai Clinical Institution of Tianjin Medical University. All treatment for animals before the experiment were done as we previously described.[15] All animal studies were approved by the Institutional Animal Use and Care Committee of Nankai Hospital (NKH-20101209), and the care and handling of the animals were in accordance with National Institutes of Health guidelines.

Electro-acupuncture pretreatment

Electro-acupuncture was initiated for 5 days consecutively before the experiment. Four stainless steel needles were inserted into the ST36 (Zusanli point) which was located between the tibia and fibular approximately 5 mm lateral to the anterior tubercle of the tibia, and the BL13 (Feishu point) which was located between T3 and T4 of the spine approximately 1.5 cm lateral to the midline in group EA and group EAZ. The parameter of electro-acupuncture was a disperse-dense wave with 10 Hz for 15 min every day.[16] In addition, electro-acupuncture was also performed throughout the experiment procedure for 6 h on the experimental day.[13] Compared with group EA and group EAZ, group AP was acupunctured at a non-acupoint with the same frequency and intensity. For acupuncture sessions, animals were lightly immobilized using hands to minimize stress. The non-treated control group was also lightly immobilized using the same method. Acupuncture points were identified by one of the authors (ZY), an experienced acupuncturist.

Experimental protocols

Rabbits were fasted for 12 h but allowed free access to water for the experiments. After the rabbits were sedated with 2 mg/kg ketamine, tracheotomy was performed and a 3.5 mm uncuffed tracheal tube was inserted and tied in place. Anaesthesia was maintained with continuous infusion of ketamine at a rate of 3 mg/kg/h. The lungs of the rabbits were ventilated using an infant ventilator (IV100B, Sechrit, Anaheim, CA) at an inspired oxygen concentration of 40%. Tidal volume was set to 10 mL/kg measured by pneumotachograph.[17] Respiratory rate was adjusted to produced initial arterial carbon dioxide tension of 35–40 mmHg. The right carotid artery was cannulated with PE-50 tubing to measure mean arterial pressure (MAP) continuously by using Hellige monitor instruments (Germany), and internal jugular vein cannulated with a 24 g cannula. The animals were placed on a heating pad so that the body temperature could be kept at 37.8–40.2°C. Lactated Ringer's solution was intravenously administered at 8 mL/kg/h.

Sixty rabbits were randomly divided into six groups: group C, group Z, group ES, group EA, group EAZ, and group AP (Figure 1). Electro-acupuncture treatment was performed in group EA and EAZ, and group AP was acupunctured at a non-acupoint as described above, and there is no electro-acupuncture treatment in group C and Z. The rabbits in group ES, EA, AP, and EAZ received 0.5 mL (5 mg/kg, intravenously[18]) LPS (L2880, Sigma, USA) to replicate the experimental model of endotoxic shock,[10] and that in group C and Z received 0.5 mL normal saline intravenously as a control. In addition, all rabbits received 0.5 mL of 30 mg/kg EB (Serva, Germany) intravenously 5 min before administering LPS or normal saline, and after 2 h of administration of LPS or normal saline, group EAZ and Z received the 1 mL (10 μmol/kg, intraperitoneally[15]) ZnPP-IX, while others received 1 mL 50 mM sodium bicarbonate vehicle instead. The experimental model of injured lung induced by endotoxic shock was defined by the lowered MAP (<75% of the baseline) and oxygenation index ($PaO_2/FiO_2 < 300$ mmHg).

Sampling and storage

The ventilation was maintained for 6 h, and then the blood samples were collected after LPS or saline administration in living animals and were centrifuged at 4000 rpm for 10 min at 4°C. The rabbits were sacrificed and the lungs were removed and quickly perfused with phosphate-buffered saline to remove the blood.

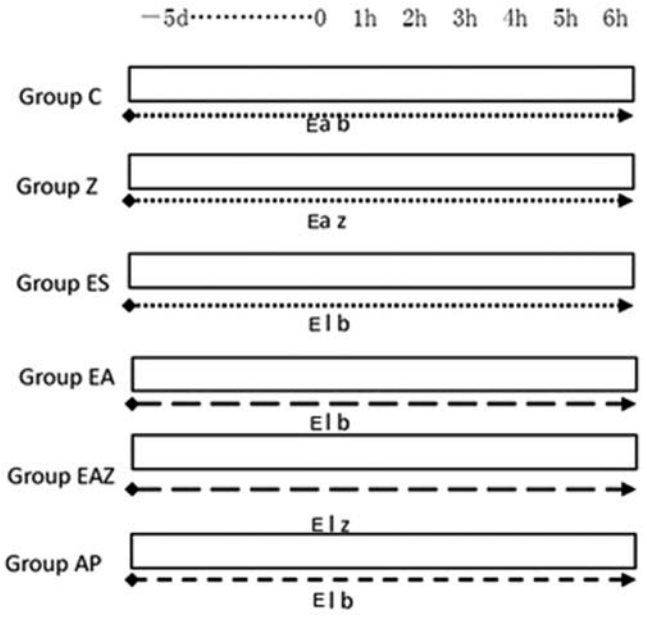

Figure 1 Experimental protocols. In the figure, 'A' presents administration of Evans blue, 'B' presents administration of NS, 'C' presents administration of sodium bicarbonate, 'Z' presents administration of zinc protoporphyrin-IX, and 'L' equals to administration of LPS, '·····' means no pretreatment, '——' means pretreatment at the ST36 and BL13 for five days,'·····▶' means no electro-acupuncture during the experiment, '— · — · —' means pretreatment at the other sites except ST36 and BL13 for five days, '——▶' means electro-acupuncture during the experiment at the ST36 and BL13, '– – – – –▶' means electro-acupuncture at the other sites except ST36 and BL13 during the experiment

Biochemical measurements

SOD activity and MDA contents in the lung tissues were determined by spectrophotometry[19] and by the method of Ohkawa *et al*.,[20] respectively. SOD activity and MDA contents were expressed as per unit of protein determined by the Lowry method.[21] The upper right lung was taken for this detection.

Measurement of CO in plasma

After 6 h of experiment, CO level in plasma was measured by the method of Chalmers[22] and was expressed as milligram of CO per liter of fresh blood.

Wet to dry ratio (W/D) weight of lung tissue

The left lung was harvested and rinsed with normal sodium, and the superfluous water was removed and then the lung was weighed as wet weight. Subsequently, the specimens were dried in an electric air blast drier at 70°C for 24 h and then weighed as dry weight. W/D ratio could be calculated.[9,15]

Measurement of EB contents

The lungs were perfused free of blood with phosphate-buffered saline containing 5-mM ethylenediaminetetraacetic acid via thoracotomy, excised *en bloc*, blotted dry, weighed, and snap frozen in liquid nitrogen. The right lower lung was weighed and then incubated in formamide for 18 h at 37°C to extract the EB dye, and centrifuged at 5000 × *g* for 30 min, the dry weight of the lower lung was estimated via the wet-to-dry weight ratio. The extracted dye was assessed in a spectrophotometer by measuring the absorbance at 620 nm and comparing to standards of EB dye dissolved in formamide. The Evans blue dye extravasation was expressed as nanograms of EB dye per milligram of dry tissue.[9,15]

Table 1 Semi-quantitative histological index for acute lung injury

Score	Hyperemia and edema	RBC and WBC infiltration	Hyaline membrane
0	No abnormalities	No RBC and WBC	No hyaline membrane
1	Mild	Very few	≤20% alveolus
2	Moderate	Part of alveolus	In 20–50% alveolus
3	Severe	Full of alveolus	≥50% alveolus

Morphology of lung tissues

The middle lobe of the right lung was fixed in 10% formalin and routinely processed into paraffin sections (4–5 μm). Sections were used for hematoxylin and eosin staining. Six slices were selected from each group of rabbits, and 10 fields of each slice were visualized by microscopy (×400). Table 1 showed the scores used to assess the degree of pathological change. The average values were taken as a semi-quantitative histological IQA of lung injury[23] (Table 1). For the lung injury score, images were evaluated by an investigator who was blinded to the identity of the slides.

TUNEL staining

An *in situ* cell death detection kit (POD; Roche, Penzberg, Germany) was used to assess cell death in 4 μm sections obtained from the lung (five animals per group). All the procedure was undergone as previously described.[24,25] All slides were subjected to the same exposure times. Ten randomly chosen fields from each animal (five animals per group) at 400 × magnification with approximately the same number of alveoli were analysed. Fields included airways or vessels were excluded from the analysis.[24] The investigator evaluating the slides was blinded to the experimental conditions associated with each slide. The TUNEL-positive cells for each optical field was quantified as the percentage of total cells counted.

Western blot analysis

The lungs frozen in liquid nitrogen and stored at −80°C were homogenized in lysis buffer solution containing 13.2 mmol/L Tris-HCl, 5.5% glycerol, 0.44% SDS, and 10% β-mercaptoethanol. An equal amount of extracted soluble protein (50 μg) was fractionated by Tris-glycine-SDS polyacrylamide gel (12%) electrophoresis. Proteins were transferred to a PVDF membrane (Bio-Rad) with a Bio-Rad transblot apparatus.[26] Blots were briefly washed with 1 × Tris-buffered saline (TBS; 200 mM Tris and 1.5 M NaCl) and then incubated overnight at 25°C with rat

anti-rabbit HO-1 IgG (Santa Cruz, USA) diluted 1:800 in blocking solution (1% nonfat milk plus 0.5% BSA in TBS-0.05% Tween 20). Blots were washed with TBS-0.05% Tween 20 and incubated for 2 h at 37°C with a 1:5000 dilution of HRP-conjugated goat anti-rat IgG (Caltag Laboratories, San Francisco, CA). The antigen-antibody signal was visualized as previously described,[27] and the quantification was performed by densitometry (Molecular Analyst Image-analysis Software, Bio-Rad). The HO-1 protein concentrations were normalized by β-actin and the IOD ratio between HO-1 and β-actin was calculated and used as the expression of HO-1.

Reverse transcription (RT)-PCR

Total RNA was extracted from the lungs by employing a Total Quick RNA kit (TA200TQR, Talent, Italy). The ethanol-precipitated RNA fraction (1 μg) was reversely transcribed using a Revert Aid^{TM} First Strand cDNA Synthesis kit (MetaBios Inc., Canada). According to the manufacturer's protocol, 50 μL of first-strand cDNA solution was obtained. The primers used in this study were 5′CCGAGGTCAAGCACAGGG3′ and 5′CACGGTCGCCA ACAGGA3′ for rabbit HO-1, 269 bp; 5′ GTAAAGACCTCT ATGCCAACA 3′ and 5′ GGACTCATCGTACTCCTGCT 3′ for rabbit β-actin, 227 bp. RT-PCR was performed as described by Suzuki *et al.*[28] The integral optical density (IOD) of the DNA bands was measured with the Quantity one 1D analysis software (Bio-Rad). The IOD ratio between HO-1 and β-actin was calculated and used as the expression of HO-1mRNA.

Statistical analysis

Values are expressed as mean ± SD or median (range). Maximum and minimum possible lung injury scores are 16 and 0, respectively. Analysis involved SPSS, version 11.5, for Windows. Repeated-measures data were statistically analysed using repeated-measures analysis of variance (ANOVA). The ALI scores were analysed using the Kruskal-Wallis rank test followed by the Dunnett test. The other data were analysed by one-way ANOVA followed by the Tukey-Kramer *post hoc* test. $P < 0.05$ was deemed statistically significant.

Results

Comparison of SOD activity, W/D ratio, MDA, EB contents, and CO

The comparison of the MDA, CO, and EB contents, SOD activity, and W/D ratio among six groups are shown in Table 2. According to the results, it is clear that the administration of LPS to the normal rabbits could induce the oxidative stress and lead to lung inflammation which reflected by increased W/D ratio, MDA, EB contents, CO, and lowered SOD activity compared to group C (all $P < 0.05$); it is also found that electro-acupuncture could reduced the lung injury induced by LPS (all $P < 0.01$). In addition, acupuncture at the other site except the ST36 and BL13 could not show the protective effect compared with group ES, and administration of HO-1 inhibitor ZnPP-IX in group EAZ could reverse the protective effects of the electro-acupuncture at the site of ST36 and BL13.

Comparison of the lung IQA score

In control rabbits, few infiltrating neutrophils were observed and there was no evidence of haemorrhage or oedema. In comparison, the lung in group ES rabbits showed diffuse oedema and severe inflammatory cell (predominantly neutrophils) infiltration in alveoli and interstitium of the lung, haemorrhage, and thickened interlobular septa (Figure 2 ES). Significantly higher IQA scores were observed in the group ES rabbits compared to the controls ($P < 0.01$) (Table 3). And pretreatment with electro-acupuncture in group EA attenuate the lung injury reflected by decreased IQA scores compared with that of group ES ($P < 0.01$) (Figure 2 EA). However, according to the results, pretreatment with ZnPP-IX in group EAZ suppressed the efficacy of electro-acupuncture preconditioning, and the IQA scores were not decreased in group EAZ (Figure 2 EAZ), and the pretreatment in group AP also did not decreased the levels of IQA scores compared with that of group ES (Figure 2 AP). In rabbits treated with ZnPP-IX alone, the IQA Score was similar to that of control rabbits, which showed that the administration of ZnPP-IX alone did not induce lung injury (Figure 2 Z and C).

Comparison of the TUNEL staining

The comparison of the percentage of the TUNEL-positive cells in total cells counted among these six groups is shown in Table 4, and the TUNEL staining is shown in Figure 3.

Table 2 Comparison of SOD, W/D ratio, MDA, CO and EB contents among these six groups

Group	SOD (NU/mg protein)	MDA (nmol/mg protein)	EB (ng/mg)	W/D ratio	CO (mg/L)
C	30.6 ± 4.39	1.81 ± 0.53	46.9 ± 9.5	3.17 ± 0.18	0.46 ± 0.04
Z	29.9 ± 3.84	1.83 ± 0.53	48.9 ± 8.2	2.98 ± 0.12	0.41 ± 0.03
ES	16.7 ± 2.03*	3.65 ± 0.93*	228.4 ± 44.7*	5.62 ± 0.19*	0.84 ± 0.14*
EA	22.7 ± 3.34*†	2.87 ± 0.72*†	180.8 ± 41.6*†	4.64 ± 0.21*†	1.18 ± 0.15*†
AP	17.3 ± 1.97*	3.47 ± 0.71*	231.2 ± 35.7*	5.59 ± 0.23*	0.84 ± 0.09*
EAZ	16.9 ± 2.01*	3.54 ± 0.82*	237.3 ± 39.1*	5.74 ± 0.42*	0.89 ± 0.13*

*$P < 0.05$ compared with control group; †$P < 0.05$ compared with ES group.

The number of TUNEL-positive cells was significantly ($P < 0.01$) increased after LPS administration compared with that of controls (Figure 3C). However, it showed a marked reduction in TUNEL-positive cells when the rabbits subjected to electro-acupuncture pretreatment in group EA ($P < 0.01$) (Figure 3 EA). The number of TUNEL-positive cells wasn't decreased in group EAZ compared with group ES, which indicated that ZnPP-IX in group EAZ suppressed the efficacy of electro-acupuncture preconditioning (Figure 3 EAZ). In addition, the number of TUNEL-positive cells wasn't decreased in group AP compared with group ES as well, which indicated electro-acupuncture at the non-acupoint couldn't inhibit the cell apoptosis (Figure 3 AP). The total number of TUNEL-positive cells in group Z was similar to that of control rabbits (Figure 3 C and Z).

Comparison of HO-1 mRNA and HO-1 protein expression

Expression of HO-1and level of HO-1 protein were investigated among these six groups, and the result was shown in Table 5 and Figure 4(a) and (b), The RT-PCR study and Western blot revealed that expression of HO-1 mRNA and level of HO-1 protein rapidly increased after administration of LPS compared with that of controls ($P < 0.01$), and electro-acupuncture in group EA could up-regulate the expression of the HO-1 mRNA and increase the level of HO-1 protein in contrast to that of group ES ($P < 0.01$). However, ZnPP-IX could abolish the effect of electro-acupuncture on up-regulation of HO-1 mRNA and increasing the level of HO-1 protein in group EAZ, and expression of HO-1 mRNA and the level of the protein in group AP did not increase in contrast to that of group ES.

Discussion

Endotoxin is one of the most common reasons leading to ALI/ARDS, in addition to direct damage to lungs, the release of a large number of inflammatory mediators, or cytokines induced by endotoxin indirectly lead to lung injury as well.[29] In addition, neutrophils activated by LPS transmigrated across vascular and into the tissues, where they exerted their toxic effects through the release of reactive oxygen metabolites, such as superoxide, hydrogen peroxide, and hydroxyl radicals, resulting in microvascular dysfunction and local inflammatory response, including alveolar capillary barrier leakage, interstitial and(or) alveolar edema, lipid peroxidation, and eventual cell death.[30] Because MDA is one of the final products of lipid peroxidation, and its concentration is directly proportional to the cell

Table 3 Comparison of the lung IQA Score among these six groups

Group	IQA score
C	0.50 ± 0.55
Z	0.60 ± 0.55
ES	6.67 ± 0.82*
EA	4.98 ± 0.89*†
AP	6.59 ± 0.74*
EAZ	6.32 ± 0.64*

*$P < 0.01$ compared with control group; †$P < 0.01$ compared with ES group.

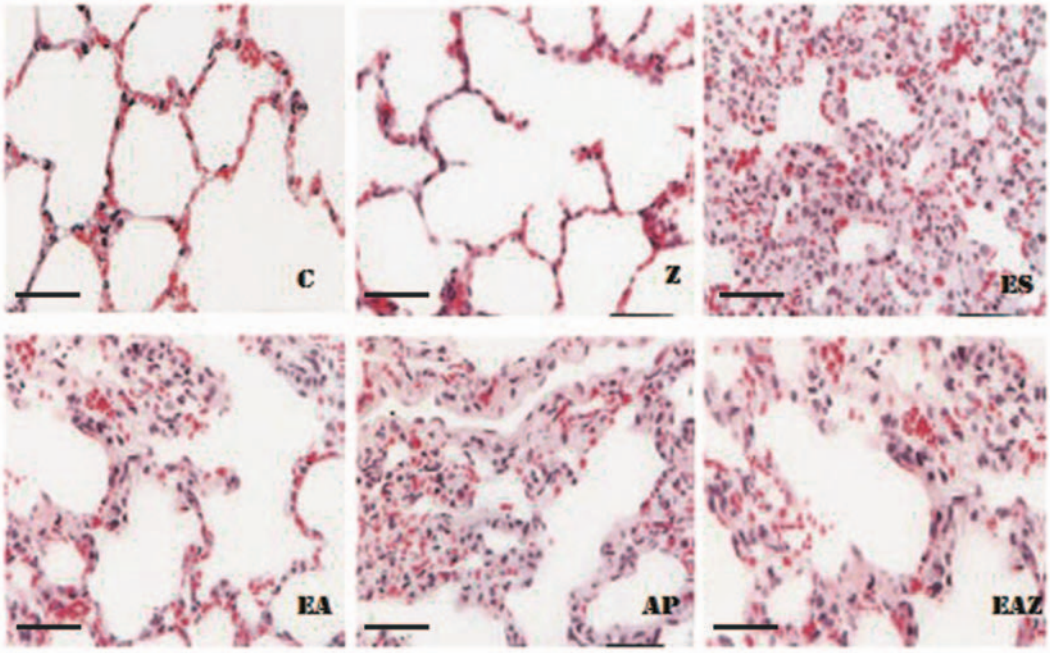

Figure 2 Microphotographs of morphological changes of lung tissues (×400): Significantly higher IQA scores were observed in the group ES rabbits compared to the controls ($P < 0.01$) (Table 3). And pretreatment with electro-acupuncture in group EA attenuate the lung injury reflected by decreased IQA scores compared with that of group ES ($P < 0.01$) (Figure 2 EA). However, pretreatment with ZnPP-IX suppressed the electro-acupuncture efficacy in group EAZ, and the IQA scores was not decreased in group EAZ (Figure 2 EAZ), and the levels of IQA scores also wasn't decreased in group AP compared with that of group ES. Scale bars: 50 μm. (A color version of this figure is available in the online journal)

damage caused by reactive oxygen metabolites,[31] we use it as a maker of oxidative stress, and Evans blue, which binds with albumin in the plasma and can leak to the interstitial when the endothelium permeability increased, was used as expression of the degree of increased endothelium permeability in the present study. Apoptosis induced by lung inflammation also contributes to the formation of ALI.[32]

It was found that the lung injury mode could be established by intratracheal/ intranasal LPS instillation,[33,34] however, it just was local LPS exposure in lungs, only could produce a controlled ALI response without causing systemic inflammation and multi-organ failure.[34] In our study, we want to establish the model of lung injury induced by endotoxic shock and investigate the effect of EA on that model. The standard model of endotoxic shock was set up by systemic LPS exposure, and it is characterized by shock, coagulopathy, and multiorgan dysfunction,[35] so we injected LPS intravenously in our study.

Previous study showed that SOD activity was significantly increased and the ROS level was significantly reduced by the EA stimulation at ST-36,[12] which strongly

Table 4 Comparison of the percent of the TUNEL-positive cells among these six groups

Group	TUNEL-positive cells (%)
C	1.32 ± 0.82
Z	1.27 ± 0.55
ES	26.3 ± 3.52*
EA	14.5 ± 5.89*†
AP	24.5 ± 5.74*
EAZ	25.7 ± 3.64*

*$P < 0.01$ compared with control group; †$P < 0.01$ compared with ES group.

Table 5 Comparison of the relative expression of HO-1 mRNA and concentration of HO-1 protein among these six groups

Group	Relative expression of HO-1 mRNA	Relative concentration of HO-1 protein
C	0.67 ± 0.09	0.15 ± 0.04
Z	0.61 ± 0.08	0.18 ± 0.05
ES	0.96 ± 0.05*	0.38 ± 0.02*
EA	1.25 ± 0.24*†	0.62 ± 0.03*†
AP	0.99 ± 0.04*	0.39 ± 0.05*
EAZ	0.90 ± 0.06*	0.41 ± 0.04*

*$P < 0.01$ compared with control group; †$P < 0.01$ compared with ES group.

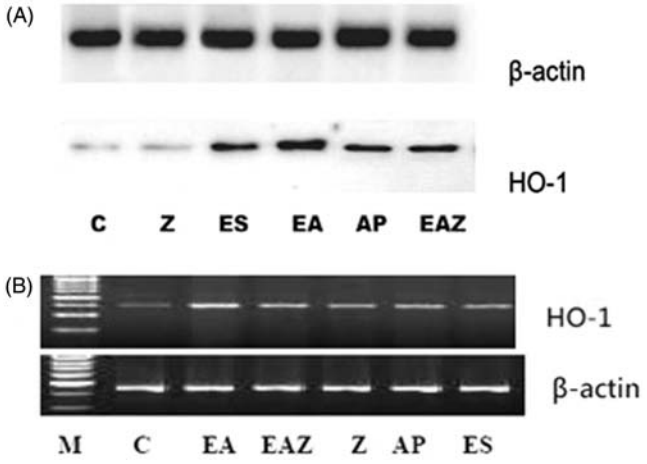

Figure 4 Results of RT-PCR and Western blot: 'A' showed the picture of Western blot, and 'B' was that of RT-PCR, 'M' presents maker, 'C' presents group C,' Z' presents group Z, 'ES' presents group ES, 'EA' presents group EA, 'AP' presents group AP, and 'EAZ' presents group EAZ

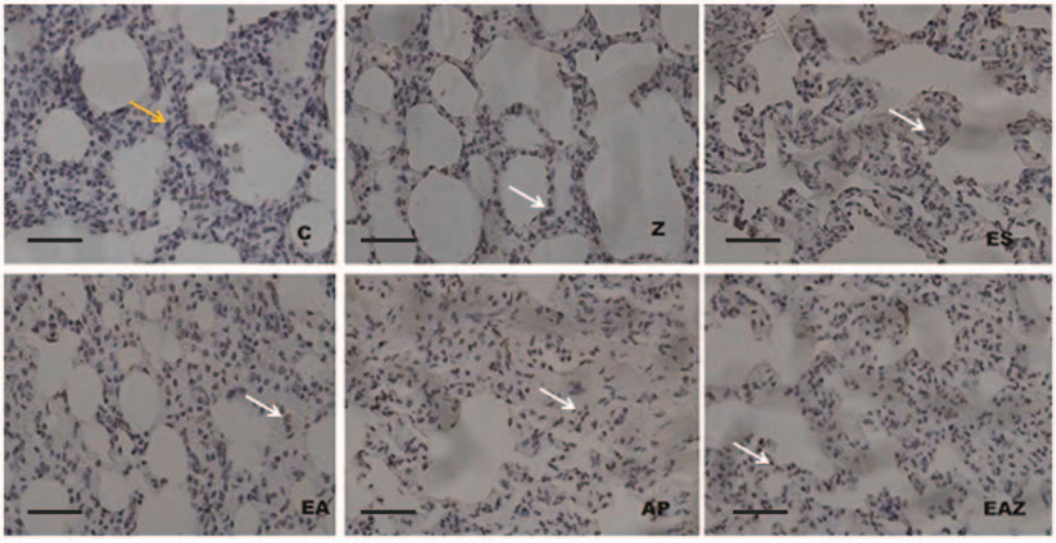

Figure 3 Results of detection of cellular DNA fragmentation using the TUNEL method as described in Methods: (C) equals to group C, (Z) equals to group Z, (ES) equals to group ES, (EA) equals to group EA, (AP) equals to group AP, (EAZ) equals to group EAZ. White arrows: the TUNEL-positive cells; yellow arrow: normal cells. Scale bars: 50 μm. (A color version of this figure is available in the online journal)

suggest that the EA treatment activates the antioxidative enzyme, resulting in a decrease of oxidative stress which is benefit to cellular protection.[36] Another research also indicated that the degree of lung injury was attenuated in the LPS plus ST-36 group compared to those in both the LPS group and the LPS plus sham group.[11] However, the molecular mechanism is unclear.

The major finding of this study is that electro-acupuncture stimulation of ST-36 and BL-13 significantly mitigates LPS-induced acute lung injury in stimulated rabbits. EA can reduce the pulmonary MDA production and EB contents induced by LPS. EA also has anti-oxidative effects by increasing SOD activity. Furthermore, the HO-1 mRNA and HO-1 protein in group ES are significantly lower than that in group EA and blockade of the HO-1 enzyme by the administration of ZnPP-IX reduced the protection in EA-treated animals, implicating a direct involvement of HO-1.

HO-1 is an inducible protein whose expression is increased several fold in response to a variety of cellular stresses and stimuli, including hypoxia, oxidative stress, and inflammatory cytokines.[37] It was said that mice deficient in HO-1 had increased susceptibility to oxidative stress,[38] and their cells were less capable to withstand an oxidative challenge than the corresponding wild type cells,[39] while HO-1 over-expression increased cellular resistance to oxidants.[40] Another studies address the molecular mechanisms responsible for the antiapoptotic effect of HO-1, activation of NF-κB signal pathway promotes a variety of genes, including pro-inflammatory as well as antiapoptotic genes that suppress apoptosis.[41]

To clarify whether EA increase the expression of HO-1 in the lung during endotoxic shock, we used the antagonist of HO-1 in group EAZ and selected ZnPP-IX instead of other metalloporphyrins to inhibit HO activity because ZnPP-IX has been shown to be selective for HO-1 over other microsomal enzymes.[9] To avoid effect of ZnPP-IX on NOS activity and soluble guanylyl cyclase activity in vascular endothelial cells, ZnPP-IX was given in a dose of 10 μmol/kg in this study,[42] and the result show that ZnPP-IX, specific inhibitor of HO-1, could abolish the effect of electro-acupuncture on up-regulation of HO-1 mRNA and increasing the level of HO-1 protein in group EAZ.

Up-regulation of HO-1 protein by electro-acupuncture in endotoxic shock rabbits was found in the lung, with increase of CO concentration and decrease of SOD, MDA contents, pulmonary vascular leakage, and the numbers of the apoptosis cell. Another research showed that EA at the Neiguan point dramatically decreased the number of apoptotic cells and the content of beta-EP and MDA, and inhibited Bax protein expression while enhancing Bcl-2 expression and GSH-PX activity in a rabbit model of myocardial ischemia-reperfusion injury.[43] EA also could suppress the increase of apoptosis and Fas protein expression in splenic lymphocytes induced by the surgical trauma stress.[44]

In summary, this study demonstrates that treatment with EA attenuates ALI induced by LPS in rabbits. In addition, EA induces HO-1, and the HO-1 inhibitor, ZnPP-IX, counteracts the protective effect of EA. Thus, the mechanism of the protective effects of EA is dependent on HO-1. The up-regulation of HO-1, in turn, results in a reduction of the production of MDA, MPO, the radio of W/D, IQA scores, the contents of EB, the number of TUNEL cells in the lungs, as well as increase in CO in the plasma. However, the mechanism of EA mediated up-regulation of HO-1 requires further research.

Author contributions: YJ, DS and LX are co-first authors. YJ designed the study. YJ, DS, and GL analysed the data. YJ and DS contributed to writing the paper. LX, ZY and LD collected data and performed experiments for this study. DS and ZY established the model together, performed surgeries, and collected all tissues together. ZY also contributed to acupuncture treatment. LX and LD were involved in pathological examination, PCR and Western blotting work.

ACKNOWLEDGEMENTS

Financial support for this research work was provided by the Natural Science Foundation of Tianjin (11JCYBJC11000) and Tianjin Science & Technology Pillar Program (12ZCZDSY013300).

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(Received October 11, 2012, Accepted December 18, 2012)