Electro-acupuncture at ‘Neiguan’ (PC6) attenuates liver injury in endotoxaemic rats

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Abstract

Background Intravenous injection of lipopolysaccharide (LPS) stimulates macrophages to release proinflammatory cytokines and nitric oxide (NO). This results in hypotension, vascular hyporeactivity and multiple organ failure (eg, liver injury) in rats. In rats with endotoxin shock, electro-acupuncture (EA) at ‘Neiguan’ (PC6) significantly attenuated LPS-induced elevated biochemical parameters of liver injury and marked infiltration of neutrophils into liver tissues caused by LPS were significantly attenuated by EA. However, hypotension, tachycardia and raised production of plasma NO were not suppressed by EA at PC6.

Methods A total of 28 male adult Wistar rats were included in this study. Rats received intravenous LPS (10 mg/kg for 4 h) or saline for 4 h followed by EA at PC6 acupuncture point.

Results Elevated biochemical parameters of liver injury and marked infiltration of neutrophils into liver tissues caused by LPS were significantly attenuated by EA. However, hypotension, tachycardia and raised production of plasma NO were not suppressed by EA at PC6.

Conclusions These results indicate that EA at PC6 should be further investigated as a possible adjuvant therapy for endotoxin-induced liver dysfunction. Its mechanism of action needs further investigation.

INTRODUCTION

Sepsis may proceed to septic shock and then multiple organ dysfunction syndrome (MODS) despite major advances in intensive care therapy. The development of MODS can increase mortality by 30% to 100% according to the number of organ systems involved.1 Sepsis-induced liver dysfunction/failure is usually attributed to systemic or microcirculatory disturbances,2 as well as the activation of inflammatory cytokines and mediators.3 Lipopolysaccharide (LPS) is an endotoxin from the cell walls of Gram-negative bacteria. Sepsis in rats induced by LPS is a well-established animal model used to investigate pathogenesis and treatment of septic shock. Intravenous injection of LPS stimulates macrophages to release systemically endogenous inflammatory mediators, including tumour necrosis factor α (TNFα), platelet-activating factor and interleukins (eg, IL-1, IL-6).4 These endogenous mediators increase nitric oxide (NO) levels via induction of NO synthase (iNOS).5 6 Overproduction of NO, a potent endogenous vasodilator, has been linked to sepsis-induced hypotension and vascular hyporesponsiveness.7 8 NO can react avidly with superoxide anion to form peroxynitrite, which leads to tissue hypoxia and liver injury.9 10

Acupuncture is used as clinical treatment or prevention for several diseases, including chronic pain, asthma, rhinitis, inflammatory bowel disease and rheumatoid arthritis in traditional Chinese medicine. Although the biological structure and mechanisms of ‘acupuncture points’ have not been established, increasing amounts of data indicate that acupuncture stimulation exhibits significant anti-inflammatory effects. For instance, acupuncture at ‘Zusanli’ (ST36) has been shown to inhibit the activity of iNOS, decrease synthesis of NO and attenuate lung and renal injury induced by LPS in rats.11 12 Following cecal ligation and puncture in rats, an acupuncture protocol has been shown to partially reverse the neutrophil migration and reduce the number of bacteria at the infection focus.13

It has been documented that acupuncture at Neiguan (PC6) can improve symptoms of angina and palpitation, and enhance left cardiac function in coronary heart disease.14 15 Li et al16 also report that electro-acupuncture (EA) at PC6 improves endotoxic shock in rats by decreasing NO and TNFα production. These results indicate that EA at PC6 could be a potential adjuvant for treatment of endotoxin shock. However, the question of whether PC6 acupuncture has similar effects against endotoxin-induced liver injuries remains unanswered. This study was designed to test the hypothesis that hepatic dysfunction can be modulated by EA at PC6 in a rat model of endotoxaemia. To extend the clinical use of PC6 acupuncture in endotoxaemia, we investigated the effects of PC6 acupuncture administered after induction of endotoxaemia.

METHODS

Animals

Male Wistar rats (250–300 g body weight) were used in this study. All experimental procedures
were approved by the institutional and local Committee on the Care and Use of Animals and all animals received humane care according to the National Institutes of Health Guidelines of USA. All rats were maintained under a 12 h light/dark cycle at a controlled temperature (21±2°C) with free access to food and tap water until the day of the experiment.

**Experimental protocols**

Twenty eight rats were anaesthetised by intraperitoneal injection of urethane (1.2 g/kg). Their tracheas were cannulated to facilitate respiration and environmental temperature was maintained at 24°C with an air-conditioning system. Their right carotid arteries were cannulated and connected to a pressure transducer (P23ID; Statham, Oxnard, California, USA) for the measurement of phasic blood pressure. This was displayed on a polygraph recorder (MacLab/4e; ADInstruments, Castle Hill, Australia). The left jugular veins were cannulated for the administration of the drugs. Rats were stabilised for 20 min after surgical preparation. The rats were randomly allocated to one of four groups as followed: (1) Control: 1 ml/kg intravenous of saline; (2) LPS: *Escherichia coli* LPS (*E coli* serotype 0127:B8, L3127) 10 mg/kg, intravenous infusion over 5 min; (3) LPS+EA: acupuncture of PC6 with electrical stimulation at 30 and 150 min following *E coli* LPS infusion; (4) LPS+ needle insertion: needle inserted at PC6 without any stimulation following *E coli* LPS infusion. The acupuncture needles were left in place after stimulation.

Mean arterial blood pressure (MAP) and arterial blood samples (0.5 ml) were obtained at baseline (ie, time 0) and specified times (1, 2 and 4 h). Each volume of blood removed was immediately replaced by the injection of an equal volume of sterile saline. Four hours after LPS infusion the rats were euthanased by intravenous administration of overdose of sodium pentobarbital and their livers were immediately harvested for histopathological study.

**Acupuncture protocols**

Rats were kept in specially designed holders with their forelimbs exposed. Pairs of stainless-steel needles (0.25 mm diameter, An Chi Handy Acupuncture Needle, Taipei, Taiwan) were inserted bilaterally into PC6 on the forelimbs to a depth of approximately 2 mm. Previous studies have documented that the PC6 in rats, analogous to humans, is located in the interosseal muscles between the radius and ulna of the forelimb at 3 mm proximal to the wrist crease.17 The needles were connected to a programmed pulse generator (Function/Arbitrary Waveform Generator HP 33120A USA) which produced constant current square-wave electrical stimulation to stimulate the acupuncture points for 10 min with parameters of 50 Hz, 4 volt and 1–3 mA (increased gradually). The electrical intensity was just strong enough to elicit slight twitches of the forelimbs. To control for the effects of needle insertion, sham acupuncture was performed by needle insertion at PC6 without electrical stimulation.

**Quantification of liver function and injury**

Using a One Touch II blood glucose monitoring system (Lifescan, Milpitas, California, USA), 10 μl of whole blood was taken and blood glucose was immediately analysed. The remaining arterial blood samples were then immediately centrifuged at 7500 g for 2 min to obtain the plasma. Approximately 50 μl of plasma was used to analyse liver function. Plasma biochemical markers of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) were analysed using Fuji DRI-CHEM 3030 (Fuji Photo Film, Tokyo, Japan).

**Measurement of plasma NO concentration**

The plasma samples (50 μl) were used to measure plasma NO concentration. In this study NO plasma concentration

![Figure 1](image_url)
refers to total plasma nitrite and nitrate concentration. Using this method, nitrate is reduced to NO via nitrite. The amount of nitrate in the plasma was measured by adding a reducing agent (0.8% VCl₃ in 1 N HCl) to the purge vessel to convert nitrate to NO, which was stripped from the plasma using helium purge gas. The NO was then drawn into a NO analyser (Sievers 280 NOA; Sievers, Boulder, Colorado, USA). Nitrate concentrations were calculated by comparison with standard solutions of sodium nitrate (Sigma Chemical, St Louis, Missouri, USA).

**Histopathological studies**

A portion of liver was fixed in 10% phosphate-buffered formaldehyde for >2 h. After washing in phosphate-buffered saline, the fixed tissues were dehydrated in graded ethanol and embedded in paraffin. Paraffin sections of 4–6 μm were sliced using a sliding microtome (Jung SM 2000; Leica Microsystems, Wetzlar, Germany), and paraffin was removed using xylene. Tissue sections were stained with H&E reagents for light microscopy. Using randomly selected high-power fields, the histopathology, including polymorphonuclear neutrophil (PMN) infiltrations, interstitial oedema and necrosis, were evaluated by a pathologist in a blinded fashion.

**Statistical analysis**

The data are presented as mean±SEM of n determinations, where n represents the number of animals studied. Statistical evaluation was performed using two-way analysis of variance followed by multiple comparison test (Scheffe test). A p value of < 0.05 was considered to be statistically significant.

**RESULTS**

No rats died during the experiment before euthanasia. The control animals exhibited stable MAP during the experiment (figure 1A). LPS led to an attenuation of MAP during the 4 h experimental period, reaching a peak at 2 h. There were no significant changes in MAP in LPS+EA and LPS+NI groups compared with the LPS group (p>0.05).

There was no significant change in blood glucose in the control group during the experiment (figure 1B). Infusion of LPS produced an increase in blood glucose at 1 and 2 h, which subsequently decreased to a level lower than baseline at 4 h. Acupuncture treatment in LPS+EA and LPS+NI groups did not significantly affect early hyperglycaemia and late hypoglycaemia in LPS rats (p>0.05).

There were no significant changes in ALT (figure 2A), AST (figure 2B) and LDH (figure 2C) levels in the control group during the experiment. Infusion of LPS produced a significant, time-dependent increase in serum ALT, AST and LDH levels (p<0.05). EA significantly attenuated the increases of these levels during the experiment (p<0.05). However, acupuncture treatment without electrical stimulation had no significant effect during the experiment (p>0.05).

**Figure 2** Effects of acupuncture on plasma levels of (A) alanine aminotransferase (ALT), (B) aspartate aminotransferase (AST) and (C) lactate dehydrogenase (LDH) in rats. Changes during the experimental period in rats that received saline alone (Control), lipopolysaccharide alone (LPS), lipopolysaccharide plus electro-acupuncture at PC6 (LPS+EA) and lipopolysaccharide plus acupuncture without electrical stimulation (LPS+NI) are shown. Data expressed as mean±SEM. n=7 in each group. *p<0.05, Control vs LPS group; †p<0.05, LPS vs LPS+EA group.
Chemiluminescence assay revealed that rats from all the groups had low systemic NO concentrations at 0 h. There was no significant change in NO concentration in the control group during the experiment (figure 3). The injection of LPS led to a significant elevation of NO concentration at 4 h (p<0.05), but not at 1 and 2 h. Acupuncture treatment with or without electrical stimulation had no significant effect on NO concentration in LPS rats during the experiment (p>0.05).

Histopathological examination showed severe congestion and swelling of hepatocytes and infiltration of PMNs among the sinusoids in the livers of LPS rats (figure 4A). In rats that received EA, only mild swelling but no evidence of congestion of hepatocytes or remarkable infiltration of PMN in sinusoids were revealed (figure 4B). Mild congestion, mild to moderate swelling of hepatocytes and occasional infiltration of PMN in the sinusoids were found in the livers of rats that received needle insertion without electrical stimulation (figure 4C).

DISCUSSION
The liver is a major organ of endotoxin detoxification, and an influx of neutrophils has been observed in the liver within 3–6 h of endotoxin administration.18 Activated neutrophils adhere to endothelial cells and induce endothelial cell injury and activation at the site of inflammation. Moreover, this process can be accompanied by hepatocellular damage, and subsequently multiple hepatic enzymes (including ALT, AST and LDH) enter the circulation. The present in vivo study demonstrated that hypotension, biphasic changes of blood glucose and acute liver injury did indeed occur when a single dose of LPS was given to rats. Hepatocytic swelling and neutrophil infiltration, the frequent histological patterns of acute liver injury, were also found after LPS administration. Moreover, EA at PC6 attenuated liver dysfunction and cytotoxicity, and appeared to suppress the increased congestion of hepatocytes and infiltration of PMN in the livers of rats treated with endotoxin. The novel observation is presented, that EA at PC6 attenuated liver injury during the LPS-induced systemic inflammatory response. However, EA at PC6 had no significant effect on the change of haemodynamics and the increase of plasma NO concentration in LPS rats.

According to figure 1A, there was no significant difference in MAP at 4 h between the control and LPS group. But significant increases of ALT, AST and LDH were found at 4 h in the LPS group compared with the control group. LPS could induce liver injury in other ways, for example, inflammation, although LPS-induced hypotension may cause liver damage by microcirculation disturbance. We used PC6 as a target to treat liver injury, and found that EA at PC6 could improve liver injury by attenuating the increases of the hepatic enzymes and infiltration of PMNs after LPS administration. In addition, Shi et al report that plasma ALT activity induced by endotoxin is decreased by EA at ST36.19 Several reviews have suggested that the potential effect...

**Figure 3** Effects of acupuncture on plasma levels of nitrate/nitrite in rats. Changes during the experimental period in rats that received saline alone (Control), lipopolysaccharide alone (LPS), lipopolysaccharide plus electro-acupuncture at PC6 (LPS + EA) and lipopolysaccharide plus acupuncture without electrical stimulation (LPS + NI) are shown. Data expressed as mean±SEM. n=7 in each group. *p<0.05, Control vs LPS group.

**Figure 4** Histopathological features of liver sections of rats from (A) lipopolysaccharide alone (LPS) group, (B) lipopolysaccharide plus electro-acupuncture at PC6 (LPS + EA) group and (C) lipopolysaccharide plus acupuncture without electrical stimulation (LPS + NI) group. (A) Severe congestion and swelling of hepatocytes and infiltration of polymorphonuclear neutrophils (PMNs) among the sinusoids were observed in the livers of the LPS alone group. (B) Mild swelling but no evidence of congestion in hepatocytes, nor remarkable infiltration of PMN in sinusoids were seen in the LPS + EA group. (C) Mild congestion, mild to moderate swelling of hepatocytes and occasional infiltration of PMN in the sinusoids were found in livers of LPS + NI group. The features were typical in >3 rats in each group. Sections were stained with H&E. ×400 (original magnification).
of acupuncture on liver injury in endotoxic rats may be associated with anti-inflammation, mediated by efferent vagus nerve activation and inflammatory macrophage deactivation as well as the release of neuuropeptides such as calcitonin gene-related peptide, substance P and -endorphin.20,21

The surge of plasma NO concentration in rats occurred at 4 h after LPS exposure, and this enhanced production of NO is largely mediated by iNOS expression.22 It has been implicated that NO is an important mediator of haemodynamic regulation in sepsis, particularly in vascular hyporesponsiveness and delayed hypotension.23 24 Several studies report that acupuncture of PC6 could reduce myocardial injury from myocardial ischaemia and protect gastric mucosa from injury possibly mediated by NO upregulation.17 25 Acupuncture at ST36 could act through inhibiting NF-κB activation to attenuate iNOS expression and resultant NO biosynthesis. In the rat model of endotoxaemia, EA at PC6 could decrease the plasma NO and TNFα levels16 and attenuate lung injury through decreasing the iNOS expression but enhancing eNOS expression in lungs.26 However, our results did not show the suppressing effect of EA at PC6 on plasma NO. The EA on PC6 appears to decrease the infiltration of PMN in endotoxic liver, which may mediate the improving effect of liver injury. However, the mechanism of EA at PC6 on liver injury should be further investigated.

CONCLUSIONS

We have demonstrated that EA at PC6 attenuates the deterioration of liver dysfunction in rats with endotoxic shock. This endotoxic shock model seems to be a useful strategy for animal research, which attempts to provide experimental findings that can be extrapolated to clinical practice. Thus, we suggest that EA at PC6 should be further investigated as a possible adjuvant therapy in patients with endotoxin-induced liver dysfunction during surgery or intensive care. However, further research is required to determine what the mechanism of acupuncture therapy seen in this study is mediated on liver injury.

Summary box

- Septic shock may lead to multiple organ failure
- We tested the effect of EA at PC6 using a rat model
- EA appears to reduce liver damage

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Competing interests None.

Ethics approval This study was conducted with the approval of the National Defense Medical Center, Taipei, Taiwan. All experimental procedures were approved by the institutional and local Committee on the Care and Use of Animals.

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