# Protective effect of electro-acupuncture on liver ischemia-reperfusion injury in rats

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Abstract. Liver ischemia-reperfusion injury is an important clinical complication in which excessive inflammation is a key factor; however, few studies have provided effective means of its regulation. As previous studies suggested that electro-acupuncture (EA) is able control excessive inflammation, the present study aimed to explore its effects on liver ischemia-reperfusion injury in experimental rats. The animals were randomly divided into surgery and sham groups, which were further divided into four sub-groups, including a non-treatment (NT), a non-point acupuncture (NPA), the non-selective nicotinic acetylcholine receptor (AChR) agonist 1,1-dimethyl-4-phenyl L-pioperazinium iodide (DMPPI) and an EA group. The alanine aminotransferase (ALT), serum cytokine and myeloperoxidase (MP) levels and the tissue pathology were evaluated after 90 min of ischemia followed by a 4, 8 or 24 h reperfusion. The results demonstrated that EA and DMPPI suppressed serum ALT elevation at 4 and 8 h reperfusion, whereas NPA did not. I/R induced hepatocellular necrosis, and cytoplasmic

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vacuolization and sinusoidal congestion was ameliorated by EA treatment after an 8 and 24 h reperfusion. In addition, EA also inhibited liver neutrophil accumulation, evidenced by a decreased MPO level at 8 h reperfusion. EA also suppressed the release of serum inflammatory factors TNF- $\alpha$  and IL-6 for the duration of reperfusion. However, little influence on IL-10 was observed. Mechanistically, vagus block by subphrenic vagotomy or mecamylamine hydrochloride abolished EA effect on liver damage, neutrophil accumulation and inflammatory factor release. In conclusion, it was demonstrated that EA protects the liver against I/R induced injury by inhibiting the inflammatory response, which is associated with the vagus.

#### Introduction

Liver ischemia-reperfusion injury (I/R) is an important clinical complication after hemorrhagic shock, hepatic trauma, resection surgery or transplantation (1,2). It has a biphasic pattern: The first 6 h of reperfusion after ischemia is the acute injury phase, which is characterized by activated massive Kupffer cells and release of pre-inflammatory cytokines, including tumor necrosis factor (TNF)- $\alpha$  and generation of reactive oxygen species. The subsequent phase is the sub-acute pattern, in which neutrophil infiltration and production of inflammatory mediators prevail, as well as inhibition of the Kupffer cell function by diminishing cytokine production and I/R injury (3-5). As excessive inflammation is one important factor of I/R injury (6), its regulation is a key approach to prevent it.

Acupuncture is one of the most ancient treatments in Traditional Chinese Medicine (TCM), which has potent effects in treating numerous diseases, including tumors and digestive tract diseases, or it may be applied for anesthesia, and previous studies reported its capacity regulate the inflammatory response (7). However, the effect of electroacupuncture (EA) and on liver I/R injury has remained to be elucidated. Hence, the aim of the present study was to explore the effect of pre-treatment by EA at the Hegu acupoint (LI-4) on I/R and to investigate the underlying mechanisms.

## Materials and methods

Establishment of the animal model of liver I/R injury. All animal experiments complied with the National Institute of Health Guide for the Care and Use of Laboratory Animals (8.9). Ethical approval was obtained from the ethics committee of Shanghai Public Health Clinical Center (Shanghai, China). A total of 96 male Sprague Dawley (SD) rats (Pharmacy College of Shanghai Jiaotong University, Shanghai, China; 6-7 weeks; 180-220 g) were acclimatized to the laboratory environment for 7 days prior to surgery. The rats were divided into two equal groups (each n=48) at random, one of which received I/R surgery and the sham group received the same surgery but without hepatic portal vessel occlusion. Animals in the two groups were further divided into four sub-groups (each n=12), namely the no treatment (NT), non-point acupuncture (NPA), 1,1-dimethyl-4-phenyl L-piperazinium iodide (DMPPI) and EA at LI-4 (EA) group. The protocol of I/R surgery was in accordance with that of previous studies (10-12). Rats were anesthetized with sodium pentobarbital (50 mg/kg) by intraperitoneal (i.p.) injection. A midline laparotomy was performed, the portal circulation to the median and left lateral lobes of the liver was carefully dissected, and a 3-cm bending atraumatic vascular clip (Surgical Instruments Factory, Shanghai, China) was placed on the vessels to obstruct the portal venous and hepatic arterial blood supply to these lobes. A total of 0.2 ml sterile saline was dripped into the abdomen to avoid the organs to dry and a simple suture was applied to prevent contamination. After 90 min of partial liver ischemia, the clamp was removed and the reperfusion began. Rats were euthanized by CO<sub>2</sub> asphyxiation (100% CO<sub>2</sub> at a fill rate of 20% cv/min) followed by cervical dislocation to ensure sacrifice following 4, 8 or 24 h of reperfusion. Blood samples as well as hepatic left lateral lobes were collected as described below for analysis.

*Vagus block*. In an additional experiment, 72 SD rats were divided into Sham, MH and SV groups (n=24). Rats in MH and SV groups were subjected to vagus nerve block prior to I/R surgery through i.p. injections of mecamylamine hydrochloride (MH) and subphrenic vagotomy (SV) respectively (13,14). Rats in these 3 groups were further divided into 2 subgroups (n=12), NPA and EA, and pretreated with NPA and EA prior to I/R or Sham surgeries as aforementioned. Following 8 h reperfusion, serum alanine aminotransferase (ALT), liver myeloperoxidase (MPO), serum TNF- $\alpha$ , interleukin (IL)-6 and IL-10 levels in MH and SV groups were determined and compared with those in Sham group.

*EA administration*. The rats in the EA and NPA groups were subjected to EA at 2/100 Hz and 3 mA for 30 min (Hans electro-stimulator; Nanjing Jisheng Medical Technology Co., Ltd., Nanjing, China). The current intensity and duration of EA were selected based on a preliminary experiment. The LI-4 is located in the forelimb, between the first and second metacarpals and needles were inserted at a straight angle at a depth of 1 mm. The non-acupoint was located at a similar site but between the fourth and fifth metacarpals (15).

*Drug administration*. The rats received i.p. administration of the nonselective nicotinic acetylcholine receptor (AChR) agonist DMPPI (2 mg/kg) at 15 min prior to ischemia (16). The rats received i.p. injection of noncompetitive nicotinic AChR antagonist MH (2 mg/kg) at 15 min prior to EA (16,17).

Blood sampling and collection of hepatic left lateral lobes. A total of 5 ml peripheral blood was obtained by inferior vena cava puncture. The blood was collected in a sterile plastic tube with coagulant, left to stand for 30 min and then centrifuged at a speed of 1,500 x g for 20 min at 4°C to obtain the serum. The serum samples were stored at -80°C until use for ALT assays. A sample from the hepatic lateral lobe was obtained for analysis. A portion of tissue was fixed in 10% buffered formalin at room temperature for 36-48 h and then embedded in paraffin for H&E staining, the procedure of which is described in the 'Histopathology' section. Other part of the tissue was immediately placed into liquid nitrogen and then stored at -80°C until use for reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

Assessment of ALT. Serum ALT was determined using an auto-bioassay at the Inspection division of Eastern Hepatobiliary Surgery Hospital (Shanghai, China). The value was expressed in international units per liter.

*Histopathology*. The liver tissue was prepared for H&E staining by a pathologist (Pathology of Eastern Hepatobiliary Surgery Hospital, Shanghai, China) according to the protocol described elsewhere (18), who was blinded to the all experimental conditions. Embedded tissue was sliced into  $4-\mu$ m-thick sections. Sections were then mounted on regular glass slides, deparaffinized in xylene at 37°C for ~15 min, rehydrated in decreasing concentrations of ethanol (100, 95, 85 and 75%, each for 5 min) at room temperature. After being rinsed with distilled water, the sections were stained with hematoxylin for 5 min at 37°C and 0.5% eosin for 1 min successively. Photomicrographs were captured under an Olympus BX51 microscope (Olympus, Tokyo, Japan) at an original magnification of x100.

*Liver neutrophil accumulation*. MPO is an index of neutrophil accumulation, which was assessed by a sandwich ELISA kit (cat. no. orb411170; Wuhan Booute Biotechnology Co., Ltd, Wuhan, China).

Assessment of serum cytokines. Serum TNF- $\alpha$ , IL-6 and IL-10 was determined in a 96-well micro-plate using a sandwich ELISA kit (cat. nos. PRTA00, PR6000B and PR1000, respectively; R&D Systems, Inc., Minneapolis, MN, USA). All serum samples were tested in duplicate.

*RT-qPCR*. Total RNA was isolated from the liver samples using a TRIzol RNA isolation system (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). An aliquot of 500 ng total RNA was reverse-transcribed to complementary cDNA, using the PrimeScript<sup>TM</sup> RT reagent kit (Takara Bio Inc., Otsu, Japan). Real-time PCR was performed using SYBR<sup>®</sup> Premix Ex Taq<sup>TM</sup> (Takara Bio Inc.) in an ABI 7300 (Applied Biosystems; Thermo Fisher Scientific, Inc.). All reactions were performed in a 50- $\mu$ l reaction system in duplicate following the manufacturer's protocol using the following conditions: 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec. Each of the samples was tested for TNF- $\alpha$ , IL-6 and IL-10 expression levels. A housekeeping gene,  $\beta$ -actin, was used to verify equal loading of RNA and cDNA in the RT and real-time PCR reactions. The threshold cycle (Cq) values were determined and used to determine the relative copy number via the  $2^{-\Delta\Delta Cq}$  formula (19). The PCR primer sequences were as follows: β-actin sense, 5'-CCA CACCCGCCACCAGTTCG-3' and antisense, 5'-CTTGCT CTGGGCCTCGTCGC-3'; TNF-α sense, 5'-TCAGTTCCA TGGCCCAGAC-3' and antisense, 5'-GTTGTCTTTGAGATC CATGCCATT-3'; IL-6 sense, 5'-TCTGCTCTGGTCTTCTGG AGTTCCG-3' and antisense, 5'-TGGATGGTCTTGGTCCTT AGCCACT-3'; IL-10 sense, 5'-GCTGCGACGCTGTCATCG ATTTCT-3' and antisense, 5'-TGTCCTGCAGTCCAGTAG ACGCC-3'.

Statistical analysis. All values are expressed as the mean  $\pm$  standard error of the mean. Differences between multiple groups were analyzed by one-way analysis of variance, followed by two-group comparison via the least significant differences test. P $\leq$ 0.05 was considered to indicate a statistically significant difference. All data were analyzed using PASW 18.0 statistics software for windows (IBM Corp., Armonk, NY, USA).

#### Results

*Effect of EA on serum ALT.* Previous studies reported that 90 min of ischemia effectively cause severe hepatocellular injury and serum ALT is significantly elevated in a time-dependent manner after reperfusion. According to the two patterns of I/R injury, the effects of EA on I/R injury were assessed after reperfusion for 4, 8 and 24 h.

As presented in Fig. 1, in the sham groups, no liver injury was encountered in the NT and different pre-treatment groups (NPA, DMPPI or EA). Subsequently, the effects of EA on I/R-associated increases in serum ALT levels were assessed after 4, 8 and 24 h of reperfusion. Compared with those in the sham group, the serum ALT levels were significantly increased in the I/R groups (NT, NPA, DMPPI or EA; n=12; P<0.05). The EA or DMPPI groups had lower ALT levels than the NT or NPA groups at 4, 8 and 24 h. A maximum in ALT levels was observed at 8 h (Fig. 1). The ALT levels in the NT group (n=12; P>0.05) reached a normal level at 48 h, while they were still elevated at 24 h (n=12; P<0.05; data not shown). Compared with the NT group, the EA group (n=12; P>0.05) had similar serum ALT levels to those in sham group.

*EA reduces I/R-associated liver tissue injury*. Liver tissue injury was determined via histopathology and assessment of neutrophil accumulation. The histopathology results were obtained by evaluating the extent of hepatocellular necrosis, cytoplasmic vacuolization and sinusoidal congestion. In the sham groups, the NPA, EA or DMPPI treatments had no obvious effects on the histopathological results (Fig. 2, first row), which was in line with the absence of effects on the ALT levels. In the NT group, the liver tissue contracted increasingly severe injury with time, displaying as hepatocellular necrosis, cytoplasmic vacuolization and sinusoidal congestion. However, in the EA and DMPPI groups, the injury was reduced after



Figure 1. EA inhibits rat serum ALT following liver I/R. Rats remained untreated (NT group) or were pre-treated with NPA, DMPPI or EA prior to the onset of 90 min ischemia, followed by 4, 8 or 24 h of reperfusion. Values are expressed as the mean ± standard error of the mean (n=12 per group). \*P<0.05 vs. sham group; \*P<0.05 vs. NT group; \$P<0.05 vs. NPA group. ALT, alanine aminotransferase; EA, electroacupuncture; I/R, ischemia/reperfusion; NT, no treatment; NPA, non-point acupuncture; DMPPI, 1,1-dimethyl-4-phenyl L-piperazinium iodide.

both 8 and 24 h reperfusion (Fig. 2), indicating EA prevented I/R induced liver injury.

Neutrophil accumulation is also an index of liver tissue injury, which was determined via the MPO concentration. The concentration of MPO in the sham group was lower than that in the other groups at 4, 8 and 24 h (Fig. 3). However, after 4 h of reperfusion, the MPO levels in the EA and DMPPI groups were merely different from those in the NT and NPA groups (P>0.05), which was inconsistent with the histopathology results. After 8 h of reperfusion, the concentration of MPO in the NT and NPA groups was much higher than that in the EA and DMPPI groups (n=6; P<0.05) and it displayed a maximum at this time-point. After 24 h of reperfusion, the concentrations of MPO in the EA group did not decline further. In addition, levels in NT and NPA groups were similar to those in the EA group at 8 h, but remained higher than those after 4 h of reperfusion (Fig. 3).

Effect of EA on serum cytokines after liver I/R. Inflammation is a key factor associated with the pathophysiology of I/R; therefore, the serum levels of inflammatory cytokines, including TNF- $\alpha$  and IL-6 and the anti-inflammatory cytokine IL-10 were assessed. There were no increases in the levels of TNF- $\alpha$ , IL-6 and IL-10 in the sham groups (Fig. 4). Compared with that in the sham groups, an increase in cytokines was detected after 4, 8 and 24 h of reperfusion in the NT and NPA groups. After 4 h of reperfusion, the inflammatory cytokines (TNF- $\alpha$ , IL-6) in the serum reached a maximum, while EA treatment had a significantly inhibitory effect on the secretion of cytokines (Fig. 4A and B), but had no effect on the secretion of the anti-inflammatory cytokine IL-10 (Fig. 4C). However, while the levels of inflammatory cytokines TNF- $\alpha$  and IL-6 in the NT an NPA groups declined rapidly after 8 and 24 h of reperfusion, they were still higher than those in the EA group. However, there were no significant differences in the levels of IL-10 among the NT, NPA and EA groups (Fig. 4A-C).



Figure 2. Pre-treatment by EA reduces I/R-induced rat liver injury-associated histopathological changes. Rats remained untreated (NT group) or were pre-treated with NPA, DMPPI or EA prior to the onset of 90 min ischemia, followed by 4,8 or 24 h of reperfusion. Histopathology was evaluated through H&E staining, original magnification, x100. EA, electroacupuncture; I/R, ischemia/reperfusion; NT, no treatment; NPA, non-point acupuncture; DMPPI, 1,1-dimethyl-4-phenyl L-piperazinium iodide.



Figure 3. EA inhibits I/R-associated neutrophil accumulation in rat livers. Rats remained untreated (NT group) or were pre-treated with NPA, DMPPI or EA prior to the onset of 90 min ischemia, followed by 4, 8 or 24 h of reperfusion. MPO in liver tissue was assessed using ELISA and indicted neutrophil accumulation. Values are expressed as the mean ± standard error of the mean (n=3 in sham group; n=6 in I/R group). \*P<0.05 vs. sham group; \$P<0.05 vs. NT group; \$P<0.05 vs. NPA group. EA, electroacupuncture; I/R, ischemia/reperfusion; NT, no treatment; NPA, non-point acupuncture; DMPPI, 1,1-dimethyl-4-phenyl L-piperazinium iodide; MPO, myeloperoxidase.

Effect of EA on the mRNA levels of cytokines in liver tissue after I/R. To investigate the suppressive effect of EA on cytokine production, the levels of inflammatory cytokines TNF- $\alpha$ , IL-6 and anti-inflammatory cytokine IL-10 mRNA in liver tissue were assessed by RT-qPCR in relativity to those in the sham group. In all I/R groups, it was demonstrated that cytokine mRNA levels were higher than those in the Sham group. No significant differences in the levels of TNF- $\alpha$  mRNA and IL-6 mRNA were identified between the EA and NT/NPA groups after 4, 8 or 24 h of reperfusion (Fig. 4D and E). Furthermore, the levels of IL-10 mRNA did not display any differences among these groups (Fig. 4F). Effect of EA on vagus blocking. To explore the mechanisms of EA, rat vagus nerves were blocked by SV or by MH injection. Following these treatments, the rats were divided into 2 subgroups (NPA or EA) at random and all received I/R surgery. The levels of ALT, liver MPO, serum TNF- $\alpha$ , IL-6 and IL-10 were significantly higher in SV and MH groups when compared to Sham group (all P<0.05; Fig. 5). No significant difference was identified in serum ALT, liver MPO and serum cytokines between NPA and EA subgroups (Fig. 5), suggesting that the protective effect of EA was abolished by vagus nerve block.

### Discussion

To the best of our knowledge, no previous studies have reported on the protective effects of EA on liver I/R, which was the aim of the present study. The results demonstrated that EA significantly attenuated liver injury, as indicated by the levels of ALT and MPO as well as histopathology. However, when the vagus nerve was blocked, EA lost its potency (16). The inflammatory cytokines assessed are correlated with the cholinergic anti-inflammatory pathway. NPA used as the negative control therapy illustrated that only EA at the specific acupoint was responsible for the specific effect. Acupuncture is a distinctive therapy in TCM and has thousands of years of history. It is increasingly prevalent to treat numerous diseases, but the mechanism has not been clarified, as it does not provide a specific cure for certain diseases and too many sites of action are present (7,15,20). LI-4 is one of the most useful acupoints in the human body and is the source point belonging to the hand yangming large intestine meridian, which is subjected to acupuncture for treating pain or inflammatory conditions (15). Hence, acupuncture at the LI-4 acupoint was performed in the present study to assess its protective effect against liver I/R injury in rats.

As in previous studies (6,16,21), the levels of ALT and MPO were assessed and liver tissues were subjected to histopathological examination following H&E staining. ALT is an index to reflect liver function and it is released into the peripheral blood upon hepatocyte injury. In the present study, the release of ALT was time-dependent, displaying a peak level at 8 h of reperfusion following liver ischemia with restoration to basal levels at 48 h without any interventional treatment of the rats. However, in the EA group, this restoration was reduced to 24 h. To the best of our knowledge, the present study was the first to demonstrate the time-dependent effect of EA on liver I/R. The results indicated that EA obviously inhibited the increase in ALT levels compared with that in the NT group in the acute phase after 4 and 8 h of reperfusion, which was similar to previously published results. However, the result that after 24 h of reperfusion (sub-acute phase), ALT levels in the EA group were still inhibited compared with those in the NT group was inconsistent with that of a previous study (16). The underlying mechanisms of action of acupuncture remain elusive, but in TCM, its long-term effects on diseases compared with those of other treatments are well-established. In the present study, H&E staining was performed to visualize tissue injury following I/R. No injury was observed in the sham operation group, while in the NT group subjected to I/R, hepatocellular breakage caused liver injury, which became increasingly severe



Figure 4. The rat serum cytokines and hepatic mRNA expression levels after I/R. Rats remained untreated (NT group) or were pre-treated with NPA or EA prior to the onset of 90 min ischemia, followed by 4, 8 or 24 h of reperfusion. Serum levels of cytokines (A) TNF- $\alpha$ , (B) IL-6 and (C) IL-10 were assessed using ELISA. \*P<0.05 vs. sham group; \*P<0.05 vs. NT group; \*P<0.05 vs. NPA group. (D-F) mRNA expression levels of (D) TNF- $\alpha$ , (E) IL-6 and (F) IL-10 in liver tissues. Values are expressed as mean ± standard error of the mean (n=10 in A-C and n=6 in D-F). TNF, tumor necrosis factor; IL, interleukin; EA, electroacupuncture; NPA, non-point acupuncture; I/R, ischemia/reperfusion; NT, not treated.

from 4 to 24 h of reperfusion, which was in accordance with a previous study (21). EA treatment had a therapeutic effect on I/R, but at 24 h, the difference between the EA and NT groups was less obvious. This meant that to a certain extent, EA inhibited the injury in the acute phase, but it did not protect against hepatocellular damage in the sub-acute phase. MPO is another index of I/R injury and reflects neutrophil accumulation (21). A difference in MPO levels between the EA and NT groups was only observed at 8 h, which may be explained by the model of the acute and sub-acute stages of I/R-induced liver injury and was in accordance with the results of the H&E staining. However, there were certain inconsistencies in the time-course of changes in the ALT and MPO levels as well as H&E staining results. This might be due to the fact that ALT is a matrix enzyme and may be gradually degraded when it is released into the blood. But the hepatocellular breaking was not a reversible procedure which was proved by H&E staining. MPO level was relatively lower after 24 h of reperfusion compared to that after 8 h of reperfusion because it was also an enzyme which would be metabolized in body.

Inflammation is one of the most complex problems in surgery. According to TCM, the central nervous system engages in cross-talk with the immune system. However, it is a slow response that does not provide any efficient protection



Figure 5. Vagus block disrupts the effects of EA on inflammatory cytokines after ischemia/reperfusion. Rats were pre-treated with NPA or EA prior to the onset of 90 min of ischemia, followed by 8 h of reperfusion and the animal model was pre-treated by SV or MH. Effects on the serum levels of (A) ALT, (B) MPO, (C) TNF- $\alpha$ , (D) IL-6 and (E) IL-10 were assessed. Values are expressed as the mean ± standard error of the mean (SV, n=10; MH, n=6). \*P<0.05 vs. sham group; #P<0.05 vs. SV group. ALT, alanine aminotransferase; MPO, myeloperoxidase; TNF, tumor necrosis factor; IL, interleukin; SV, subphrenic vagotomy; MH, mecamylamine hydrochloride; EA, electroacupuncture; NPA, non-point acupuncture.

from acute injury, including liver I/R promoted by excessive inflammation. Certain studies reported on the involvement of the cholinergic anti-inflammatory pathway (CAIP), which has shorter response times than the humoral anti-inflammatory pathways (22). The stimulated vagus releases Ach, which inhibits cytokines released from macrophages, but this effect is diminished or eliminated when the  $\alpha$ -7 receptor of nicotine is blocked (17). Another study reported that early-phase I/R-associated liver injury in rats was mitigated when the nicotine receptor of the vagus nerve was stimulated by certain drugs (16). However, the drug has various known and unknown side effects and vagus electro-stimulation is likely to be harmful for the human body (23).

In addition, inflammation is the key promoting factor of I/R-associated injury and has important roles in the whole process (24,25). However, the acute phase features a massive release of cytokines, particularly pro-inflammatory cytokines including TNF- $\alpha$  or IL-6, which are later degraded (26). In the present study, the cytokines were assessed in the serum by ELISA and in liver tissues at the mRNA level, but they were not in parallel. No significant difference was present in the liver mRNA levels of cytokines between the EA and NT

groups. However, the I/R-associated increases in the serum levels of these cytokines were effectively inhibited by EA (16). Therefore, the inhibitory effect of EA on I/R-associated liver injury may be due to the attenuation of the release of inflammatory cytokines into the serum, particularly of TNF- $\alpha$ , which is the core cytokine to stimulate various cell types, including Kupffer cells or hepatocytes, to produce various other cytokines. The response to EA was displayed in the ALT and MPO levels, as well as in the histopathology results, indicating that I/R-induced injury was reduced. Another cytokine, IL-10, which is considered to be an anti-inflammatory index, was assessed in the present study (27,28). However, the results indicated that EA had no evident effect on it at the serum or tissue mRNA level, which was similar to the results of previous studies. It was therefore demonstrated that EA did not inhibit I/R-induced liver injury through the anti-inflammatory system (26).

The effect of EA on the inflammatory system attracts numerous interests. The CAIP has been in the focus of research since 2000. Studies have indicated that direct electro-stimulation of the vagus nerve antagonized inflammation caused by sepsis and inhibited macrophages incubated with cytokines in vitro (13). Another study revealed that EA at the Zusanli (ST36) point had an anti-sepsis effect via the CAIP. It was reported that activation of the vagus CAIP protected against I/R at the early phase (29). The present study therefore hypothesized that EA protects the liver from I/R-associated injury through the CAIP. To prove this, the vagus potential was first tested under EA and the potential pulse was more obvious with a stronger current (data not shown). Subsequently, DMPPI, a nonselective nicotinic AChR agonist, was used as a positive control treatment for comparison with the EA group, which was proved to effectively activate vagus CAIP (16). The results indicated no differences between the EA group and the DMPPI group, indicating that the mechanism of EA to protect the liver from I/R was similar to that of DMPPI, namely via CAIP; however, this mechanism remains to be fully confirmed. Following this, the vagus was blocked by SV or i.p. injection of MH prior to I/R and liver injury and serum cytokines were determined after 8 h of reperfusion (13). The protective effect of EA against I/R induced liver injury was ameliorated following vagus block, indicating the primary role of the vagus in EA. A limitation of the present study is that the effect of SV and MH on liver injury was not assessed in Sham rats. As relatively little information exists regarding the effect of SV or MH on liver injury, it is hypothesized that SV and MH themselves may have little effect on liver injury and serum cytokine levels, which requires further experimental verification.

To demonstrate that only EA performed at specific acupoints, including LI-4, had a protective effect, the experimental design of the present study included an NPA group. All indexes of the NPA group were indifferent from those of the NT group, while those in the EA group exhibited a marked difference. This was supported by the ancient concept of TCM that a point with a non-meridian location, known as the 'ah-shi' point, is considered to only treat pain that does not concentrate in one area but does not have any specific curative effect as acupuncture performed at a meridian point such as LI-4 (15).

In TCM, LI-4 is an important acupoint, as is ST-36 and the two are always combined. Thus, as acupuncture on LI-4 affects

ST-36; the LI-4 acupoint was selected for the present study. LI-4 belongs to the hand yangming large intestine meridian, which is connected with the foot yangming stomach meridian, on which ST-36 is located. Therefore, acupuncture on LI-4 has part of the effect of that on ST-36. ST-36 is always used to exert anti-inflammatory effects and a previous study reported that the CAIP constituted the major mechanism of the effect of acupuncture on ST-36 against lipopolysaccharide-induced sepsis in rats (15). This study demonstrated the power of acupuncture, but it only determined that it reduced the sepsis, but did not fully prevent its occurrence. In the present study, the anti-inflammatory function of EA at LI-4, an important acupoint with strong therapeutic efficacy, was investigated in association with its protective effect against liver I/R. EA is safer for humans than drugs or direct electro-stimulation of the vagus nerve, as it has barely any side effects and has been used for thousands of years in China, while drugs are currently not approved for clinical use and direct vagus stimulation is obviously dangerous.

In conclusion, the present study indicated that the EA effectively protects the liver from I/R injury and the major mechanism of action was to reduce pro-inflammatory cytokines through activation of the CAIP. However, anti-inflammatory cytokines did not have any important role. Acupuncture is an ancient TCM treatment with significant efficacy, although the underlying mechanisms remain elusive. The function of acupuncture was explored and further details require to be assessed in-depth in the future.

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