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Research Paper

# Cepharanthine alleviates liver injury in a rodent model of limb ischemia–reperfusion

Yin-Kuang Chang [1], Su-Cheng Huang [1], Ming-Chang Kao [2, 3 †], Chun-Jen Huang [2, 3 * †]

[1] Department of Obstetrics/Gynecology, Taipei Tzu Chi Hospital, Taipei, Taiwan
[2] Department of Anesthesiology, Taipei Tzu Chi Hospital, Taipei, Taiwan
[3] School of Medicine, Tzu Chi University, Hualien, Taiwan

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ABSTRACT

*Objectives:* Limb ischemia–reperfusion (I/R) causes remote organ injury (e.g., liver injury). Oxidation and inflammation are crucial mechanisms. We investigated the effects of cepharanthine, a potent anti-oxidative and anti-inflammatory drug, on alleviating liver injury induced by limb I/R.

*Methods:* Twenty-four adult male Sprague-Dawley rats were randomized to receive sham operation (Sham), Sham plus cepharanthine, I/R, or I/R plus cepharanthine and designated as the Sham, Sham+Cep, I/R, or I/R+Cep group, respectively ($n = 6$ in each group). I/R was induced by applying rubber band tourniquets high around each hind limb for 3 hours followed by reperfusion for 24 hours.

*Results:* The plasma levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) of the Sham and Sham+Cep groups were low, and the levels of AST and ALT of the I/R group were significantly higher than those of the Sham group (both $p < 0.001$). By contrast, the AST and ALT of the I/R+Cep group were significantly lower than those of the I/R group (both $p < 0.001$). The hepatic levels of nitric oxide (NO), malondialdehyde (MDA), macrophage inflammatory protein 2 (MIP-2), interleukin-6 (IL-6), and cyclooxygenase-2 (COX-2)/prostaglandin $E_2$ ($PGE_2$) of the Sham and Sham+Cep groups were also low. As expected, the NO, MDA, MIP-2, IL-6, and COX-2/$PGE_2$ of the I/R group were significantly higher than those of the Sham group (all $p < 0.001$). By contrast, the NO, MDA, MIP-2, IL-6, and COX-2/$PGE_2$ of the I/R+Cep group were significantly lower than those of the I/R group (all $p < 0.05$).

*Conclusion:* Cepharanthine alleviates liver injury in a rodent model of limb I/R. The mechanisms may involve reducing oxidation and inflammation.



## 1. Introduction

Vital organs (e.g., the liver) are susceptible to the influence of limb ischemia and subsequent reperfusion (I/R).[1–6] The underlying mechanisms may involve oxidation and inflammation.[3–6] Experimental data have demonstrated that antioxidation and/or anti-inflammation therapies may alleviate the remote organ injury induced by limb I/R.[3–5]

Cepharanthine is an alkaloid extract from *Stephania cepharantha* Hayata.[7] Cepharanthine possesses therapeutic potential and has been used in treatments of clinical conditions, including idiopathic thrombocytopenic purpura, refractory anemia, radiation-induced leukopenia, alopecia areata, and sarcoidosis.[8,9] Cepharanthine possesses potent antioxidative and anti-inflammatory capacity.[8–10] In line with this notion, we speculate that cepharanthine may exert therapeutic effects and alleviate liver injury induced by limb I/R.

To elucidate further, we conducted this study. Our hypothesis was that cepharanthine can mitigate liver injury induced by limb I/R in rats. This study employed a rodent model of bilateral hind limb I/R to facilitate investigations. Assays of histology and liver function as well as oxidation and inflammation were preformed to confirm the therapeutic effects of cepharanthine and the possible underlying mechanisms.

## 2. Methods

This study employed a total of 24 adult male Sprague-Dawley rats (200–250 g; BioLASCO Taiwan Co., Ltd., Taipei, Taiwan). All animal experiments were approved by the Institutional Animal Use and Care Committee, Taipei Tzu Chi Hospital, Taipei, Taiwan

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* Corresponding author. Department of Anesthesiology, Taipei Tzu Chi Hospital, 289 Jianguo Road, Sindian District, New Taipei City 231, Taiwan.
*E-mail address:* huangcj1112@gmail.com (C.-J. Huang).

† These two authors contributed equally to this study.

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(103-IACUC No.020). Care and handling of the rats were performed in accordance with the guidelines of the National Institutes of Health. All rats were supplied with standard laboratory chow and water ad libitum until the day of the experiment.

### 2.1. Anesthesia and hind limb I/R protocol

All rats were anesthetized with a mixture of zoletil/xylazine (30/10 mg/kg, i.m.) and placed supine on a heating pad. Supplemental doses of the zoletil/xylazine mixture (10/3 mg/kg) were administered hourly until the end of each experiment. To induce hind limb I/R, rubber band tourniquets were applied high around each hind limb for 3 hours followed by removal of the rubber bands for 24 hours, according to previous reports.[4,11] During the reperfusion period, the rats were returned to their cages and supplied with standard laboratory chow and water ad libitum, as mentioned above.

### 2.2. Experimental protocols

The rats were randomly allocated to the sham operation (Sham), Sham plus cepharanthine (Sham+Cep), limb I/R (I/R), or I/R plus cepharanthine (I/R+Cep) group ($n = 6$ in each group). The Sham group received sham operation plus vehicle injection [30 μL of dimethyl sulfoxide (DMSO), i.p.; Sigma–Aldrich, St. Louis, MO, USA]. The Sham+Cep group received sham operation plus cepharanthine (10 mg/kg, i.p.; LKT Laboratories, Inc., St. Paul, MN, USA). The I/R group received I/R plus vehicle (30 μL of DMSO, i.p.). The I/R+Cep group received I/R plus cepharanthine (10 mg/kg, i.p.). Administration of vehicle or cepharanthine was performed immediately before reperfusion or at a comparable time point in the Sham groups. The dose of cepharanthine was determined according to a previous report.[10]

### 2.3. Collections of blood and tissue samples and liver function assay

At the end of the reperfusion period, the rats were anesthetized again to facilitate blood drawing. Then, all rats were euthanized with a high-dose pentobarbital (100 mg/kg, i.p.). The liver was removed, snap-frozen, and then stored at −80°C for subsequent analysis.

The blood samples (5 mL) were then centrifuged (1500 g × 5 minutes) to separate plasma. The plasma samples (1 mL) were then analyzed using a chemistry analyzer (Roche Reflotron 1 Chemistry Analyzer; Roche Diagnostic Corp., Indianapolis, IN, USA) to determine the plasma concentrations of aspartate aminotransferase (AST) and alanine aminotransferase (ALT).

### 2.4. Assays of nitric oxide and malondialdehyde

The snap-frozen liver tissue samples were processed, as previously reported,[12] to facilitate oxidative status evaluation. In brief, the concentrations of nitrite and nitrate [i.e., the stable nitric oxide (NO) metabolites] of the liver tissue samples were measured using a colorimetric assay kit (Cayman Chemical, Ann Arbor, MI, USA) to measure the concentrations of reactive nitrogen species. The malondialdehyde (MDA) concentrations of the liver tissue samples were also measured with a thiobarbituric acid reactive substance assay kit (Cayman Chemical) to assay the status of lipid peroxidation.

### 2.5. Assays of inflammatory molecules

The liver tissue samples were processed, also as previously reported.[12] The inflammatory molecule concentrations of the liver tissue samples, including chemokine (e.g., macrophage inflammatory protein-2, MIP-2), cytokine (e.g., interleukin-6; IL-6) and prostaglandin $E_2$ ($PGE_2$), were measured using enzyme-linked immunosorbent assay kits (Enzo Life Science, Farmingdale, NY, USA) according to the manufacturer's instructions.

### 2.6. Assay of cyclooxygenase-2 expression

Cyclooxygenase-2 (COX-2) tightly regulates the production of $PGE_2$.[13] The expression of COX-2 mRNA in the liver tissue samples was measured using reverse transcription and polymerase chain reaction (RT–PCR). Tissue processing was performed as previously reported.[4] The primer sequences of COX-2 and β-actin (as an internal standard) and RT–PCR protocols were also adapted from our previous report.[4] After separation, the PCR-amplified cDNA band densities were quantified using densitometric techniques (Scion Image for Windows, Scion Corp., Frederic, MD, USA).

### 2.7. Statistical analysis

One-way analysis of variance with Tukey post-hoc test was used for multiple comparisons. Data were presented as means ± standard deviations. The significance level was set at 0.05. A commercial software package (SigmaStat for Windows, SPSS Inc., Chicago, IL, USA) was used for data analysis.

## 3. Results

### 3.1. Liver function assay

The plasma levels of AST and ALT of the Sham and Sham+Cep groups were low (Figure 1). As expected, the plasma levels of AST and ALT of the I/R group were significantly higher than those of the Sham group (both $p < 0.001$; Figure 2). However, the plasma levels of AST and ALT of the I/R+Cep group were significantly lower than those of the I/R group (both $p < 0.001$; Figure 1).

### 3.2. Oxidative status assays

As expected, the hepatic levels of NO of the Sham and Sham+Cep groups were low, and the hepatic level of NO of the I/R group was significantly higher than that of the Sham group ($p < 0.001$; Figure 2). By contrast, the hepatic level of NO of the I/R+Cep group was significantly lower than that of the I/R group ($p < 0.001$; Figure 2).

The hepatic MDA data basically paralleled the hepatic NO data. The hepatic MDA level of the Sham group was low and the hepatic MDA level of the I/R group was significantly higher than that of the Sham group ($p < 0.001$; Figure 2). Similarly, the hepatic MDA level of the I/R+Cep group was significantly lower than that of the I/R group ($p = 0.039$; Figure 2). The hepatic MDA levels of the Sham+Cep and I/R+Cep groups were significantly lower than that of the Sham group ($p = 0.007$ and $p = 0.017$, respectively; Figure 2).

### 3.3. Assays of inflammatory molecules

The hepatic levels of MIP-2 and IL-6 of the Sham and Sham+Cep groups were low, and the hepatic levels of MIP-2 and IL-6 of the I/R group were significantly higher than those of the Sham group (both $p < 0.001$; Figure 3). The hepatic levels of MIP-2 and IL-6 of the I/R+Cep group were significantly lower than those of the I/R group ($p < 0.001$ and $p = 0.039$, respectively; Figure 3).

Similarly, the hepatic levels of COX-2 mRNA and $PGE_2$ of the Sham and Sham+Cep groups were low, and the hepatic levels of COX-2 mRNA and $PGE_2$ of the I/R group were significantly higher