EXPERIMENTAL STUDY

Relationship between iNOS expression and apoptosis in cerebral tissue, and the effect of Sini injection in endotoxin shock rats

Hui Pei, Yuzhong Zhang, Haiyan Wu, Liwei Ren, Xu Jia, Yinzhu Zhang, Ming Chen

**Abstract**

**OBJECTIVE:** To investigate the dynamic changes and relationship of inducible nitric oxide synthase (iNOS) and apoptosis in endotoxin shock rats, as well as the effects of Sini injection.

**METHODS:** In total, 102 Sprague-Dawley (SD) rats were randomly divided into a normal group (n=6, NG), sham operation group (n=24, OG), model group (n=24, MG), dexamethasone group (n=24, DG), and Sini group (n=24, SG). The endotoxin shock model was induced by an intravenous injection of lipopolysaccharide (LPS) (8 mg/kg). Rats in the OG, MG, DG, and SG groups were further divided into 4 groups: 1, 2, 3 and 6 h after shock groups (n=6 per group). iNOS expression was detected by immunohistochemistry. Terminal Deoxynucleotidyl Transferase Mediated Deoxyuridine Triphosphate-biotin Nick End Labeling was employed to measure apoptosis.

**RESULTS:** No iNOS expression was found in the OG group. Compared with the OG group, iNOS expression in the MG group was markedly elevated, reached a peak at 1 h (P<0.01), decreased at 2 and 3 h, and rebounded at 6 h. Compared with the MG group, iNOS expression decreased significantly in both the DG (P<0.05) and SG (P<0.01) groups at 6 h. The number of apoptotic cells in the MG group was markedly increased than that in the NG and OG (P<0.01) groups, and reached a peak at 6 h. The number of apoptotic cells in the DG group at 1 and 2 h (P<0.01) and SG group at 2, 3 and 6 h (P<0.01) decreased when compared with the MG group.

**CONCLUSION:** Sini injection can significantly inhibit NO generation, which decreases apoptosis and subsequently protects the brain from endotoxic shock.

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**Key words:** Septic shock; Nitric oxide synthase type II; Apoptosis; Sini injection

**INTRODUCTION**

Lipopolysaccharide (LPS)-induced endotoxic shock has been characterized by multiple organ failure, low blood pressure, and high mortality. Our study used endotoxic shock to simulate septic shock. The ultimate consequence of septic shock is a systemic inflammatory response generated by infection. The increasing morbidity and mortality associated with septic shock is a major public health problem. Currently, controlling blood pressure, infection, and blood clotting are the principle treatments, however, these strategies are limited with medical level. In Traditional Chinese Medicine (TCM), Sini decoction has been used to treat septic shock effectively for many years.
aimed to investigate the dynamic changes and relationship of inducible nitric oxide synthase (iNOS) and apoptosis in endotoxin shock rats, and disclose the protective mechanism of Sini decoction.

MATERIALS AND METHODS

Animals and grouping

One hundred and two Sprague-Dawley (SD) rats (51 males and 51 females; 6-8 weeks of age; body weight 180-200 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. Quote the agreement of Local Ethics Committee of the Institution on Laboratory Animal Care in Methods. All animal studies were approved by the Medical Ethics Committee of Xiyuan Hospital of China Academy of Chinese Medical Sciences. All rats were randomly divided into 4 groups: 1, 2, 3 and 6 h after shock divided into 5 groups: normal group (n=6, NG); sham-operated group (n=24, OG); model group (n=24, MG); dexamethasone group (n=24; DG); Sini group (n=24, SG). Rats in the OG, MG, DG, and SG groups were further divided into 4 groups: 1, 2, 3 and 6 h after shock n=6 rats per group. All rats had free access to food and drink and were housed at room temperature (23°C-27°C) at a humidity of 50%-60% for 3 days. Rats had no access to food or drink the night before surgery. Rats were anesthetized by injection with 3% (v/v) pentobarbital sodium solution (1 mL/kg) into the abdominal cavity. Under sterilized conditions, a PE 50 tube with heparin was intubated into the left common carotid artery. The tube was connected to a BL-420E+ organism function experiment system through a YH-4 pressure transducer. After blood pressure stabilized for 20 min, rats in the MG group received LPS (8 mg/kg) through the femoral vein and then normal saline (50 mL/kg) by intravenous drip. Successful model establishment was obtained when mean arterial blood pressure (MABP) decreased by more than 25%. The OG group received normal saline (20 mL/kg). The DG group received 1/3 of the total amount of dexamethasone (10 mL/kg) first by intravenous infusion, LPS (8 mg/kg) by intravenous infusion, and 2/3 of dexamethasone by intravenous drip. The injection method for the SG group was same for DG rats. The dose of transfusion for each rat was 50 mL/kg, and the injection was completed within 30 min. Brain tissues from rats were obtained at 1, 2, 3, and 6 h. Rats were fixed and their heart and liver were explored after a vertical incision to the middle of the chest. The left cardiac apex was cut, inserted and fixed with a tube, and then the right atrium was cut. The rat was quickly perfused with warm normal saline solution until the liver turned white, followed by 150 mL of 4% (w/v) paraformaldehyde solution. After completion of the perfusion, rats were decapitated and brains, without the olfactory bulb, cerebellum and lower brainstem, were post-fixed in 4% (w/v) paraformaldehyde solution and embedded in paraffin. Coronal sections were cut at 5 μm.

Reagents and instruments

iNOS I-Ab was purchased from Biosen Biotechnology Company (Beijing, China); Immunohistochemical staining and DAB kit was from Boster Biological Technology Company (Wuhan, China); lipopolysaccharide was from Sigma Biotechnology Company (NJ, USA). Pentobarbital sodium was purchased from Fuchen Chemical Reagent Factory (Tianjin, China), saline from Shuanghe Pharmacological Group (Beijing, China). Sini injections from the College of Pharmacy of Beijing University of Traditional Chinese Medicine [chief elements: Fuzi (Radix Aconiti Lateralis Preparata) 15 g, Ganjiang (Rhizoma Zingiberis) 9 g, Gancao (Radix Glycyrrhizae) 6 g, size: 10 mL/cig]; dexamethasone injection was from Pharmaceutical Industry Limited Company (Tianjin, China, size: 10 mL/cig); heparin sodium injection was from Biochemistry Chemical Pharmaceutical Factory (Tianjin, China). The BL-420E+ organism function experiment system was from Taimeng Technology Co., Ltd. (Chengdu, China).

Immunohistochemical staining

Sections underwent routine deparaffinization, followed by antigen retrieval using 0.01 mol/L (pH 6.0) citrate buffer in a microwave (mid-range heat, 8-10 min, with cooling to room temperature; repeated 3 times). Endogenous peroxidase activity was inhibited with 3% (v/v) H2O2, then the sections were washed by serum PBS (3 min and repeated 3 times). After excess serum was removed, sections were incubated with the primary antibody in humidified boxes at 37°C for 60 min (1:200). They were washed once again and incubated with biotin conjugated secondary antibodies working solution at 37°C for 3 min and repeated 3 times. After excess serum was removed, sections were incubated with the primary antibody in humidified boxes at 37°C for 60 min (1:200). They were washed once again and incubated with biotin conjugated secondary antibodies working solution at 37°C for 30 min, and then washed again and incubated with Streptavidin-Biotin Comlex (SABC) (Wuhan Boster Bioengineering Co., Ltd., China) for 30 min at 37°C. Subsequently, sections were colored with DAB (3, 3-diaminobenzidine), dehydrated, and then coverslipped. Five visual fields (×200) were randomly taken for each slice and images were analyzed using the MiEV 2.0 Analysis system (Yichuang Electronics Co., Ltd., Shangdong, China). The numbers of positive cells was expressed as a percentage of the total number of cells counted.

Terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL)

After deparaffinization, sections underwent TUNEL according to the manufacturer’s instructions and observed under the microscope. Five high-power images from each slice were selected and the numbers of positive cells were counted. The apoptosis index using propidium iodide (PI) was calculated as follows: PI (%) = numbers of positive nuclei/total number of nu-
Statistical analysis

All data were analyzed using SAS (vers 8.2, SAS Institute, Chicago, IL, USA) software. Quantitative data are expressed as the mean ± standard deviation \( (\bar{x} \pm s) \). The single factor analysis of variance of completely randomized design was used for parameter comparison between groups. \( P<0.05 \) was considered statistically significant.

RESULTS

Changes of general living states and mean arterial blood pressure

Endotoxin shock rats appeared to have poor mental condition, with reduced activity, listlessness, dull fur, cold limbs, and diarrhea, which belonged to a lesser Yin pattern with declined Yang in TCM. There was no marked change in MABP in the OG group. Compared with the OG group, MABP in the MG group decreased significantly after injection of LPS \((P<0.01)\) and was reduced by 40.8% of the initial value at 6 h. Compared with the OG group, MABP in the DG group decreased significantly after injection of LPS + DEX \((P<0.05)\) and reduced by 45.3% of the initial value at 6 h. However, MABP in the DG group was higher than that in the MG group \((P<0.05)\). Compared with the OG group, MABP of the SG group decreased significantly after injection of LPS + SINI \((P<0.01)\) and reduced by 67.7% of the initial value at 6 h. However, the MABP in the SG group was significantly higher than that in the MG group \((P<0.01)\).

iNOS expression

Immunohistochemical staining revealed that no iNOS expression was found in the OG and NG groups. iNOS expression in the MG group reached a maximum at 1 h (Figure 1). There were no significant differences between the OG and NG groups \((P>0.05)\). Compared with the OG group, there were significant differences among groups, except in the NG group \((P<0.01)\), at 1 h; however, no significant differences were observed between the MG and SG groups \((P>0.05)\). Compared with the OG group, changes were the same at 2 h as at 1 h \((P<0.01)\); however, there were no significant differences in each group when compared with the MG group \((P>0.05)\). Changes at 3 h were similar to 2 h. Compared with the OG group, changes were the same at 6 h as at 1 h \((P<0.01)\). Compared with the MG group, iNOS expression was significantly lower in the DG \((P<0.05)\) and SG groups \((P<0.01)\) (Figure 1).

Cell apoptosis

The extent of rat myocardial cell apoptosis was not significantly different between the OG and NG groups \((P>0.05)\). There were significant difference between the MG group at 2 h and MG group at 1, 3 and 6 h \((P<0.01)\) (Figure 2). Apoptosis in the DG group reached a maximum at 3 h, and there was no significant difference between the 1 and 2 h subgroups \((P>0.05)\). Compared with the SG group at 1 h, there was a significa-

Table 1 MABP levels in each rat endotoxic shock group (KPa, \( \bar{x} \pm s \))

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>0 h</th>
<th>1 h</th>
<th>2 h</th>
<th>3 h</th>
<th>6 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>OG</td>
<td>6</td>
<td>106.6±4.2</td>
<td>100.2±2.0</td>
<td>96.4±1.8</td>
<td>92.6±1.5</td>
<td>90.5±1.1</td>
</tr>
<tr>
<td>MG</td>
<td>6</td>
<td>104.2±4.8</td>
<td>88.5±1.7</td>
<td>69.1±1.9</td>
<td>55.0±3.1</td>
<td>42.4±1.5</td>
</tr>
<tr>
<td>DG</td>
<td>6</td>
<td>102.2±4.2</td>
<td>97.1±2.4(^a)</td>
<td>77.3±2.8(^b)</td>
<td>56.8±2.8(^c)</td>
<td>46.3±2.8(^d)</td>
</tr>
<tr>
<td>SG</td>
<td>6</td>
<td>102.8±5.1</td>
<td>88.8±1.5(^c)</td>
<td>68.9±1.3(^e)</td>
<td>63.8±3.6(^e)</td>
<td>69.6±2.0(^e)</td>
</tr>
</tbody>
</table>

Notes: OG: sham-operated group, rats received normal saline (20 mL/kg) by intravenous infusion; MG: model group, rats received LPS (8 mg/kg) through the femoral vein and then normal saline (50 mL/kg) by intravenous drip; DG: dexamethasone group, rats received 1/3 of the total amount of dexamethasone (10 mL/kg) first by intravenous infusion, LPS (8 mg/kg) by intravenous infusion, and 2/3 of dexamethasone by intravenous drip; SG: Sini group, rats received 1/3 of the total amount of Sini injection (10 mL/kg) first by intravenous infusion, LPS (8 mg/kg) by intravenous infusion, and 2/3 of Sini injection by intravenous drip. SABC: streptavidin-biotin complex; iNOS: inducible nitric oxide synthase.

Figure 1 Myocardial immunohistochemical staining of rats (SABC×200)

A: NG; B: OG; C: MG at 1 h; D: DG at 1 h; E: SG at 6 h. NG: normal group, rats received normal diet; OG: sham-operated group, rats received normal saline (20 mL/kg) by intravenous infusion; MG: model group, rats received LPS (8 mg/kg) through the femoral vein and then normal saline (50 mL/kg) by intravenous drip; DG: dexamethasone group, rats received 1/3 of the total amount of dexamethasone (10 mL/kg) first by intravenous infusion, LPS (8 mg/kg) by intravenous infusion, and 2/3 of dexamethasone by intravenous drip; SG: Sini group, rats received 1/3 of the total amount of Sini injection (10 mL/kg) first by intravenous infusion, LPS (8 mg/kg) by intravenous infusion, and 2/3 of Sini injection by intravenous drip. SABC: streptavidin-biotin complex; iNOS: inducible nitric oxide synthase.
Recently, researchers have used LPS to establish animal models to study septic shock. Compared with sham group, intravenous infusion, and 2/3 of dexamethasone by intravenous drip; SG: Sini group, rats received 1/3 of the total amount of Sini injection (10 mL/kg) first by intravenous infusion, LPS (8 mg/kg) by intravenous infusion, and 2/3 of Sini injection by intravenous drip. TUNEL: terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate-biotin nick end labeling; SABC: streptavidin-biotin complex; iNOS: inducible nitric oxide synthase.

In our study, we injected different doses of LPS (4, 6, 8, 10 mL/kg) into the rat via the femoral vein and then normal saline (50 mL/kg) by intravenous drip. Compared with sham group, there were no significant differences among other groups (P > 0.05). However, there were significant differences among other groups (P < 0.01). At 1 h, there were no significant differences between the DG and SG groups, and the MG and SG groups (both P > 0.05). Compared with the OG group, there was a significant difference in the DG and SG groups at 2 h (P < 0.05). Compared with the MG group, there was a significant difference in the DG and SG groups (P < 0.01). Compared with the MG group, there was no significant difference in the DG group (P > 0.05) at 3 h. A similar result was found in the SG group when compared with the OG group (P > 0.05). However, there were significant differences among other groups (P < 0.01). The changes at 6 h were similar to 3 h (Table 2).

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>1 h</th>
<th>2 h</th>
<th>3 h</th>
<th>6 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>NG</td>
<td>6</td>
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<td>0</td>
<td>0</td>
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<tr>
<td>OG</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MG</td>
<td>6</td>
<td>66.67*</td>
<td>20.42*</td>
<td>24.50*</td>
<td>61.00*</td>
</tr>
<tr>
<td>DG</td>
<td>6</td>
<td>46.17*</td>
<td>13.92*</td>
<td>32.00*</td>
<td>50.83*</td>
</tr>
<tr>
<td>SG</td>
<td>6</td>
<td>62.33*</td>
<td>11.33*</td>
<td>29.00*</td>
<td>19.83*</td>
</tr>
</tbody>
</table>

Notes: NG: normal group, rats received normal diet; OG: sham-operated group, rats received normal saline (20 mL/kg) by intravenous infusion; MG: model group, rats received LPS (8 mg/kg) through the femoral vein and then normal saline (50 mL/kg) by intravenous drip; DG: dexamethasone group, rats received 1/3 of the total amount of dexamethasone (10 mL/kg) first by intravenous infusion, LPS (8 mg/kg) by intravenous infusion, and 2/3 of dexamethasone by intravenous drip; SG: Sini group, rats received 1/3 of the total amount of Sini injection (10 mL/kg) first by intravenous infusion, LPS (8 mg/kg) by intravenous infusion, and 2/3 of Sini injection by intravenous drip. Compared with sham group, *P < 0.01; compared with model group, †P < 0.01; ‡P < 0.05.

**DISCUSSION**

The use of an effective, reproducible, reliable, and stable animal model is a prerequisite to study septic shock. Recently, researchers have used LPS to establish a septic shock model in animals due to its good controllability. In our study, we injected different doses of LPS (4, 6, 8, 10 mL/kg) into the rat via the femoral vein, tail vein and abdominal cavity, and continuously monitored blood pressure for 6 h. We found that tail vein injection was ineffective and that the effects of LPS following abdominal cavity injection were too slow. If 4 or 6 mL/kg of LPS were injected into the femoral vein, blood pressure showed a transient fall after 3-5 min, then recovered to normal range quickly. If 8 or 10 mL/kg of LPS was injected into the femoral vein, blood pressure dropped by 25% after 3-5 min, then decreased from 1 to 6 h. Rat mortality was virtually 100% until 12 h after 8 mL/kg LPS injection or until 6 h after 10 mL/kg LPS injection. Therefore, our study used 8 mL/kg of LPS. After LPS injection, endotoxin shock rats appeared to be in poor metal condition, had reduced activity, had dull fur, cold limbs, and diarrhea. MABP in the model group dropped by 40.8% of the initial value at 6 h, which showed that the septic shock model was established successfully.

Sini decoction from *Shang Han Lun*, is a basic prescription for curing Shaoyin disease. It can increases Yang and reduces Yin, thereby restoring the balance declines. During the course of disease, ‘cold’ injuring Yang is the main feature. Sini decoction plays an important role in warming Yang, dispelling cold, and preventing Yang decline. In this study, we chose Sini decoction for the treatment of rats with septic shock as it has a more Yin pattern with declined Yang. Sini decoction is made up of Fu (Radix Aconiti Lateralis Preparata) 15 g, Ganjiang (Rhizoma Zingiberis) 9 g, Gancao (Radix Glycyrrhizae) 6 g. Fu (Radix Aconiti Lateralis Preparata) is the principal herb in the formula, acts on the heart, spleen, kidney meridians and can warm Yang, dissipate cold and restore Yang stop collapse. Ganjiang (Rhizoma Zingiberis), another important herb in the formula, acts on the heart, spleen, and lung meridians and can warm the middle, dissipate cold, support Yang and promote blood circulation. The mutual promotion of Fu (Radix Aconiti Lateralis Preparata) and Ganjiang (Rhizoma Zingiberis) can warm to generate nurture and foster nature. Gancao (Radix Glycyrrhizae), the adjuvant and conductant herb in the formula, can supply the center and replenish Qi, ease the drastic nature of Ganjiang (Rhizoma Zingiberis) and Fu (Radix Aconiti Lateralis Preparata), and harmonize the medicinal properties.
Nitric oxide (NO) is a known signaling molecule that has neuroprotective and neurotoxic effects during cerebral ischemic injury. Because of its rapid degradation, quantitative determination is difficult. Therefore, NO synthase has been used to monitor NO production during ischemic cerebrovascular disease in recent years. NO affects blood vessel function and has cytotoxic effects during septic shock. In some experiments, LPS acts on endotheliocytes to enhance iNOS activity and increase NO release. In rats lacking the iNOS gene, a decline in vascular contractile function following LPS exposure was relatively mild, indicating that its lethality rate was relatively low. It has been suggested that iNOS plays an important role in endotoxic shock. iNOS is not expressed in the normal brain, but during pathogenesis, such as inflammation, after LPS and cytokine stimulation, some cells can induce gene transcription, induce protein biosynthesis, and create iNOS, which produces more NO than constitutive nitric oxide synthase. Lee et al found that NO produced by iNOS plays an important role in neuron death in culture media of rat hippocampal slices after treatment with LPS. Our experimental results showed that there was no expression of iNOS in the NG and OG groups, which is consistent with previous reports. In the MG group, expression levels reached a peak at 1 h, then fell at 2 and 3 h, and rose at 6 h. This could be because of a transient increase in iNOS in the brain. Along with progressive brain tissue injury, L-Arginine supplies decrease and iNOS activity is inhibited. As a result, iNOS expression fell at 2 and 3 h. At 6 h, the brain generated massive amounts of cytokines to activate iNOS, thus iNOS increased again. iNOS expression decreased in the DG group at 1 and 2 h and in the SG group at 6 h. The Sini decoction took effect slowly but evidently, and showed strong inhibition on iNOS expression. Brain injury during septic shock can lead to apoptosis, which is affected by apoptosis-related gene expression and can be adjusted by many internal and external factors. NO produced by iNOS is related to apoptosis.

In our study, we found that rat brain tissue had few apoptotic cells in the NG and OG groups. Compared with the OG group, apoptotic cells in the MG group were significantly increased and peaked at 6 h. NO growth induced rat brain apoptosis following endotoxic shock. Compared with the MG group, the apoptotic reduction in the DG group at 1 and 2 h showed that dexamethasone had rapid onset. Apoptotic cells in the SG group decreased at 2, 3 and 6 h, indicating a slower but durable effect on apoptosis.

Overall, Sini injection had a certain therapeutic effect on shock as it could both raise blood pressure and decrease the expression of iNOS and apoptosis. Sini decoction took effect slowly but lasted longer, while the action of dexamethasone injection was early, its effects were unsustainable.

### REFERENCES


**Table 3** PI-positive cell number in each rat endotoxic shock group ( $\bar{x} \pm s $ )

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>1 h</th>
<th>2 h</th>
<th>3 h</th>
<th>6 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>NG</td>
<td>6</td>
<td>2.31±0.37</td>
<td>2.31±0.37</td>
<td>2.31±0.37</td>
<td>2.31±0.37</td>
</tr>
<tr>
<td>OG</td>
<td>6</td>
<td>2.34±0.55</td>
<td>2.29±0.53</td>
<td>2.50±0.32</td>
<td>3.05±0.93</td>
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<tr>
<td>MG</td>
<td>6</td>
<td>16.53±2.34</td>
<td>9.48±0.42</td>
<td>12.03±1.86</td>
<td>17.17±1.63</td>
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<tr>
<td>DG</td>
<td>6</td>
<td>2.55±0.15‘</td>
<td>2.17±0.30‘</td>
<td>19.97±2.78‘a</td>
<td>13.32±3.09‘a</td>
</tr>
<tr>
<td>SG</td>
<td>6</td>
<td>8.47±1.47‘a</td>
<td>2.30±0.55‘</td>
<td>2.30±0.41‘</td>
<td>2.07±0.53‘</td>
</tr>
</tbody>
</table>

Notes: NG: normal group, rats received normal diet; OG: sham-operated group, rats received normal saline (20 mL/kg) by intravenous infusion; MG: model group, rats received LPS (8 mg/kg) through the femoral vein and then normal saline (50 mL/kg) by intravenous drip; DG: dexamethasone group, rats received 1/3 of the total amount of dexamethasone (10 mL/kg) first by intravenous infusion, LPS (8 mg/kg) by intravenous infusion, and 2/3 of dexamethasone by intravenous drip; SG: Sini group, rats received 1/3 of the total amount of Sini injection (10 mL/kg) first by intravenous infusion, LPS (8 mg/kg) by intravenous infusion, and 2/3 of Sini injection by intravenous drip.

PI: neurocyte apoptosis index. Compared with model group, ‘P<0.01; compared with sham group, ‘P<0.05.
2731-2737.


