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Sodium Tanshinone IIA Sulfonate Improves Hemodynamic Parameters, Cytokine Release, and Multi-Organ Damage in Endotoxemia Rabbits

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Data Interpretation D
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Background: The aim of this study was to evaluate the protective effects of sodium tanshinone IIA sulfonate (STS) on hemodynamic parameters, cytokine release, and multiple organ damage in an animal model of lipopolysaccharide (LPS)-induced endotoxemia.

Material/Methods: Twenty-four rabbits were randomly divided into 3 groups: control (n=8), LPS (n=8), and STS pretreatment + LPS (n=8) groups. With arterial invasive monitoring, hemodynamic variables were observed at 30 min before and at 0, 10, 20, 30, 60, 120, 180, 240, and 300 min after LPS injection. Circulatory inflammatory cytokines, including tumor necrosis factor- α (TNF- α) and interleukin-10 (IL-10), and relevant biochemical markers, including arterial partial pressure of oxygen (PaO₂), plasma cardiac troponin I (cTnI), alanine aminotransferase (ALT), and creatinine (Cr), were measured at each time point. At the end of the experiment, all rabbits were sacrificed; histopathological examination of the heart, lung, liver, and kidney tissue was performed and organ injury was semi-quantitatively scored for each organ.

Results: Mean arterial pressure (MAP) and heart rate (HR) significantly decreased within 30 min and again after 120 min following LPS injection. However, STS pretreatment gradually normalized MAP and HR after 120 min following LPS injection. In addition, STS ameliorated LPS-induced decrease of PaO₂, LPS-induced increase of TNF- α , cTnI, and ALT, and enhanced LPS-induced increase of IL-10. Moreover, STS reduced heart, lung, and liver histopathologic injury.

Conclusions: STS can significantly stabilize LPS-induced hemodynamic deterioration, regulate inflammatory cytokine secretion, and protect heart, lung, and liver in rabbits.

MeSH Keywords: **LPS • Multiple Organ Injury • Protective Effect • STS**

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Background

The inflammatory response is a complex biological response of body tissues to harmful stimuli, such as pathogens, damaged cells, or irritants. While this response provides a protective effect for the body, it also leads to inflammation and tissue damage. Macrophages play a key role in inflammatory response and, when excessively activated, can cause the aberrant release of inflammatory cytokines such as TNF- α , which induces IL-1 β and IL-6 production, creating a cascading effect that amplifies the inflammatory response to toxic stimuli. In contrast, high expression of IL-10 inhibits inflammation and enhances immunity, revealing IL-10 as an anti-inflammatory cytokine [1]. An imbalance between pro-inflammatory and anti-inflammatory responses can damage the structure and function of vital organs such as lungs, heart, liver, and kidneys [2–5], and even progress to multiple organ dysfunction syndrome (MODS) and death. Thus, regulating cytokines release and protecting vulnerable organs at the initial phase of an inflammatory response is of clinical significant.

Salvia miltiorrhiza Bunge, also known as Danshen in Chinese, has been used historically in traditional Chinese medicine (TCM) to activate blood circulation and eliminate toxic materials [6]. Many drugs containing Danshen, such as Tanshinone capsule and Fufang Danshen dripping pill, are in clinical use in China, and the former has been approved for phase III clinical trials in the United States. Tanshinone IIA is the primary active component extracted from the dry root of *Salvia miltiorrhiza* Bunge. Sodium tanshinone IIA sulfonate (STS), a derivative of tanshinone IIA, was developed for increased water solubility and bioavailability compared to tanshinone IIA. STS has been suggested to have certain pharmacological properties, including anti-inflammatory and antioxidant effects, and inhibition of apoptosis [7]. In recent years, the anti-inflammatory property of STS has attracted increasing attention. Several *in vitro* experiments have shown a protective effect of tanshinone IIA or its derivative STS on the release of inflammatory cytokines in RAW264.7 macrophages [8–10]. In addition, *in vivo* experiments have explored the efficacy of tanshinone IIA in diverse inflammatory diseases, including encephalomyelitis, atherosclerosis, and chronic arthritis [11–13]. However, whether STS provides multi-organ protective effects in response to systematic inflammation is still unclear. This study was designed to detect the effects of STS injection on hemodynamics, release of inflammatory cytokines, and multiple organ injury in endotoxemic rabbits.

Material and Methods

Animals

Twenty-four male New Zealand rabbits, weighing (mean $\pm$ standard deviation) 2.35 ± 0.31 kg, were obtained from the

Nanjing Qinglong Mountain Rabbit Facility (Nanjing, China). Rabbits were housed with a 12: 12 h light: dark cycle in a climate-controlled room with *ad libitum* access to food and water. Experiments were conducted in accordance with animal care guidelines, using approved protocols (IACUC-1704019) from the Institutional Animal Care and Use Committee of Nanjing Medical University.

Experimental design and hemodynamic monitor

Twenty-four rabbits were randomly divided into 3 groups: a control group (n=8), a lipopolysaccharide (LPS) group (n=8), and a sodium tanshinone IIA sulfonate (STS) pretreatment + LPS group (n=8). All rabbits were fasted 1 night before the experiment. The animals were anesthetized with intraperitoneal injection of pentobarbital sodium (40 mg/kg; Shanghai Haling Biotechnology Co., Ltd., Shanghai, China), and positioned supine, with the right inguinal region sterilized with 0.5% iodine. After regional infiltration with 1% lidocaine, the right femoral vein was cannulated for medication and the right femoral artery was cannulated and connected to a multifunctional monitor (Siemens 1260, Germany). Adequate anesthesia was maintained throughout the experiment with an additional 30 mg/kg (2/3 of initial dose) pentobarbital supplement according to pain reflexes induced by a tail flinch. All rabbits breathed air spontaneously throughout the experiment. Operation duration was limited to less than 10 min, and no rabbit died due to the surgical operation.

Rabbits in the LPS group were injected intravenously with 10 ml saline and 30 min later were injected with an endotoxemia dose of LPS (60 μ g/kg; *Escherichia coli*, serotype 0111: B4, DIFCO, USA) dissolved in 10 ml of saline. Rabbits in the STS+LPS group received a pretreatment of an STS injection (20 mg/kg; Shanghai No1 Biochemical & Pharmaceutical Co., Ltd., Shanghai, China), supplemented with saline to a total volume of 10 ml, and 30 min later, 10 ml saline followed. Rabbits in the control group received 10 ml saline twice with a 30-min interval. Saline, STS, or LPS was bolus injected over a duration of 30 s. A detailed flowchart of the experiment is provided in Figure 1.

Hemodynamics, including mean arterial pressure (MAP) and heart rate (HR), were observed 30 min before, and at 0, 10, 20, 30, 60, 120, 180, 240, and 300 min after the second injection described above. At the same time points, arterial blood samples (2 ml) were collected for blood gas analysis of PaO₂ (GEM/Premier 3000, Werfen, USA). The blood was then centrifuged at 3000 rpm for 10 min, and the resulting plasma stored at -70°C for detection of cytokines, including tumor necrosis factor- α (TNF- α) and interleukin-10 (IL-10), as well as biochemical markers, including plasma cardiac troponin I (cTnI), alanine aminotransferase (ALT), and creatinine (Cr). At the end of the experiment, rabbits were sacrificed with a lethal

Figure 1. Detailed flow chart of our study.

All rabbits were anesthetized with an intraperitoneal injection of pentobarbital sodium (40 mg/kg). Then, the right femoral vein and artery were cannulated within an operation duration of 10 min. Thereafter, rabbits in LPS, STS+LPS, and control groups received saline + LPS, STS + LPS, and saline + saline, respectively. Hemodynamic monitoring and biochemical analysis were performed at various time points. At the end of the experiment, all rabbits were sacrificed and histopathologic examination of organ tissues was performed.

dose of pentobarbital, and heart, lung, liver, and kidney tissue sections were collected; tissue sample sizes were 0.5×0.5×0.5 cm and tissues were fixed in 4% paraformaldehyde for subsequent histological analysis.

Inflammatory cytokine and biochemical marker measurement

Plasma levels of TNF- α and IL-10 were measured using commercially available ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA) according to manufacturer's instructions. An automatic biochemical analyzer (OlympusAU2700, Tokyo, Japan) was used to measure cTnI, ALT, and Cr concentrations.

Histological examination

Organ tissue sections were stained with hematoxylin and eosin and observed under a light microscope (Olympus, Tokyo, Japan). Pathologic examinations were performed in the Pathology Department of the First Affiliated Hospital of Nanjing Medical University. Two observers blind to group assignments analyzed the samples (n=8 in each group) and determined levels of heart, lung, liver, and kidney injury according to semi-quantitative scoring, as shown in Tables 1–4, respectively. The mean score was used for comparison among groups.

Statistical analysis

All values are expressed as the mean $\pm$ standard deviation and treatment effects were analyzed using a one-way analysis of variance (ANOVA) with Bonferroni post hoc comparison using the Graph Pad Prism 7.0 software (Graph Pad Software Inc., San Diego, CA). $P<0.05$ was considered statistically significant.

Table 2. Lung injury score parameters [15].

Changes of lung tissues structure	Index
None	0
Focal interstitial congestion and inflammatory cell infiltration <50%	1
Diffuse interstitial congestion and inflammatory cell infiltration >50%	2
Focal consolidation and inflammatory cell infiltration <50%	3
Diffuse consolidation and inflammatory cell infiltration >50%	4

Results

STS stabilizes hemodynamic changes after LPS injection

After LPS intravenous injection, MAP significantly decreased from 95 ± 12 mmHg to 37 ± 6 mmHg at 10 min, progressively recovered from 30–60 min, and then dropped again for the remaining observation period. STS pretreatment reduced the decrease of MAP at 60 min after LPS injection (Figure 2A). Similarly, HR decreased from 267 ± 32 bpm to 96 ± 15 bpm at 10 min after injection, progressively recovered to 259 ± 30 bpm at 60 min, and then started decreasing again and continued to decrease thereafter. The STS pretreatment also led

Table 1. Heart injury score parameters [14].

Index	0	1	2	3
Myocardial edema	None	Mild	Median	Severe
Bleeding	None	<30%	30–50%	>50%
Infiltration	None	2- to 3-fold	3- to 10-fold	>10-fold

Table 3. Liver injury score parameters [16].

Index	0	1	2	3
Necrosis	None	Focal piecemeal	Continuous <50%	Continuous >50%
Bleeding	None	<30%	30–50%	>50%
Infiltration	None	2- to 3-fold	3- to 10-fold	>10-fold

Table 4. Kidney injury score parameters [17].

Injured renal tubules and shrunk glomerulus	Score
None	0
<10%	1
11–25%	2
26–45%	3
46–75%	4
>76%	5

HR change to stabilize at 60 min onward after LPS administration (Figure 2B).

STS inhibits pro-inflammatory cytokine TNF- α and induces IL-10 in rabbits with LPS-induced endotoxic shock

Serum levels of the pro-inflammatory cytokine TNF- α increased starting at 30 min after LPS injection, reaching a nearly 20-fold increase at 120 min, after which it decreased again; however,

it remained significantly higher than normal at the end of the experiment. STS had an inhibitory effect on TNF- α release from 120 min to 300 min following LPS injection (Figure 3A). IL-10 levels increased 30 min after LPS injection, peaked at 240 min, and decreased thereafter. STS injection led to a greater increase in IL-10 levels at 60–180 min following LPS injection compared to the LPS-only group (Figure 3B).

STS ameliorates PaO₂ and inhibits increase of organ injury-related biochemical markers in rabbits with LPS-induced endotoxic shock

LPS injection induced an abrupt and consistent decrease of PaO₂ from 30 min to the end of the experiment, when it reached nearly one-third of its baseline value. STS pretreatment decreased this response; however, PaO₂ remained significantly lower than the values in the control group at corresponding time points (Figure 4A).

In the organ injury-related biochemical markers, cTnI increased at 30 min after LPS stimulation; this was inhibited by STS pretreatment at 60–180 min (Figure 4B). ALT was upregulated at 60 min after LPS stimulation; STS pretreatment significantly inhibited ALT upregulation at 180–300 min (Figure 4C). In

Figure 2. STS stabilizes the hemodynamic changes after LPS injection. Mean arterial pressure (MAP; **A**) and heart rate (HR; **B**) changes at 30 min before, and 0, 10, 20, 30, 60, 120, 180, 240, and 300 min after LPS injection with or without STS pretreatment in rabbits. Data are presented as the mean $\pm$ standard deviation. * $P < 0.05$ vs. control group; # $P < 0.05$ vs. LPS group at corresponding time point; LPS – lipopolysaccharide; STS – sodium tanshinone IIA sulfonate.

Figure 3. STS inhibits pro-inflammatory cytokine TNF- α and induces IL-10 in rabbits with LPS-induced endotoxemia. Serum TNF- α (A) and IL-10 (B) plasma concentration at 30 min before, and 0, 30, 60, 120, 180, 240, and 300 min after LPS injection with or without STS pretreatment in rabbits. Data are presented as mean $\pm$ standard deviation. * $P < 0.05$ vs. control group; # $P < 0.05$ vs. LPS group at the corresponding time point; TNF- α – tumor necrosis factor- α ; IL-10 – interleukin-10; LPS – lipopolysaccharide; STS – sodium tanshinone IIA sulfonate.

Figure 4. STS ameliorates PaO₂ and inhibits increase of organ injury-related biochemical markers in rabbits with LPS-induced endotoxemia. Partial pressure of oxygen (PaO₂; A), and serum cardiac troponin I (cTnI; B), alanine aminotransferase (ALT; C), and creatinine (Cr; D) levels at 30 min before, and 0, 30, 60, 120, 180, 240, and 300 min after LPS injection with or without STS pretreatment in rabbits. Data are presented at the mean $\pm$ standard deviation. * $P < 0.05$ vs. control group; # $P < 0.05$ vs. LPS group at corresponding time point; LPS – lipopolysaccharide; STS – sodium tanshinone IIA sulfonate.

Figure 5. STS pretreatment attenuates pathological injury of heart, lung, and liver in LPS-induced endotoxemia rabbits. Representative histopathological images of heart (A), lung (B), and liver (C) tissue from rabbits in control, LPS, and STS+LPS groups. Statistical analysis of organ injury scores (D). Images in A–C were magnified by 200 $\times$. * $P < 0.05$ vs. control group; # $P < 0.05$ vs. LPS group; LPS – lipopolysaccharide; STS – sodium tanshinone IIA sulfonate.

contrast, Cr level was not affected by LPS injection, with or without STS pretreatment (Figure 4D).

STS pretreatment attenuates pathological injury of heart, lung, and liver in rabbits with LPS-induced endotoxic shock

The histopathological findings revealed considerable damage in the heart, lung, and liver tissue after an endotoxic shock dose of LPS; however, a marked decrease in such damage was observed with STS pretreatment (Figure 5A–5D). Heart tissue after LPS stimulation exhibited median myocardial edema, dispersed bleeding, and inflammatory cells infiltration. The lung tissue exhibited obvious interstitial congestion and inflammatory cells infiltration. The liver injury was multifocal, including hemorrhage and increased inflammatory cells. No obvious histopathological injury of kidney tissue was observed after LPS injection during the observation period, with or without STS pretreatment (data not shown).

Discussion

This study reveals that STS improves hemodynamics, limits release of inflammatory cytokines, and ameliorates heart, lung, and liver injury in an experimental endotoxemia rabbit model.

A single bolus of LPS (60 $\mu\text{g}/\text{kg}$) was used in our study to induce endotoxemia. Of note, this dose is significantly different from that used in other reports. For example, Nakamura et al. [19] gave rabbits 3 sequential intravenous boluses of 10 $\mu\text{g}/\text{kg}$ LPS

at 0, 5, and 24 h to induce endotoxemia. Larocca et al. [20] found a single intraperitoneal bolus of 100 $\mu\text{g}/\text{kg}$ LPS prominently increased TNF- α and IL-6 at 90 min post-injection. In Wiel et al. [18], a single bolus of intravenous 500 $\mu\text{g}/\text{kg}$ LPS injected to rabbits generated a nearly 25% decrease in mean blood pressure at 4 h post-injection. However, none of these reports examined hemodynamic changes immediately after LPS injection. We observed, for the first time, an obvious double-phase hemodynamic collapse at the initial period post-LPS exposure, accompanying a prominent upregulation in inflammatory cytokines. An endotoxemia dose of LPS 60 $\mu\text{g}/\text{kg}$ may partially explain why a transient but not consistent elevation of TNF- α and IL-10 was observed within 300 min after LPS injection.

The double-phase hemodynamic collapse included an initial acute shock in the first 30 min, perhaps owing to the acute cardiovascular toxicity of LPS. A subsequent circulatory failure happened mainly at 60 min after LPS injection, which might be caused by inflammatory response, organ injury, or other mechanisms not explored in this experiment. STS induces vasodilation in the coronary artery, but not in resistance and conduit arteries [21,22]. This partially explains why STS pretreatment had no further worsening effect on hemodynamics in the first 30 min after LPS treatment. In this study, we observed only a protective effect of STS pretreatment on MAP and HR after 60 min after LPS injection; this effect, not observed in other studies to date, may be partially explained by STS promoting a balancing of pro- and anti-inflammatory cytokines, as well as inflammation cell clearance from organ tissues.

Cytokines are a class of small molecular proteins with extensive biological activity and complex functions. TNF- α and IL-10 were measured 30 min prior to LPS injection to exclude the possible influence of a long duration of immobilization (40 min) before the LPS injection. Mainly produced by monocyte/macrophages, TNF- α is considered the most important cytokine that promotes inflammatory response and tissue damage [23]. LPS stimulation increased circulatory TNF- α and IL-10 levels, reflecting an innate immune response to harmful stimuli. More importantly, STS pretreatment not only ameliorated TNF- α production, but also potentiated IL-10 secretion, to maintain a pro- and anti-inflammatory balance. Usually produced by monocytes/lymphocytes, IL-10 acts as a general inhibitor to suppress lymphocyte proliferation and cytokine responses. IL-10 infusion can prevent lethal endotoxic shock and endogenous IL-10 determines the amount of LPS that can be tolerated without death [24]. In contrast to numerous prior studies on the anti-inflammatory effects of STS for TNF- α [8–10,25,26], the stimulating effect of STS for IL-10 has seldom been reported [27]; this study thus broadens our knowledge about the inflammation regulation ability of STS.

Associated with the significant reduction of pro-inflammatory cytokine TNF- α and a boost of IL-10, pathological results revealed that STS also remarkably mitigated the pathologic damage to the heart, lungs, and liver, but not kidneys, with less tissue injury and inflammatory cell infiltration in endotoxemic rabbits. Consistent with pathology results, STS improved PaO₂, cTnI, and ALT, but not kidney function-related Cr level. Similarly, Chen et al. [25] observed STS improved inflammation, apoptosis, and coagulopathy disorder, and provided lung, spleen, liver, kidney, and small intestine protection in heat-shock rats after recovery for 6 h, while Gao et al. [8] reported tanshinone (the main component of which is tanshinone IIA) exerted anti-inflammatory effects and improved acute kidney injury in mice after LPS stimulation for 12 h. Of note, no protective benefit of STS on kidneys was observed in our study, likely due to our relatively short (300 min) experiment. As PaO₂ consistently decreased after LPS exposure throughout the experiment and concurrent inflammation and progressive deterioration

in HR and MAP occurred, inevitably, this will lead to organ hypoperfusion and hypoxia, and theoretically will eventually result in kidney injury. However, the exact mechanisms of kidney injury are not fully understood, and other mechanisms are at play, such as microvascular dysfunction or a metabolic response to inflammatory injury [5]. Taken together, these results indicate that STS is a potent agent for regulating inflammatory responses and protecting rabbits with endotoxemia from an over-inflammatory reaction that could lead to serious organ damage and even death.

Several limitations in our study must be acknowledged. First, all rabbits were anesthetized with pentobarbital bolus injection, a paradigm that cannot provide stable anesthesia throughout the experiment. Second, the dose of 20 mg/kg was chosen based on our pilot study of STS on hemodynamics in rabbits and the dose that appeared to effectively improve MAP and HR; however, whether the therapeutic effect of STS on inflammatory cytokines and biochemical marker levels in an endotoxemia model are dose-dependent has not been explored. Third, the experiment lasted only 300 min, and survival rate discrepancy in response to LPS with or without STS was not followed up. Finally, although different mechanisms underlying how STS regulates inflammation have been explored, such as inhibiting intracellular chloride channel 1 expression and membrane translocation [12], modulating neutrophils activities [13], and suppressing NF- κ B signaling pathway in endothelial cells [26], the exact regulation mechanism in this study is unexplored and needs further investigation.

Conclusions

STS, a water-soluble derivative of tanshinone IIA, one of the most pharmacologically active monomers extracted from *Salvia miltiorrhiza* Bunge, effectively improves LPS-induced hemodynamics, regulates excessive inflammation through inhibiting TNF- α and stimulating IL-10, attenuates heart, lung, and liver damage, and provides a promising alternative for initial-phase therapy of endotoxemia.

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