Dihydromyricetin alleviates endothelial inflammatory response through IRE1α/NF-κB signaling pathway in sepsis

Abstract

Introduction
The high mortality of sepsis is closely related to disorder of coagulation induced by endothelial inflammatory response. Our aim is to investigate the protective effects of Dihydromyricetin (DHM) on endothelial cells in sepsis and the endoplasmic reticulum (ER) stress mechanism.

Material and methods
In vivo, we conducted an animal study for which fifty male Wistar rats were randomly and equally divided into five groups: sham group, cecal ligation and puncture (CLP) group and three CLP + DHM (50, 100, 150 mg/kg) groups, the DHM was orally administered 2 h after CLP for 3 days (once per day). In vitro, human umbilical vein endothelial cells (HUVECs) were treated with DHM (50μmol) for 24 h after stimulation by lipopolysaccharide (LPS). In the inhibition groups, reactive oxygen species (ROS) inhibitor N-acetylcysteine (NAC, 3 mmol) and endoplasmic reticulum (ER) stress inhibitor (STF-083010, 10 μmol) were incubated prior to LPS.

Results
Our results indicated that DHM (150 mg/kg) alleviated the histopathological injury of endothelium, decreased the release of inflammatory cytokines and adhesion molecules such as interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), vascular cell adhesion molecule 1 (VCAM-1) and endothelin-1 (ET-1), and inhibited the production of ROS production. In addition, we found that DHM ameliorated ER damage, significantly decreased the protein expressions of IRE1α/NF-κB signaling pathway.

Conclusions
DHM treatment alleviated inflammatory response of endothelial cells in sepsis through the IRE1α/NF-κB signaling pathway triggered by oxidative stress. This study provided experimental rationale for the treatment of DHM on therapy of sepsis.
Dihydromyricetin alleviates endothelial inflammatory response through IRE1α/NF-κB signaling pathway in sepsis

Xifeng Wang¹, Xiaomin Xu², Yu Peng Yang³, Xin Xin², Zekang Li², Qimeng Wang², Xiaoli Li¹, Yun Hou⁴, Lianshuang Zhang⁴*

¹ Department of Critical Care Medicine, Yu Huang Ding Hospital, Qingdao University, Yantai, P. R. China
² College of Clinical Medicine, Bin Zhou Medical University, Yan Tai, P. R. China
³ Department of Common surgery, Ji Nan Zhang Qiu District Hospital of Traditional Chinese Medicine, Zhang Qiu, China
⁴ Department of Histology and Embryology, Binzhou Medical University, Yantai, P. R. China

*Corresponding author:
Associate Prof. Lian-Shuang Zhang
Department of Histology and Embryology, Bin Zhou Medical University, Yantai 264003, P.R. China
Tel: +86 0535-6913212
Email: zls197600@126.com
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ABSTRACT
The high mortality of sepsis is closely related to disorder of coagulation induced by endothelial inflammatory response. Our aim is to investigate the protective effects of Dihydromyricetin (DHM) on endothelial cells in sepsis and the endoplasmic reticulum (ER) stress mechanism. In vivo, we conducted an animal study for which fifty male Wistar rats were randomly and equally divided into five groups: sham group, cecal ligation and puncture (CLP) group and three CLP+ DHM (50, 100, 150 mg/kg) groups, the DHM was orally administered 2 h after CLP for 3 days (once per day). In vitro, human umbilical vein endothelial cells (HUVECs) were treated with DHM (50 μmol) for 24 h after stimulation by lipopolysaccharide (LPS). In the inhibition groups, reactive oxygen species (ROS) inhibitor N-acetylcysteine (NAC, 3 mmol) and endoplasmic reticulum (ER) stress inhibitor (STF-083010, 10 μmol) were incubated prior to LPS. Our results indicated that DHM (150 mg/kg) alleviated the histopathological injury of endothelium, decreased the release of inflammatory cytokines and adhesion molecules such as interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), vascular cell adhesion molecule 1 (VCAM-1) and endothelin-1 (ET-1), and inhibited the production of ROS. In addition, we found that DHM ameliorated ER damage, significantly decreased the protein expressions of IRE1α/NF-κB signaling pathway. DHM treatment alleviated inflammatory response of endothelial cells in sepsis through the IRE1α/NF-κB signaling pathway triggered by oxidative stress. This study provided experimental rationale for the treatment of DHM on therapy of sepsis.

Keywords: Dihydromyricetin; Inflammatory response; Endothelial cell; IRE1α/NF-κB signaling pathway; Sepsis.

1. Introduction
Sepsis, as a high morbidity and mortality disease in the clinic, is extensively perceived as mortal organ dysfunction induced by the disturbance of host reaction during the process of infection [1], and it is the leading cause of death in intensive care patients [2]. Its occurrence involves a variety of pathophysiological mechanisms, including abnormal coagulation caused by infectious factors [3]. Accumulating studies have shown that a variety of cytokines induced by inflammation such as TNF-α and interleukin-1 (IL-1) are released from damaged endothelial cells [4, 5] and further promote disorders of coagulation, which leads to diffuse intravascular coagulation (DIC) and subsequent organ dysfunction. Current reports support that all these affect patient outcomes [6].

The disorder of coagulation is associated with endothelial cells [7, 8]. As barrier between blood flow and vascular wall during sepsis, endothelial cells are the valuable targets during inflammation reaction [9-13]. Bacteria endotoxin such as LPS could lead to a rise of ROS and then contribute to oxidative stress during the development of sepsis associated with endothelial cells, and further activate downstream pathways. ER is the critical organelle, which is responsible for protein synthesis as well as protein processing. In addition, it produces a marked effect in maintaining intracellular homeostasis [14]. Various extracellular stresses allow misfolded proteins to accumulate in ER and then activate ER stress signals including inositol-requiring enzyme 1α (IRE1α) [15, 16]. ER stress takes a prominent effect on the pathological mechanism of vascular inflammation-induced endothelial dysfunction [17] and IRE1α is the most conserved ER stress transducer in evolution [18]. Thus, the inhibition of IRE1α signaling pathway may have a protective effect on endothelial cells in sepsis.

DHM is extracted from the tender stem and leaves of Ampelopsis grossedentata [19]. Ampelopsis is used for the treatment of liver disorders in Chinese traditional medicine [20]. Previous studies have revealed that DHM has antioxidation effects among other pharmacological activities [21]. Current studies have demonstrated that DHM improved vascular hyporesponsiveness and alleviated injuries of vascular
endothelial cells in sepsis [22, 23]. However, whether and how DHM ameliorates the inflammatory response of endothelial cells in sepsis requires further elucidation.

So, we investigated whether DHM alleviated endothelial cell inflammatory response in sepsis through modulating the IRE1α/NF-κB signaling pathway in this study. The current results provided novelty evidence for elucidating the effects of DHM on the therapy of sepsis and its potential mechanism.

## 2. Methods

### 2.1. Experimental design in vivo

Operating procedures and animal use were approved by the Animal Ethics Committee of Binzhou Medical University. Fifty Wistar rats (male, 8 weeks old) were purchased from Ji'nan Peng Yue (Shandong, China). The rats were given adequate food and water, day/night cycle at 12:12 h for one week before experiments [24]. They were divided into five groups, control group, CLP (cecal ligation perforation) model group, and three CLP+DHM treatment groups randomly, and ten rats in each group. The CLP model refers to our previous experiments [25]. We used 0.5% sodium carboxymethylcellulose (CMC-Na) to dissolve DHM and diluted it to a final concentration at 100 mg/mL with ultrapure water, the DHM treatment groups received daily administration of DHM at a different dose (50, 100 and 150mg/Kg) 2h after CLP operation [26]. Three days after DHM treatment, 4% chloral hydrate (5 mL/kg) was used to euthanize rats in each group. Because of the close relationship between endothelial injury of artery and coagulation abnormalities in our previous studies [27], we still took abdominal aorta as the research object. Blood and abdominal aorta were removed from each animal with blood being allowed to clot naturally, and then it was centrifuged, the serum was collected and stored for treatment. In addition, vascular tissues were fixed into the 4% paraformaldehyde for further tests. The histopathological changes of endothelium were evaluated to reveal the most effective concentration of DHM for subsequent experiments.
2.2. Hematoxylin and eosin (HE) staining of artery

The abdominal aorta was dehydrated with alcohol at different concentrations, and embedded in paraffin, and then it was sectioned at 5μm (RM2245 Leica, Germany). After staining with HE, arteries were observed under a light microscope (Echo, USA).

2.3. TUNEL staining of artery

The abdominal aorta was embedded in paraffin, dewaxed with xylene and hydrated in alcohol. Then the tissue was stained by the TUNEL kit (Beyotime, China) according to previous research [28]. Finally, after staining with 3-3-diaminobenzidine substrate, it was observed under microscopy and analyzed with a computer image analyzing system (Product model: Motic Images Advanced 3.2).

2.4. HUVECs culture and treatment

HUVECs were cultured in culture media which contains high-glucose DMEM (Haclon, USA), 1% penicillin-streptomycin and 10% fetal bovine serum at 37℃ in 5% CO₂. There are control group, lipopolysaccharide (LPS) group, LPS+DHM treatment group, LPS+STF-083010 treatment group or LPS+N-acetyl-L-cysteine (NAC, Sigma, USA) treatment group. In LPS group, cells were stimulated with LPS (Sigma, USA) at concentration of 1 μg/mL for 24 hours. NAC was used as the inhibitor of ROS and was incubated at 3mmol 4h prior to LPS stimulation, and STF-083010 group was incubated with STF-083010 at the concentration of 10 μmol 4h prior to LPS stimulation. In DHM treatment group, HUVECs were pretreated with 50 μmol DHM before stimulated by 1μg /mL of LPS for 24 h. This dose-dependent on the highest cell viability of different concentrations of DHM on HUVECs. After these treatments, HUVEC or its supernatant were collected for subsequent test. At least three wells were used for each treatment group.

2.5. Detection of IL-1β, TNF-α, IL-6, ET-1 and VCAM-1 levels in serum and supernatant

Concentrations of TNF-α, IL-1β, IL-6, ET-1 and VCAM-1 in rat serum or HUVEC
supernatant were measured by ELISA Kits (Mlbio, China) according to the standard procedures. The microplate reader is used to detect the absorbance value of these factors at the wavelength of 450 nm (Thermo Multiskan™ MK3; Thermo Fisher Scientific).

2.6. **ROS and MDA determination**

Among all groups, the cells were collected, centrifuged, resuspended and incubated with 10 μmol DCFH-DA probe (Jiancheng, China) in dark for 20 min, and then the cells were resuspended with 350 μl PBS and the average fluorescence intensity of DCF was detected by Flow cytometry (BD FACS Canto II USA). The MDA concentrations were measured using a specific kit (Jiancheng, China) [21] and the absorbance was recorded at 532 nm wavelength with a microplate reader (DNM-9602G, China).

2.7. **Transmission electron microscope (TEM) detection**

TEM was performed according to experimental procedures. After ethanol washing and dehydration, cells were fixed, embedded and were cut at 60 nm, stained, then observed under the TEM (JEM 2100, JEOL, Japan).

2.8. **IRE1α/NF-κB signaling pathway proteins detection**

The proteins were extracted and quantified according to the kit (Jiancheng, China). Lysate proteins (20 μg) were loaded onto 10% SDS-polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were incubated with IRE1α (1:800, Proteintech, China), p-IRE1α (1:400, CST, USA), GRP78 (1:500, Proteintech, China), XBP1 (1:400, Boster, China), NF-κB (1:800, Proteintech, China), and p-NF-κB (1:1000, Beyotime, China) overnight at 4°C after being blocked. And then the membranes were washed by Tris Buffered Saline Tween (TBST) and incubated with IgG secondary antibody (1:5000, Boster, China). The protein bounds were measured by ECL chemiluminescence reagent (Beyotime, China), and the densitometric analyses were performed using the Image Lab™ Software (Bio-Rad Laboratories Inc.,
2.9. Data analysis

SPSS 21.0 software was used for statistical analysis. The results were analyzed by one-way analysis of variance followed by the least significant difference method. Data were expressed as mean ± standard deviation (SD). The results were considered significant at P<0.05. Three to five replicate wells were used in each treatment group.

3. Results

3.1. The protective effects of DHM on endothelial cells in rats

We used H&E staining to explore the protection of DHM on endothelial cells. The nucleus and cytoplasm are normal (Figure 1A a) in sham group, while in CLP group, the damage cells (nuclear pyknosis) were observed, and some cells were exfoliated from the intima (Figure 1A b). Compared with CLP group, 150 mg/Kg DHM treatment group showed improvement in endothelial injury (Figure 1A e), while there were no obvious improvement in 50 and 100 mg/Kg groups (Figure 1A c, d). The percentage of damage cells in each group were measured (Figure 1B) and the results showed that it was higher in CLP group than that in sham group (P<0.05). In contrast, 150 mg/Kg DHM treatment attenuated damage cells percentage compared with CLP group (P<0.05). So we selected 150 mg/Kg DHM treatment for further experiments.
**Figure 1** Effects of DHM on histopathology in rat arteries. A (a) Sham group, (b) CLP group, (c) CLP+DHM 50mg/Kg group, (d) CLP+DHM 100mg/Kg group, (e) CLP+DHM 150mg/Kg group. Black arrow indicates pyknosis nucleus cells, HE × 400. B Percentage of abnormal cells. Data are expressed as means ± SD, ** p < 0.01 versus sham group, # p < 0.05 versus CLP group.

3.2. The effects of DHM on apoptosis of endothelial cells in rats

Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) staining was used to detect apoptotic cells. As shown in Figure 2A, TUNEL positive staining endothelial cells were observed in vascular as arrow denoted. The gray values of each group were measured, and it was higher in CLP group than that of control group (P<0.05), while DHM treatment decreased it significantly than CLP group (P<0.05) (Figure 2B).

**Figure 2** Effects of DHM on apoptosis of endothelial cells in rat artery. A TUNEL staining of artery. (a) Control group, (b) CLP group, (c) CLP+DHM 150mg/Kg group. Black arrows indicate TUNEL positive staining endothelial cells. ×400. B Gray value in each group. Data are expressed as means ± SD, ** p < 0.01 versus sham group, # p < 0.05 versus CLP group.

3.3. The effects of DHM on the levels of TNF-α, IL-6, IL-1β, ET-1 and VCAM-1 in serum

We assayed levels of adhesion molecules and pro-inflammatory cytokines
respectively in serum. As shown in Figure 3, the levels of TNF-α, IL-6, IL-1β (Figure 3A a b c) and adhesion molecules including ET-1, VCAM-1 (Figure 3B a b) in CLP group and in LPS group were both higher than those in control group (P<0.05), while pro-inflammatory cytokines and adhesion molecules levels were evidently decreased after DHM pretreatment (P<0.05).

Figure 3  Effects of DHM on pro-inflammatory and adhesion factor expressions in rat plasma. A The expressions of pro-inflammatory factors. (a) TNF-α, (b) IL-1β, (c) IL-6. B The expressions of adhesion factor factors. (a) ET-1, (b) VCAM-1. Data are expressed as means ± SD, ** p < 0.01 versus sham group, # p< 0.05 versus CLP group.

3.4. The effects of DHM on ROS and MDA levels in HUVECs

We used NAC as an antioxidant to explore the effects of DHM on oxidative stress induced by LPS in HUVECs (Figure 4). The results indicated that the mean fluorescence of DCF and concentration of MDA in LPS group were higher than those of sham group (P<0.05). In contrast, pretreatment with DHM prior to LPS obviously suppressed the production of ROS and MDA compared with LPS group (P<0.05), the
effect was similar to the NAC treatment.

Figure 4  Effects of DHM on ROS and MDA levels. A Flow cytometry for detection of ROS. (a) Control group, (b) LPS group, (c) LPS+DHM group, (d) LPS+NAC group. B Median fluorescence of DCF in each group. C Concentration of MDA levels in each group. Data are expressed as means ± SD, * p < 0.05 versus control group, # p < 0.05 versus LPS group.

3.5. The effects of DHM on the levels of TNF-α, IL-6, IL-1β, ET-1 and VCAM-1 in HUVEC supernatant

We assayed levels of adhesion molecules and pro-inflammatory cytokines in HUVEC supernatant. As shown in Figure 5, the levels of TNF-α, IL-6, IL-1β (Figure 5A a b c) and adhesion molecules including ET-1, VCAM-1 (Figure 5B a b) in CLP group and in LPS group were both higher than those in control group (P<0.05), while pro-inflammatory cytokines and adhesion molecules levels were evidently decreased after DHM pretreatment (P<0.05), which showed the similar effects compared with pretreatment of ER stress inhibitor STF-083010.
Figure 5 Effects of DHM on pro-inflammatory and adhesion factor expressions in supernatant of HUVEC. A The expression of pro-inflammatory factors. (a) TNF-α, (b) IL-1β, (c) IL-6. B The expression of adhesion factor factors. (a) ET-1, (b) VCAM-1. Data are expressed as means ± SD, ** p < 0.01 versus control group, # p < 0.05 versus LPS group.

3.6. The effects of DHM on ER in HUVECs

As shown in Figure 6, in control group, a well-arranged rough endoplasmic reticulum (RER) was observed, and the ribosome was abundantly attached (Figure 6a). But in LPS group, the swelling and degranulation of rough ER were found (Figure 6b). However, these were ameliorated by DHM (Figure 6c) as well as NAC treatment (Figure 6d).
Figure 6 Effect of DHM on endoplasmic reticulum ultrastructure in HUVECs. (a) Control group, (b) LPS stimulation group, (c) DHM treatment group, (d) NAC pretreatment group. Transmission electron microscope ×8000. Black arrow denotes rough endoplasmic reticulum.

3.7. The effects of DHM on protein expressions in HUVECs

As shown in Figure 7A, B, C, LPS stimulation up-regulated the expression levels of phosphorylated IRE1α (p-IRE1α) and IRE1α, while treatment of DHM decreased these expressions as well as NAC group when compared to LPS group (P<0.05). Moreover, the expressions of GRP78, XBP-1, phosphorylated nuclear factor-kappa B (p-NF-κB) and nuclear factor-kappa B (NF-κB) increased in LPS group compared to control group (P<0.05). However, after treatment with DHM or STF-083010, these proteins was decreased compared with LPS-treated group (P<0.05, Figure 7 D, E, F, G, H).
Figure 7 Effects of DHM on protein expressions of IRE1α/NF-κB signaling pathway. A, D Effects of DHM on the expressions of p-IRE1α, IRE1α, GRP78, XBP-1, p-NF-κB and NF-κB. B, C, E, F, G, H DHM inhibited ER stress through the down-regulation of IRE1α/NF-κB signaling pathway. Data are expressed as means ± SD, * p < 0.05 versus control group, # p < 0.05 versus LPS group.

4. Discussion

Coagulation disorder is a significant cause of the high mortality of sepsis. Our previous study showed that coagulation disorder could be triggered by endothelial injuries [29] which is induced by endotoxemia in sepsis [30]. When endothelial cells were stimulated by LPS, a large amount of inflammatory factors such as TNF-α, IL-6, IL-1β and adhesion molecules such as ET-1 and VCAM-1 are released, which will further promote and aggravate coagulation disorder, as well as lead to organ dysfunction [31]. Consequently, the alleviation of inflammatory response can effectively protect the endothelial function and ameliorate the coagulation disorder in sepsis.
In the present study, the DHM treatment improved endothelial histological structure, reduced the releases of TNF-α, IL-6, IL-1β, ET-1 and VCAM-1 of endothelial cells. These results indicated that DHM alleviated the inflammatory response of endothelial cells in sepsis, which consistent with the study that DHM inhibited inflammatory reactions of lung in septic mice [26]. In addition, DHM down-regulated the expressions of GRP78 and XBP1, which were consistent with the effect of ER stress inhibitor STF-083010. The results displayed that the treatment of DHM alleviated the inflammatory response of endothelial cells through inhibiting ER stress. ER is an important place for protein synthesis. Proper folding and assembly of polypeptide chains in eukaryotic cells is associated with a variety of metabolic activities. Various extracellular stresses could lead to misfolded proteins to accumulate in ER and activate stress signals such as IRE1α [15, 16]. Activated IRE1α reduces the accumulation of unfolded/misfolded proteins by increasing the expressions of GRP78 and XBP1 [32]. Our results showed that LPS induced ER stress which was corroborated by the ultrastructural changes of ER and the upregulated proteins expressions of IRE1α and p-IRE1α, GRP78 and XBP1.

ER stress could be activated by oxidative stress [33]. Consistent with previous studies that LPS induced strong oxidative stress and a decrease in endogenous antioxidant defenses [34-36], our current research results suggested that ROS and MDA levels in HUVECs treated with LPS have up-regulated. However, the treatment of DHM markedly reduced these levels of these in HUVECs, these findings indicated that DHM can alleviate LPS-induced oxidative stress in sepsis. Furthermore, treatment with DHM ameliorated the LPS-induced ER injury, and remarkably down-regulated the expression of IRE1α and p-IRE1α as well as NAC treatment. All these present studies have shown that DHM has anti-oxidative stress properties which also has been proven by previous study in a rat model of sepsis [37]. All the above results indicated that DHM alleviated IRE1α mediated ER stress through inhibiting oxidative stress.

To gain a deep analysis of the detailed mechanisms of DHM protection for endothelial cells, ER-stress-mediated IRE1α/NF-κB pathway was examined in
HUVECs. Tam et al has emphasized the functional crosstalk between ER stress and the activation of NF-κB [38]. Another study showed that ER stress could result in excessive activation of NF-κB [39]. Accompanied by the NF-κB phosphorylation, pro-inflammatory factors, such as IL-1β, TNF-α as well as IL-6, took an ascending trend in sepsis [40]. And then, the endothelial dysfunction was down-regulated [40].

In terms of the present research, the expressions of NF-κB and p-NF-κB which were associated with NF-κB signaling pathway were up-regulated, and the levels of ET-1, VCAM-1, IL-1β, TNF-α and IL-6 were all increased after LPS stimulation. However, all these were decreased after intervention with DHM and ER stress inhibitor STF-083010 (Figure 4). These findings indicate that DHM alleviate inflammatory response by regulating IRE1α/NF-κB signaling pathway.

Taken together, our results indicated that DHM protected endothelial cells against inflammatory response via IRE1α/NF-κB signaling pathway induced by oxidative stress in sepsis, which provided an experimental rationale for the treatment of clinical sepsis. In addition, there are some limitations in our study, such as the use of inhibitor is STF-083010, not gene knockdown.

5. Conclusion

DHM treatment could ameliorate endothelial function through alleviating inflammatory response mediated by IRE1α/NF-κB pathway in sepsis. This provides a promising therapeutic method for patients with sepsis in the clinic.

Declaration of interest statement
None.

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Figure 2

A

B

Figure 2
Figure 3

A

**a**

TNF-α levels (ng/L)

Sham group  CLP  CLP+DHM (150mg)

b

IL-1β levels (ng/L)

Sham group  CLP  CLP+DHM (150mg)

c

IL-8 levels (ng/L)

Sham group  CLP  CLP+DHM (150mg)

B

**a**

ET-1 levels (pg/mL)

Sham group  CLP  CLP+DHM (150mg)

b

VCAM-1 levels (ng/L)

Sham group  CLP  CLP+DHM (150mg)
Figure 4
