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

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Abstract

Introduction: The high mortality of sepsis is closely related to a disorder of coagulation induced by the 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 in which fifty male Wistar rats were randomly and equally divided into five groups: a sham group, a cecal ligation and puncture (CLP) group and three CLP + DHM (50, 100, 150 mg/kg) groups. The DHM was orally administered 2 hours after CLP for 3 days (once per day). *In vitro*, human umbilical vein endothelial cells (HUVECs) were treated with DHM (50 μmol) for 24 hours after stimulation by lipopolysaccharide (LPS). In the inhibition groups, the reactive oxygen species (ROS) inhibitor N-acetylcysteine (NAC, 3 mmol) and 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 the endothelium, decreased the release of inflammatory cytokines and adhesion molecules such as interleukin-1β (IL-1β), IL-6, tumor necrosis factor α (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, and significantly decreased the protein expression of the IRE1α/NF-κB signaling pathway.
Conclusions: Dihydromyricetin treatment alleviated the inflammatory response of endothelial cells in sepsis through the IRE1α/NF-κB signaling pathway triggered by oxidative stress. This study provided an experimental rationale for the treatment of DHM in therapy of sepsis.

Key words: dihydromyricetin, inflammatory response, endothelial cell, IRE1α/NF-κB signaling pathway, sepsis.

Introduction

Sepsis, as a high morbidity and mortality disease in the clinic, is extensively perceived as mortal organ dysfunction induced by disturbance of the 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 tumor necrosis factor α (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. Recent reports suggest that all these affect patient outcomes [6].

The disorder of coagulation is associated with endothelial cells [7, 8]. As a barrier between blood flow and the vascular wall during sepsis, endothelial cells are valuable targets during an inflammatory reaction [9–13]. Bacterial endotoxins such as lipopolysaccharide (LPS) could lead to a rise of reactive oxygen species (ROS) and then contribute to oxidative stress during the development of sepsis associated with endothelial cells, and further activate downstream pathways. Endoplasmic reticulum (ER) is a 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]. Endoplasmic reticulum stress exerts 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, inhibition of the IRE1α signaling pathway may have a protective effect on endothelial cells in sepsis.

Dihydromyricetin (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 antioxidant effects among other pharmacological activities [21]. Recent studies 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.

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

Material and methods

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 hours for one week before experiments [24]. They were randomly divided into five groups: a control group, a CLP (cecal ligation perforation) model group, and three CLP + DHM treatment groups, with 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 of 100 mg/ml with ultrapure water. The DHM treatment groups received daily administration of DHM at a different dose (50,100 and 150 mg/kg) 2 hours after the 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 the artery and coagulation abnormalities in our previous studies [27], we still took the abdominal aorta as the research object. Blood and the abdominal aorta were removed from each animal with blood being allowed to clot naturally, then it was centrifuged, and the serum was collected and stored for treatment. In addition, vascular tissues were fixed in 4% paraformaldehyde for further tests. The histopathological changes of endothelium were evaluated to reveal the most effective concentration of DHM for subsequent experiments.

Hematoxylin and eosin staining of artery

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

TUNEL staining of artery

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

HUVEC culture and treatment

HUVECs were cultured in culture media which contains high-glucose DMEM (Shaclone, USA), 1% penicillin-streptomycin and 10% fetal bovine serum at 37°C in 5% CO_2. There was a control group,

a lipopolysaccharide (LPS) group, an LPS + DHM treatment group, an LPS + STF-083010 treatment group and an LPS + N-acetyl-L-cysteine (NAC, Sigma, USA) treatment group. In the LPS group, cells were stimulated with LPS (Sigma, USA) at a concentration of 1 μg/ml for 24 hours. NAC was used as the inhibitor of ROS and was incubated at 3 mmol 4 hours prior to LPS stimulation, and the STF-083010 group was incubated with STF-083010 at the concentration of 10 μmol 4 hours prior to LPS stimulation. In the DHM treatment group, HUVECs were pretreated with 50 μmol DHM before stimulated by 1 μg/ml of LPS for 24 h. This dose was dependent on the highest cell viability of different concentrations of DHM on HUVECs. After these treatments, HUVECs or their supernatants were collected for the subsequent test. At least three wells were used for each treatment group.

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 vascular cell adhesion molecule 1 (VCAM-1) in rat serum or HUVEC supernatant were measured by ELISA Kits (Mlbio, China) according to the standard procedures. A microplate reader was used to detect the absorbance value of these factors at the wavelength of 450 nm (Thermo Multiskan MK3; Thermo Fisher Scientific).

ROS and MDA determination

Among all groups, the cells were collected, centrifuged, resuspended and incubated with 10 μmol DCFH-DA probe (Jiancheng, China) in the dark for 20 min, and then the cells were resuspended with 350 μl of 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).

Transmission electron microscope detection

Transmission electron microscope (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).

Detection of IRE1α /NF-κB signaling pathway proteins

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. Then the membranes were washed with Tris Buffered Saline Tween (TBST) and incubated with IgG secondary antibody (1:5000, Boster, China). The protein bonds were measured with an ECL chemiluminescence reagent (Beyotime, China), and the densitometric analyses were performed using the Image Lab Software (Bio-Rad Laboratories Inc., Hercules, CA, USA).

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.

Results

Protective effects of DHM on endothelial cells in rats

We used HE staining to explore the protective effect of DHM on endothelial cells. The nucleus and cytoplasm are normal (Figure 1 A, a) in the sham group, while in CLP group, damaged cells (nuclear pyknosis) were observed, and some cells were exfoliated from the intima (Figure 1 A, b). Compared with the CLP group, the 150 mg/kg DHM treatment group showed improvement in endothelial injury (Figure 1 A, e), while there was no obvious improvement in 50 and 100 mg/kg groups (Figure 1 A, c, d). The percentage of damaged cells in each group was measured (Figure 1 B) and the results showed that it was higher in the CLP group than in the sham group ($p < 0.05$). In contrast, 150 mg/kg DHM treatment attenuated the damaged cell percentage compared with the CLP group ($p < 0.05$), so we selected 150 mg/kg DHM treatment for further experiments.

Effects of DHM on apoptosis of endothelial cells in rats

Terminal deoxynucleotidyl transferase-mediated nick end labeling staining was used to detect apoptotic cells. As shown in Figure 2 A, TUNEL positive staining endothelial cells were observed in the vasculature, as indicated by an arrow. The gray values of each group were measured, and

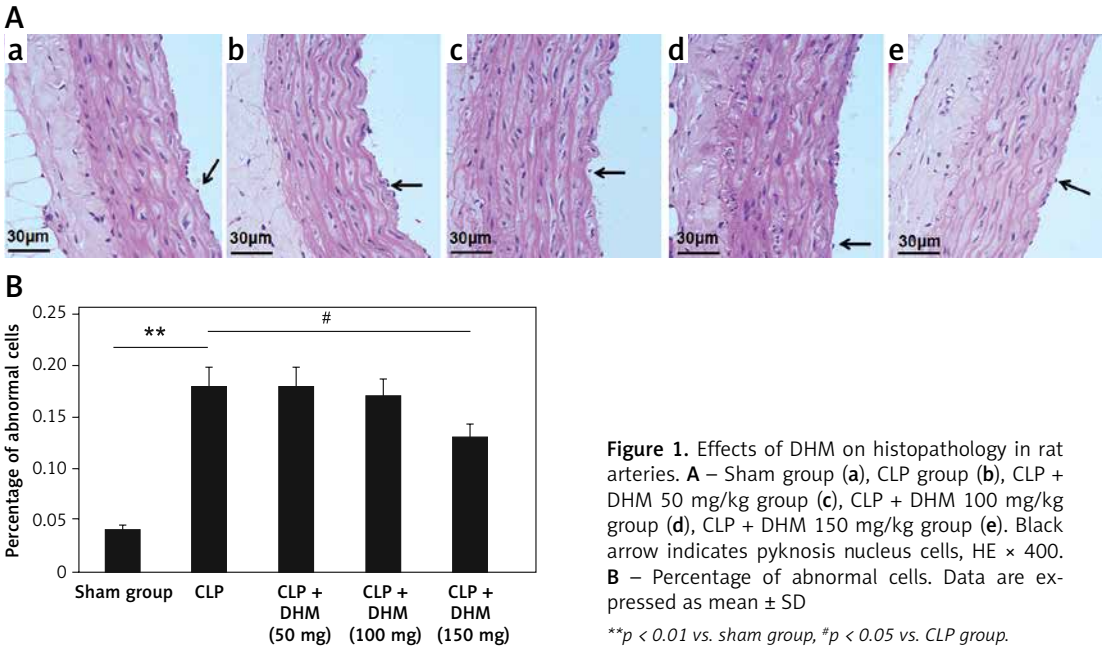

Figure 1. Effects of DHM on histopathology in rat arteries. **A** – Sham group (**a**), CLP group (**b**), CLP + DHM 50 mg/kg group (**c**), CLP + DHM 100 mg/kg group (**d**), CLP + DHM 150 mg/kg group (**e**). Black arrow indicates pyknosis nucleus cells, HE × 400. **B** – Percentage of abnormal cells. Data are expressed as mean ± SD

***p < 0.01 vs. sham group, #p < 0.05 vs. CLP group.*

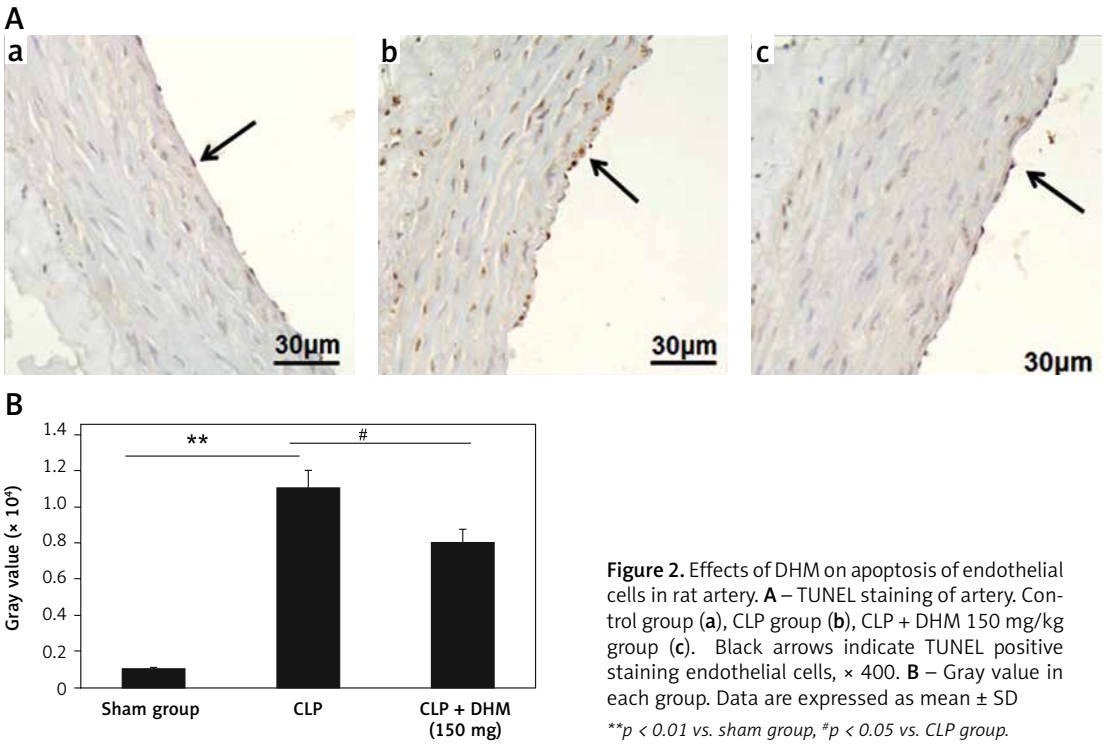

Figure 2. Effects of DHM on apoptosis of endothelial cells in rat artery. **A** – TUNEL staining of artery. Control group (**a**), CLP group (**b**), CLP + DHM 150 mg/kg group (**c**). Black arrows indicate TUNEL positive staining endothelial cells, × 400. **B** – Gray value in each group. Data are expressed as mean ± SD

***p < 0.01 vs. sham group, #p < 0.05 vs. CLP group.*

it was higher in the CLP group than the control group ($p < 0.05$), while DHM treatment decreased it significantly compared to the CLP group ($p < 0.05$) (Figure 2 B).

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

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

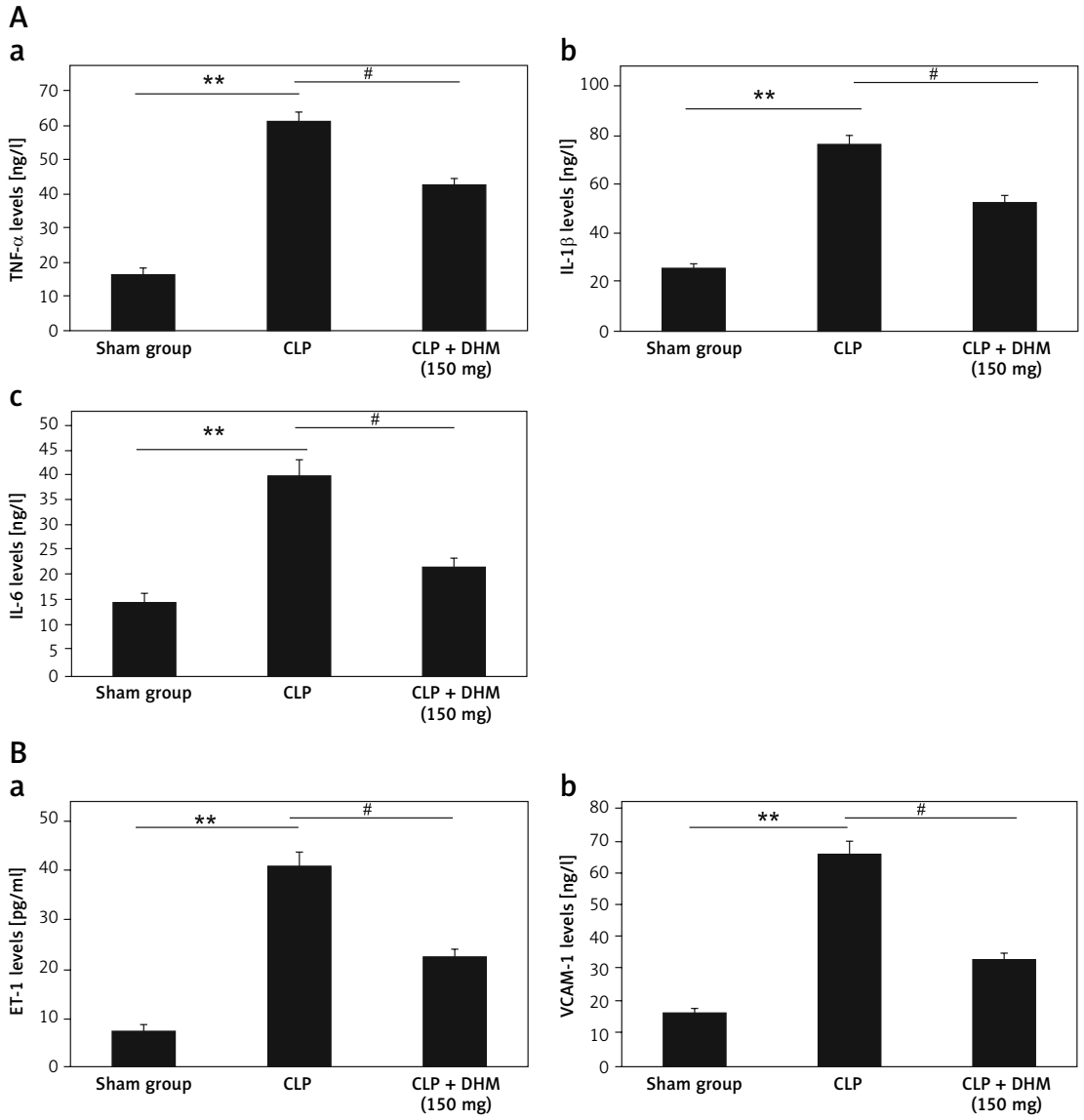

Figure 3. Effects of DHM on pro-inflammatory and adhesion factor expression levels in rat plasma. **A** – Expression of pro-inflammatory factors. TNF-α (**a**), IL-1β (**b**), IL-6 (**c**). **B** – Expression of adhesion factor factors. ET-1 (**a**), VCAM-1 (**b**). Data are expressed as mean ± SD

***p < 0.01 vs. sham group, #p < 0.05 vs. CLP group.*

in HUVECs (Figure 4). The results indicated that the mean fluorescence of DCF and concentration of MDA in the LPS group were higher than in the sham group ($p < 0.05$). In contrast, pretreatment with DHM prior to LPS obviously suppressed the production of ROS and MDA compared with the LPS group ($p < 0.05$), and the effect was similar to the NAC treatment.

Effects of DHM on 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 5 A, a–c) and adhesion molecules including ET-1 and VCAM-1 (Figure 5 B, a, b) in the CLP group and in the LPS group were both higher than those in the control group ($p < 0.05$), while pro-inflammatory cytokine and adhesion molecule levels were evidently decreased after DHM pretreatment ($p < 0.05$), which showed similar effects compared with pretreatment with the ER stress inhibitor STF-083010.

Effects of DHM on ER in HUVECs

As shown in Figure 6, in the control group, a well-arranged rough endoplasmic reticulum (RER) was observed, and the ribosome was abundantly attached (Figure 6 A). However, in the LPS

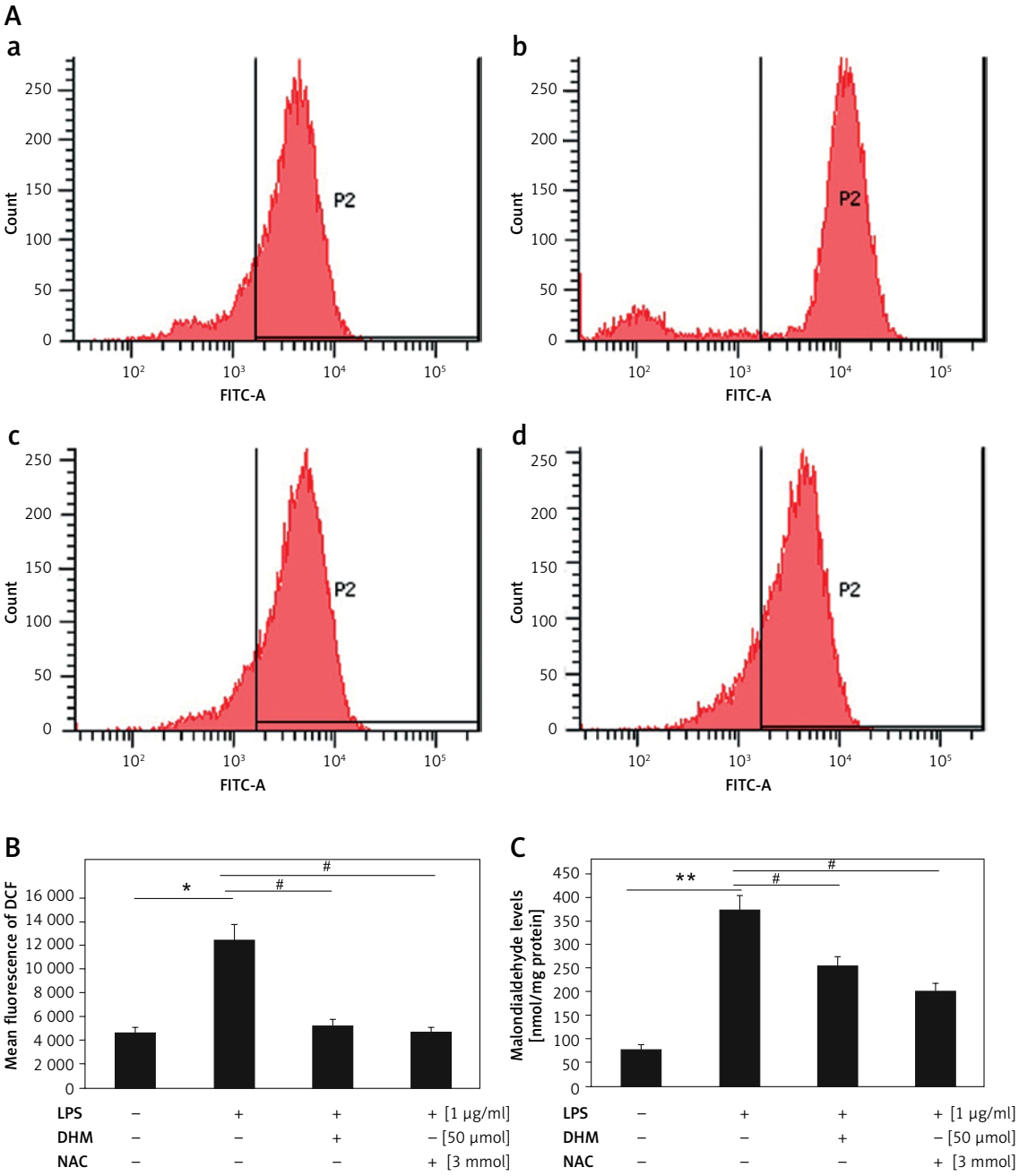

Figure 4. Effects of DHM on ROS and MDA levels. **A** – Flow cytometry for detection of ROS. Control group (**a**), LPS group (**b**), LPS + DHM group (**c**), LPS + NAC group (**d**). **B** – Median fluorescence of DCF in each group. **C** – Concentration of MDA levels in each group. Data are expressed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

**p < 0.05 vs. control group, #p < 0.05 vs. LPS group.*

group, swelling and degranulation of the rough ER were found (Figure 6 B), but these were ameliorated by DHM (Figure 6 C) as well as NAC treatment (Figure 6 D).

Effects of DHM on protein expression in HUVECs

As shown in Figure 7 A–C, LPS stimulation up-regulated the expression levels of phosphorylated IRE1α (p-IRE1α) and IRE1α, while treatment with DHM decreased their expression levels as well as the NAC group when compared to the LPS group ($p < 0.05$). Moreover, the expression of GRP78, XBP-1, phosphorylated nuclear factor-kappa B (p-NF-κB) and nuclear factor-kappa B (NF-κB) increased in the LPS group compared to the control group ($p < 0.05$). However, after treatment with DHM or STF-083010, these proteins were decreased compared with the LPS-treated group ($p < 0.05$, Figure 7 D–H).

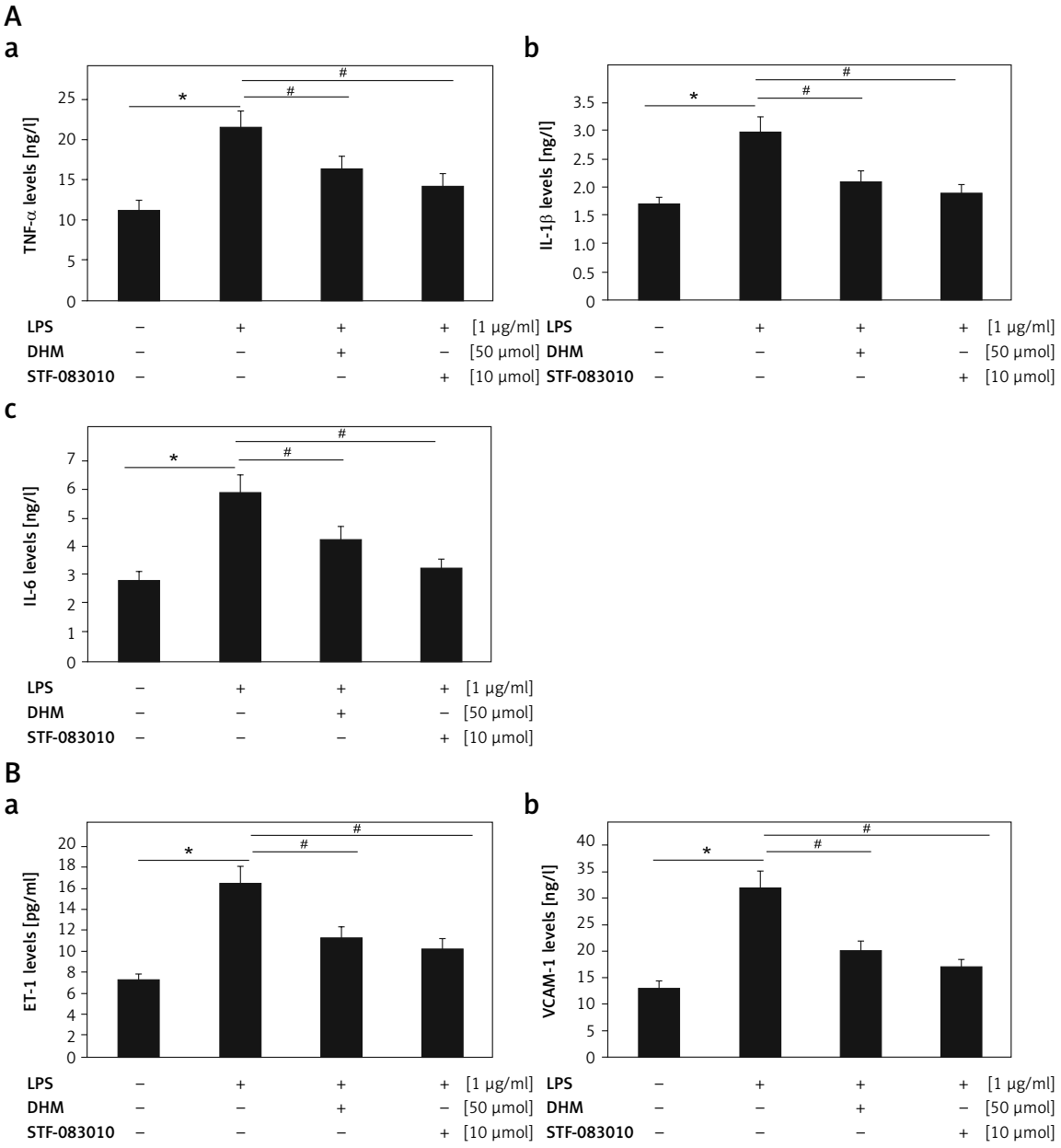

Figure 5. Effects of DHM on pro-inflammatory and adhesion factor expression levels in supernatant of HUVEC. **A** – Expression of pro-inflammatory factors. TNF-α (a), IL-1β (b), IL-6 (c). **B** – Expression of adhesion factor factors. ET-1 (a), VCAM-1 (b). Data are expressed as mean ± SD

**p < 0.05 vs. control group, #p < 0.05 vs. LPS group.*

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 are 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 leading to organ dysfunction [31]. Consequently, alleviation of the 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 and reduced the release 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 is consistent with a study showing that DHM inhibited inflammatory reactions of the lung in septic mice [26]. In addition, DHM down-regulated the expression of GRP78 and XBP1, which was consistent with the effect of the ER stress inhibitor STF-083010. The

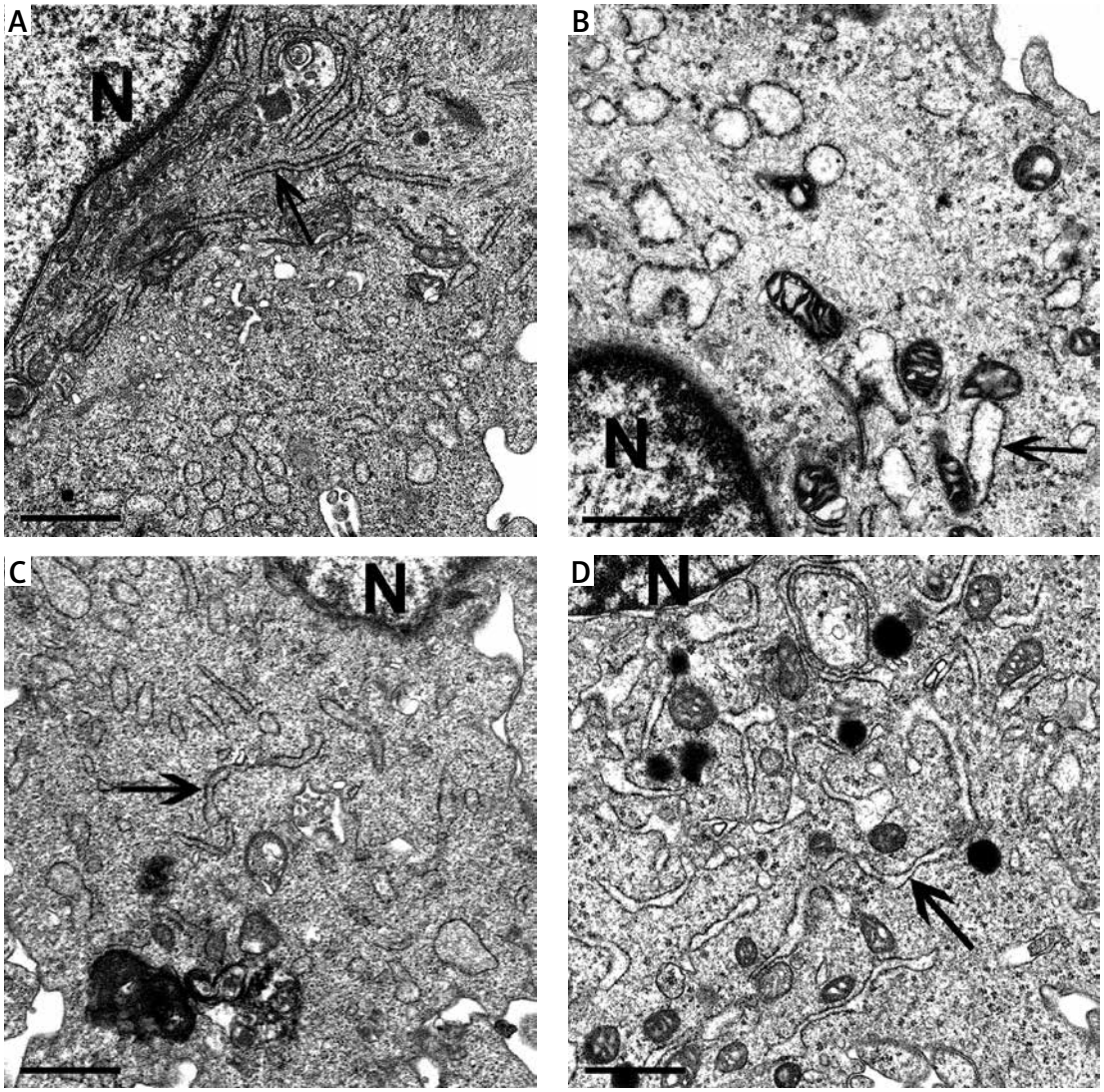

Figure 6. Effect of DHM on endoplasmic reticulum ultrastructure in HUVECs. Control group (**A**), LPS stimulation group (**B**), DHM treatment group (**C**), NAC pretreatment group (**D**). Transmission electron microscope × 8000. Black arrow denotes rough endoplasmic reticulum

results indicated 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 accumulating in ER and activation of stress signals such as IRE1α [15, 16]. Activated IRE1α reduces the accumulation of unfolded/misfolded proteins by increasing the expression of GRP78 and XBP1 [32]. Our results showed that LPS induced ER stress, which was corroborated by the ultrastructural changes of ER and upregulated expression of the proteins IRE1α and p-IRE1α, GRP78 and XBP1.

Endoplasmic reticulum stress could be activated by oxidative stress [33]. Consistent with previous studies in which 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 were up-regulated. However, the treatment of DHM markedly reduced these levels 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 studies have shown that DHM has anti-oxidative stress properties, which has also been proven by a 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 achieve a deep analysis of the detailed mechanisms of DHM protection for endothelial

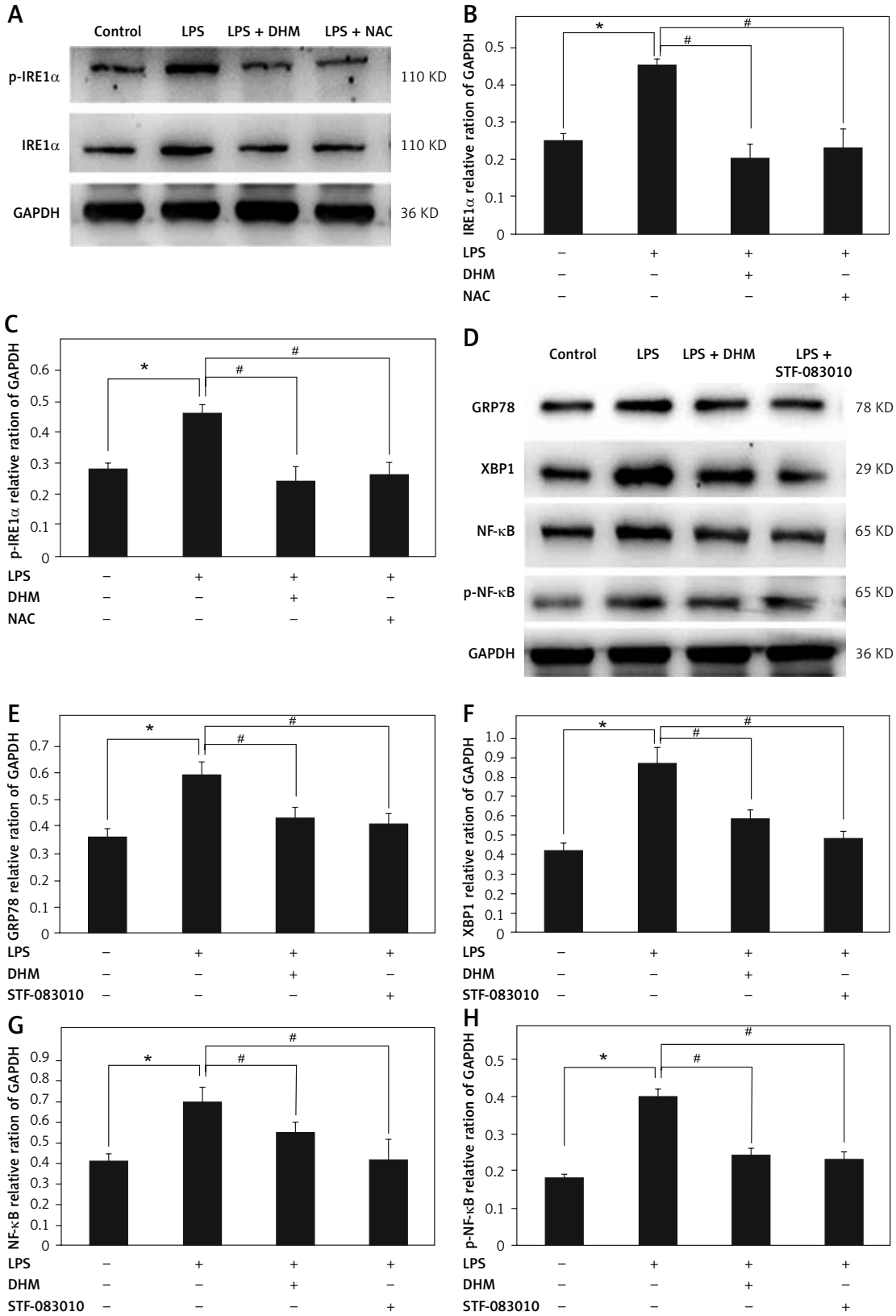

Figure 7. Effects of DHM on expression of proteins of the IRE1α/NF-κB signaling pathway. **A**, **D** – Effects of DHM on expression of p-IRE1α, IRE1α, GRP78, XBP-1, p-NF-κB and NF-κB. **B**, **C**, **E**, **F**, **G**, **H** – DHM inhibited ER stress through down-regulation of the IRE1α/NF-κB signaling pathway. Data are expressed as mean ± SD

**p < 0.05 vs. control group, #p < 0.05 vs. LPS group.*

cells, the ER-stress-mediated IRE1α/NF-κB pathway was examined in HUVECs. Tam et al. 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 NF-κB phosphorylation, pro-inflammatory factors, such as IL-1β, TNF-α and IL-6, showed an ascending trend in sepsis [40]. Then the endothelial dysfunction was down-regulated [40]. In terms of the present research, the expression levels of NF-κB and p-NF-κB, which were associated with the 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 the ER stress inhibitor STF-083010 (Figure 4). These findings indicate that DHM alleviates the inflammatory response by regulating the IRE1α/NF-κB signaling pathway.

Taken together, our results indicated that DHM protected endothelial cells against the inflammatory response via the 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 the inhibitor STF-083010, not gene knockdown.

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

Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.

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