

Septic serum mediates inflammatory injury in human umbilical vein endothelial cells via reactive oxygen species, mitogen activated protein kinases and nuclear factor- κ B

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Abstract. Sepsis-induced blood vessel dysfunction is mainly caused by microvascular endothelial cell injury. However, the mechanism underlying sepsis-induced endothelial cell injury remains unclear. The present study hypothesized that sepsis-induced inflammatory injury of endothelial cells may be the first step of endothelial barrier dysfunction. Therefore, the present study aimed to uncover the mechanism underlying the inflammatory effects of sepsis. A rat model of cecal ligation and puncture-induced sepsis was established, and septic serum was collected. Subsequently, human umbilical vein endothelial cells (HUVECs) were treated with the isolated septic or normal serum. HUVEC viability was assessed using a Cell Count Kit-8 assay. Furthermore, transmission electron microscopy and reverse transcription-quantitative PCR (RT-qPCR) analysis were carried out to observe the cell morphology and determine the mRNA expression levels in septic serum-induced HUVECs. The protein expression levels were evaluated by western blot analysis, and the secretion of the inflammatory factors interleukin (IL)-1 β , IL-6 and tumor necrosis factor (TNF)- α was determined by ELISA. Additionally, reactive oxygen species (ROS) generation and nuclear factor (NF)- κ B nuclear translocation were observed under a fluorescence microscope. The results of the present study demonstrated that HUVEC viability was significantly decreased following 12- or 24-h treatment with septic serum. In addition, chromatin condensation, mitochondrial vacuolization and endoplasmic reticulum degranulation were observed following treatment with septic serum. Furthermore, the

secretion levels of IL-1 β , IL-6 and TNF- α were increased in septic serum-stimulated HUVECs. Septic serum treatment also enhanced superoxide anion generation, promoted extracellular signal regulated kinase 1/2 (ERK1/2), N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38) phosphorylation, and increased NF- κ B levels in the nuclei of HUVECs. Finally, pre-treatment of HUVECs with the antioxidant N-acetylcysteine, the ERK1/2 inhibitor PD98059, the p38 inhibitor SB203580, the JNK inhibitor SP610025 or the NF- κ B inhibitor pyrrolidine dithiocarbamate restored the septic serum-induced IL-1 β , IL-6 and TNF- α expression. In conclusion, the results of the current study suggested that the septic serum-induced endothelial cell injury may be mediated by increasing ROS generation, activation of mitogen-activated protein kinases and NF- κ B translocation.

Introduction

Sepsis may promote systemic inflammatory injury of the blood vessels, resulting in microvascular endothelial cell dysfunction and injury (1-3). Microvascular dysfunction is of great importance in the clinic and has been associated with increasing mortality when the dysfunction persists for a long time (4-7). Endothelial dysfunction is considered to be an early event for a range of vascular diseases (such as atherosclerosis, hypertension and myocardial ischemia) and it has been reported that inflammation is involved in this pathological process (8,9). Results from clinical and scientific studies have demonstrated that septic microvascular dysfunction may be mediated by a number of factors and processes, including the activation of leukocytes (10), the secretion of inflammatory cytokines (11) and the exposure of microvascular cells to harmful leukocyte-derived molecules (12). In addition, it has been suggested that the local production of inflammatory factors in vascular cells may exert a direct and significant effect on the pathological process of sepsis (13). The expression of several types of inflammatory cytokines, including interleukin (IL)-1 β , IL-6 and tumor necrosis factor (TNF)- α may be induced by sepsis (14,15). Furthermore, inflammatory cell infiltration and the oxidative stress-mediated generation of reactive oxygen species (ROS) may promote blood vessel damage,

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activation of mitogen-activated protein kinases (MAPKs) and translocation of nuclear factor- κ B (NF- κ B) into the cell nucleus (16,17). However, the specific mechanism underlying the sepsis-induced pro-inflammatory responses remains unclear. Therefore, the present study aimed to determine the mechanism underlying the sepsis-induced inflammatory injury of HUVECs by focusing on the effects of ROS, MAPKs and NF- κ B.

Materials and methods

Reagents. Dulbecco's modified Eagle's medium (DMEM) was obtained from HyClone (Cytiva), and fetal bovine serum (FBS) was purchased from Gibco. TRIzol[®] was obtained from Invitrogen (Thermo Fisher Scientific, Inc.). PrimeScript[™] 1st strand cDNA Synthesis kit and SYBR[®] Premix Ex Taq were obtained from Takara Biotechnology Co., Ltd. The extracellular signal-regulated kinase 1/2 (ERK 1/2) inhibitor PD98059, the p38 inhibitor SB203580, the antioxidant N-acetylcysteine (NAC) and the NF- κ B inhibitor pyrrolidine dithiocarbamate (PDTC) were purchased from Sigma-Aldrich, Merck KGaA. The following antibodies were used in the present study: Rabbit monoclonal anti- β -actin (cat. no. NC021; Zhuangzhi Biotech), anti-NF- κ B (cat. no. ab16502; Abcam), anti-lamin B1 (cat. no. AF1408), anti-NF- κ B p65 (cat. no. SN368), anti-ERK1/2 (cat. no. AF1051), anti-phospho-ERK1/2 (cat. no. AF5851), anti-JNK (cat. no. AJ518), anti-phospho-JNK (cat. no. AJ516) (all from Beyotime Institute of Biotechnology), anti-phospho-p38 (cat. no. 9216), anti-p38 (cat. no. 8690) (both from Cell Signaling Technology, Inc.). The HRP-conjugated anti-mouse IgG (cat. no. CW0102S) and anti-Rabbit IgG (cat. no. CW0103S) secondary antibodies were obtained from CW Biotech, Co., Ltd. Furthermore, 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA) was obtained from Beyotime Institute of Biotechnology, and ELISA kits for detecting human IL-1 β (cat. no. F01220), IL-6 (cat. no. F01310) and TNF- α (cat. no. F02810) were obtained from Westang. All other chemicals used in the experiments were of analytical grade.

Isolation of septic serum. Male Sprague-Dawley (SD) rats (age, 6 weeks; n=16; weight, 150–170 g; purchased from Chengdu Dossy Experimental Animals Co., Ltd.) were randomly divided into two groups. The animals were maintained under pathogen-free conditions (temperature, 25°C; humidity, 50%; 12-h light/dark cycle) and had free access to food and water. A cecal ligation and puncture-induced sepsis rat model was established as previously described (18). Briefly, rats were fasted overnight (12 h) one day prior surgery (the body weight loss after fasting was 3–8 g) and anesthetized by intraperitoneal injection of 10% chloral hydrate (200 mg/kg body weight). None of the rats presented with signs of peritonitis following injection. Once the rats appeared unconscious with normal breath, the lower abdomen was incised, and the cecum was ligated with 2-0 surgical silk, pierced with an 18-gauge needle, gently compressed until fecal matter was extruded, and returned to the abdominal cavity. Finally, the abdomen was completely closed with 2-0 surgical silk. Animals in the sham group underwent exactly the same procedure without the cecal puncture. After 24 h, the rats were euthanized with intraperitoneal injection of pentobarbital sodium (200 mg/kg);

death was confirmed by the occurrence of cardio-respiratory arrest, and ~10 ml of serum was collected from the abdominal aorta. In the current study, symptoms such as pain, weight loss, loss of appetite or weakness were set as humane endpoints; however, no animal was sacrificed prior the completion of the experiments due to reaching these endpoints. All experimental procedures in animals were carried out according to international, national and institutional regulations, and were approved by the Shaanxi University of Chinese Medicine (approval no. 201801115).

Cell culture and treatment. HUVECs were obtained from Cobioer Biosciences Co., Ltd. (lot no. CBP60340) and cultured in DMEM supplemented with 10% FBS at 37°C with 5% CO₂. Prior to treatment, cells (1–1.5 \times 10⁷) were cultured in serum-free medium for an additional 12 h. HUVECs in the septic serum-treatment group were cultured in DMEM supplemented with 10% septic serum for 12 or 24 h, whereas those in the control group were cultured in DMEM with 10% control serum. For the cell signaling pathway investigation, the cells were pre-treated with specific inhibitors for 1 h, followed by treatment with 10% septic serum. The concentrations of the specific inhibitors were as follows: 20 μ M ERK 1/2 inhibitor PD98059; 10 μ M p38 inhibitor SB203580; 20 μ M JNK inhibitor SP600125; 10 μ M antioxidant NAC; and 10 μ M NF- κ B inhibitor PDTC, as previously described (19).

Cell viability assay. Following treatment, HUVEC viability was assessed using a Cell Counting Kit (CCK-8; cat. no. C0037; Beyotime Institute of Biotechnology). Briefly, cells (1–1.5 \times 10⁵) were seeded into a 96-well plate, and 10 μ l CCK-8 solution was added into each well, followed by incubation at 37°C for an additional 4 h. Subsequently, the optical density (OD) of each well was measured at 450 nm using a microplate reader (Molecular Devices, LLC). The mean OD value from six wells was obtained, and the cell viability was calculated as the percentage relative to the OD values in the control group.

Cell morphology analysis. Cell morphology was examined using a JEM-101 (Jeol Electron Inc.) transmission electron microscope (TEM). At 12 h following treatment, cells were collected by centrifugation (150.3 \times g; 5 min), washed three times with PBS and fixed in 1% paraformaldehyde supplemented with 2% glutaraldehyde for 24 h at 4°C. Fixed cells were further treated with 1% osmium tetroxide for 2 h at 25°C, dehydrated in graded ethanol (50, 70, 80, 90 and 100% for 10 min/step) and embedded in araldite. Ultrathin sections (70 nm) were cut, stained with uranyl acetate for 30 min at 25°C, washed three times with double distilled water, and stained with lead citrate for 10 min at 25°C, followed by washing three times with double distilled water. Finally, the cell morphology was observed under a JEM-101 TEM (x8,000 magnification; JEOL Ltd.), and three fields were observed per sample.

Reverse transcription-qPCR (RT-qPCR). Following treatment, HUVECs (1 \times 10⁵ cells/well) were washed twice with ice-cold PBS, and total RNA was extracted using TRIzol[®] reagent according to the manufacturer's instructions. The concentration of the total RNA was determined by measuring the absorbance at 260 nm. Subsequently, total RNA was reverse-transcribed

into cDNA using a PrimeScript™ 1st strand cDNA Synthesis Kit. The cDNA of the target genes was amplified using the SYBR® Premix Ex Taq on the Mx3000P quantitative PCR system (Stratagene; Agilent Technologies, Inc.). The primer sequences used were as follows: Human IL-1 β forward, 5'-CATTGAGCCTCATGCTCTGTT-3' and reverse, 5'-CGC TGTCTGAGCGGATGAA-3'; human IL-6 forward, 5'-TTC GGTCCAGTTGCCTTCTC-3' and reverse, 5'-TCACCAGGC AAGTCTCTCA-3'; human TNF- α forward, 5'-GCTGCA CTTTGGAGTGATCG-3' and reverse, 5'-GCTTGAGGGTTT GCTACAACA-3'; and human GAPDH forward, 5'-TGTGGG CATCAATGGATTGG-3' and reverse, 5'-ACACCATGT ATTCCGGGTCAAT-3'. The expression levels of the target mRNAs were normalized to those of GAPDH. All samples were run in triplicate and analyzed using the $2^{-\Delta\Delta C_q}$ method as previously described (20).

ELISA. The current knowledge of the pathophysiology of sepsis suggests that patients present with hyperinflammation, and excessive production of inflammatory markers (such as IL-1 β , IL-6 and TNF- α) occurs throughout the course of sepsis (21). In the current study, HUVECs ($1-1.5 \times 10^5$) were cultured in 96-well plates and stimulated with septic serum for 12 h. Subsequently, the supernatant was collected by centrifugation ($900 \times g$; 10 min at 4°C), and the serum levels of IL-1 β , IL-6 and TNF- α were evaluated using specific ELISA kits according to the manufacturer's instructions. Subsequently, the optical density (OD) of each well was measured at 490 nm using a microplate reader (Molecular Devices, LLC).

Measurement of superoxide anion generation. HUVECs ($1-1.5 \times 10^7$) were cultured in 6-well plates and treated with septic serum for 12 h. Subsequently, the cells were supplemented with 10 μ M H₂DCF-DA for 1 h and washed with ice-cold PBS three times. Fluorescence images were acquired at an excitation wavelength of 488 nm and an emission wavelength of 525 nm under a fluorescence microscope (x200 magnification; Olympus Corporation), and six fields were observed per well. Fluorescence intensity was calculated and analyzed from the fluorescence images with the Image-pro plus software (Version X; Media Cybernetics, Inc.). The relative fluorescence intensity was calculated as the mean value of six independent experiments.

Western blotting. Following treatment with septic or normal serum in 6-well plates, cells were washed twice with ice-cold PBS and lysed using a lysis buffer (100 μ l/well; Beyotime Institute of Biotechnology) supplemented with a protease inhibitor cocktail and phosphatase inhibitors (Roche Diagnostics). The nuclear proteins were extracted using a NE-PER Nuclear Cytoplasmic Extraction Reagent kit (Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Briefly, treated cells were lysed with 200 μ l cytoplasmic extraction reagent I followed by the addition of 11 μ l cytoplasmic extraction reagent II for 5 sec. Subsequently, the cells were incubated on ice for 1 min, centrifuged ($10,000 \times g$) at 4°C for 5 min, and the supernatant fractions (cytoplasmic extracts) were transferred into a prechilled tube. The insoluble pellet fraction was resuspended in 100 μ l nuclear extraction reagent followed by vortexing for 15 sec, incubation on ice for

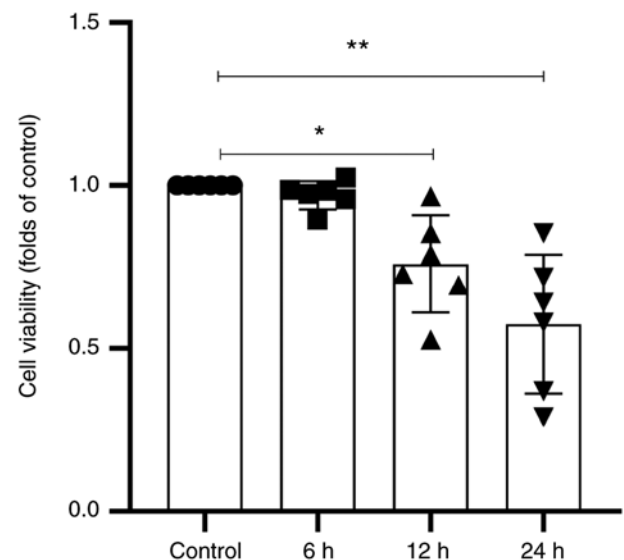


Figure 1. Septic serum decreases the viability of human umbilical vein endothelial cells. The cells were incubated with septic serum for 0-24 h, and the cell viability was determined by Cell Counting Kit-8 assay. The data from six independent experiments are expressed as the mean \pm SEM. *P<0.05 and **P<0.01.

10 min and centrifugation ($12,000 \times g$) at 4°C for 10 min. The resulting supernatant was used for subsequent experiments. Protein concentration was determined with a BCA protein assay kit (Bio-Rad Laboratories, Inc.). Equal amounts of protein extracts (30 μ g) were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes (pore size, 0.45 μ m; Cytiva). The membranes were incubated with antibodies against β -actin (1:2,000), NF- κ B (1:2,500), lamin B1 (1:1,500), JNK (1:1,500), phospho-JNK (1:800), p38 (1:1,000), phospho-p38 (1:500), ERK1/2 (1:1,000) or phospho-ERK1/2 (1:800) overnight at 4°C. Following washing with TBS + 0.25% Tween-20 three times, the membranes were incubated with the corresponding secondary antibody (1:2,500) for 3 h at 25°C, and the immune complexes were enhanced by chemiluminescence (Merck Life Science UK, Ltd.). The intensity of the bands was determined by scanning and quantification using the Bio-Rad Gel Doc™ 2000 imaging system (Bio-Rad Laboratories, Inc.).

Statistical analysis. The data are presented as the mean \pm standard error of the mean. The normality and homogeneity of these data were tested, and the differences among groups were assessed with one-way ANOVA followed by Dunnett's or non-parametric Kruskal-Wallis analysis followed by Dunn's test using GraphPad Prism 8.3 software (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Septic serum attenuates HUVEC viability. In the present study, cell viability was assessed to evaluate the toxic effects of septic serum. As demonstrated in Fig. 1, the viability of HUVECs was significantly decreased following treatment with septic serum for 12 and 24 h. This suggested that

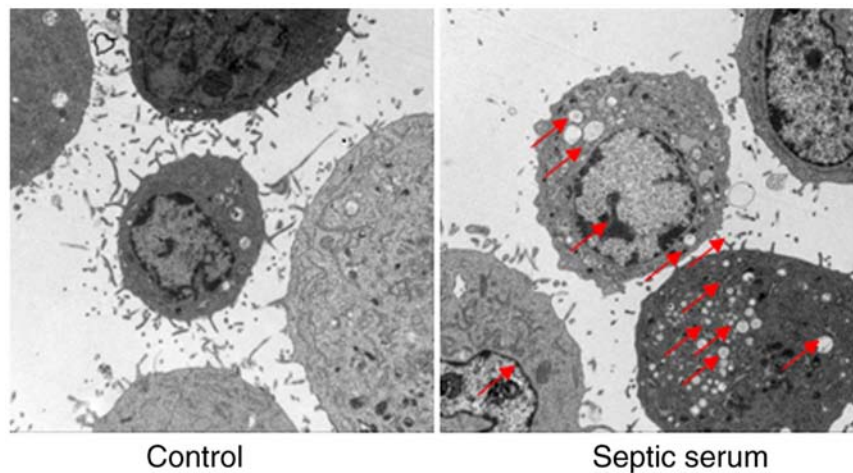


Figure 2. Septic serum induces injury in human umbilical vein endothelial cells. The cells were subjected to septic serum treatment for 12 h, and the morphological changes were observed by transmission electron microscopy. Magnification, x8,000.

sepsis exerted harmful effects on vascular endothelial cells; according to these results, 12 h was selected the treatment time for subsequent experiments.

Septic serum induces HUVEC injury. To evaluate the effects of septic serum on endothelial cell injury, HUVECs were observed under TEM following treatment with septic serum. As presented in Fig. 2, cells treated with the normal serum exhibited normal morphology. However, when cells were treated with septic serum for 12 h, harmful morphological changes were identified, including chromatin condensation, mitochondrial vacuolization and endoplasmic reticulum degranulation. These results further demonstrated that septic serum mediated HUVEC injury.

Septic serum stimulates the expression of inflammatory cytokines by HUVECs. In the present study, the mRNA and protein levels of IL-1 β , IL-6 and TNF- α were determined by RT-qPCR and ELISA, respectively. The results demonstrated that the mRNA expression levels of IL-1 β , IL-6 and TNF- α were significantly increased following treatment of HUVECs with septic serum compared with those in the normal serum-treated cells (Fig. 3A-C). Consistent with these results, ELISA demonstrated that the protein secretion levels of IL-1 β , IL-6 and TNF- α in the culture medium were significantly increased following treatment of HUVECs with septic serum compared with those in the medium collected from cells treated with normal serum (Fig. 3D-F).

Septic serum promotes ROS generation, ERK1/2 and p38 phosphorylation, and the translocation of NF- κ B in HUVECs. As demonstrated in Fig. 4A, the intracellular ROS generation was notably enhanced in HUVECs treated with septic serum compared with that in the control cells. Furthermore, western blotting results demonstrated that the levels of phosphorylation of ERK1/2, p38 and JNK, and the protein levels of NF- κ B p65 in the cell nuclei were markedly increased after cell stimulation with septic serum for 12 h compared with those in the control group (Fig. 4B-E). Immunofluorescence staining also revealed that septic serum promoted NF- κ B translocation into HUVEC nuclei (Fig. 4F). Additionally, ELISA results

demonstrated that the secretion levels of IL-1 β , IL-6 and TNF- α were significantly decreased in the culture medium isolated from HUVECs pre-treated with the ERK1/2 inhibitor PD98059, the p38 inhibitor SB203580, the JNK inhibitor SP610025, the NF- κ B inhibitor PDTC or the antioxidant NAC for 1 h compared with those in cells treated with septic serum alone (Fig. 5). These results indicated that ROS, MAPKs and the NF- κ B signaling pathway may be involved in septic serum-induced inflammation in HUVECs.

Discussion

Endothelial cells are considered to be a crucial link between the cardiovascular and immune systems, and an essential and active component of the immune response (22). In sepsis, multiple organ dysfunction is partially caused by systemic inflammation-mediated microvascular endothelial cell injury (23-25). Furthermore, it has been revealed that the high levels of circulating endothelial cells and soluble markers associated with endothelial cell damage may indicate vascular injury, and are highly associated with severe sepsis and high mortality (26). In the current study, a sepsis rat model was established to investigate the effects and mechanism of sepsis on endothelial cell injury. The results demonstrated that treatment of HUVECs with septic serum induced the expression of IL-1 β , IL-6 and TNF- α , suggesting that the *in vitro* model mimicked the *in vivo* processes.

Sepsis is characterized by the activation of inflammation via several mechanisms, including severe oxidative stress-induced endothelial cell damage (27-29), and ROS-associated molecular signature predicts survival in patients with sepsis (30). ROS generation occurs during the onset of the inflammatory cascade (31). The results of the current study demonstrated that ROS generation was involved in the inflammatory effects of septic serum, as high levels of ROS were detected in HUVECs following treatment with septic serum, whereas pre-treatment with NAC significantly attenuated the sepsis-mediated expression of the inflammatory factors IL-1 β , IL-6 and TNF- α . It has been suggested that ROS serves a crucial role in the activation of proinflammatory mediators such as MAPKs, NF- κ B and NLRP3 inflammasomes (32,33). Dysregulation of ROS generation

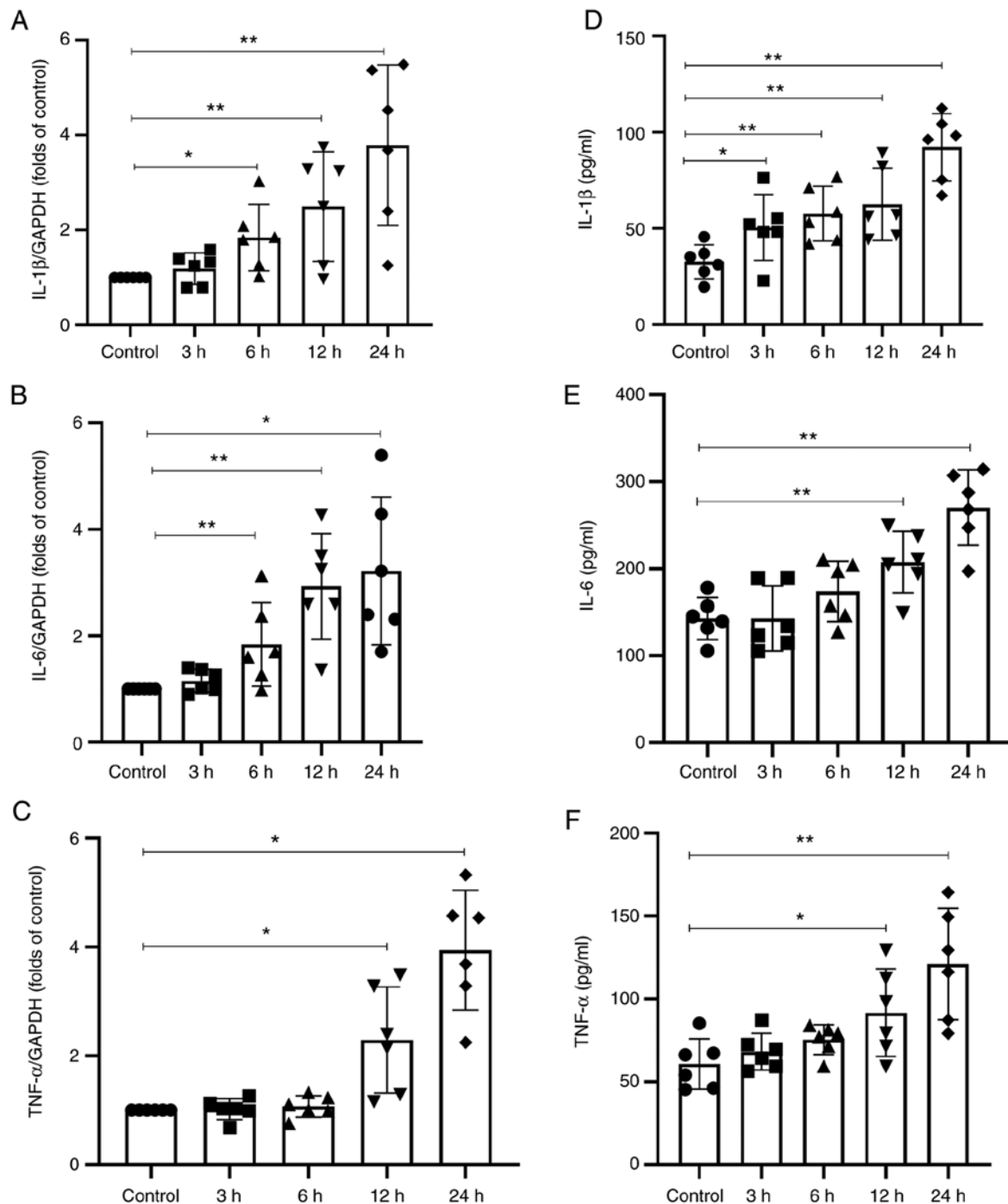


Figure 3. Septic serum increases mRNA and protein expression levels of IL-1 β , IL-6 and TNF- α in human umbilical vein endothelial cells. The cells were subjected to septic serum treatment for 0-24 h. (A) IL-1 β , (B) IL-6 and (C) TNF- α mRNA expression levels were determined by reverse transcription-quantitative PCR. (D) IL-1 β , (E) IL-6 and (F) TNF- α protein levels were determined by ELISA. The data from six independent experiments are expressed as the mean \pm SEM. *P<0.05 and **P<0.01.

or insufficient ROS scavenging may result in the oxidation of a range of biomolecules, such as hypoxia-inducible factor 1 α , AMPK and NF- κ B inducing kinase, and the structural modification of proteins triggering signaling cascades, including the MAPK, NF- κ B and PI3K/AKT signaling pathways, leading to the progression of inflammatory diseases (34,35). In the present study, increased levels of phosphorylated ERK1/2 and p38, and translocation of NF- κ B to the nucleus were observed following stimulation of HUVECs with septic serum compared with those observed in the control cells. MAPKs and the NF- κ B signaling

serve a pivotal role in inflammation (17,36-40), whereas the activation of NF- κ B regulates the expression of a number of inflammatory cytokines (41,42). In the present study, treatment of HUVECs with selective ERK1/2, p38 MAPK and NF- κ B inhibitors significantly suppressed the septic serum-induced secretion of inflammatory factors.

In conclusion, the results of the present study demonstrated that septic serum mediated endothelial cell injury via increasing ROS generation, activating MAPKs and promoting NF- κ B translocation (Fig. 6). These results may provide a

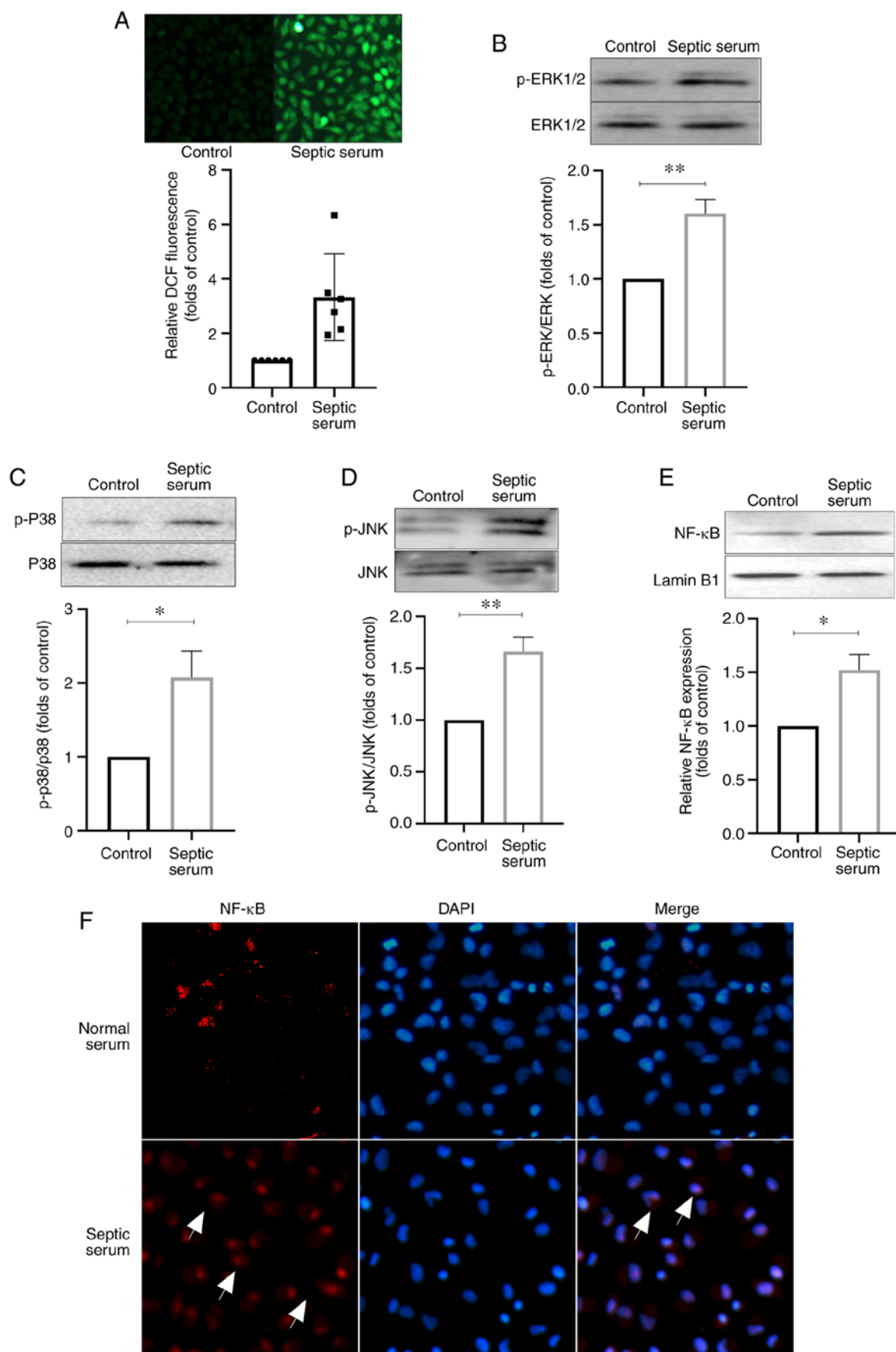


Figure 4. Septic serum increases the generation of ROS, activates MAPKs and promotes NF- κ B transduction in HUVECs. (A) The cells were subjected to septic serum treatment for 12 h, and ROS generation was determined by fluorescence microscopy. Magnification, x200. (B-E) Protein phosphorylation levels of (B) ERK1/2, (C) p38, (D) JNK and (E) NF- κ B were assayed by western blotting. (F) The NF- κ B level in the nuclei of HUVECs was detected by immunofluorescence staining. Red, NF- κ B subunit p65; blue, nucleus. Data are expressed as the mean \pm SEM from three independent experiments. * P <0.05 and ** P <0.01. DCF, 2',7'-dichlorodihydrofluorescein diacetate; ROS, reactive oxygen species; p-, phosphorylated; HUVECs, human umbilical vein endothelial cells.

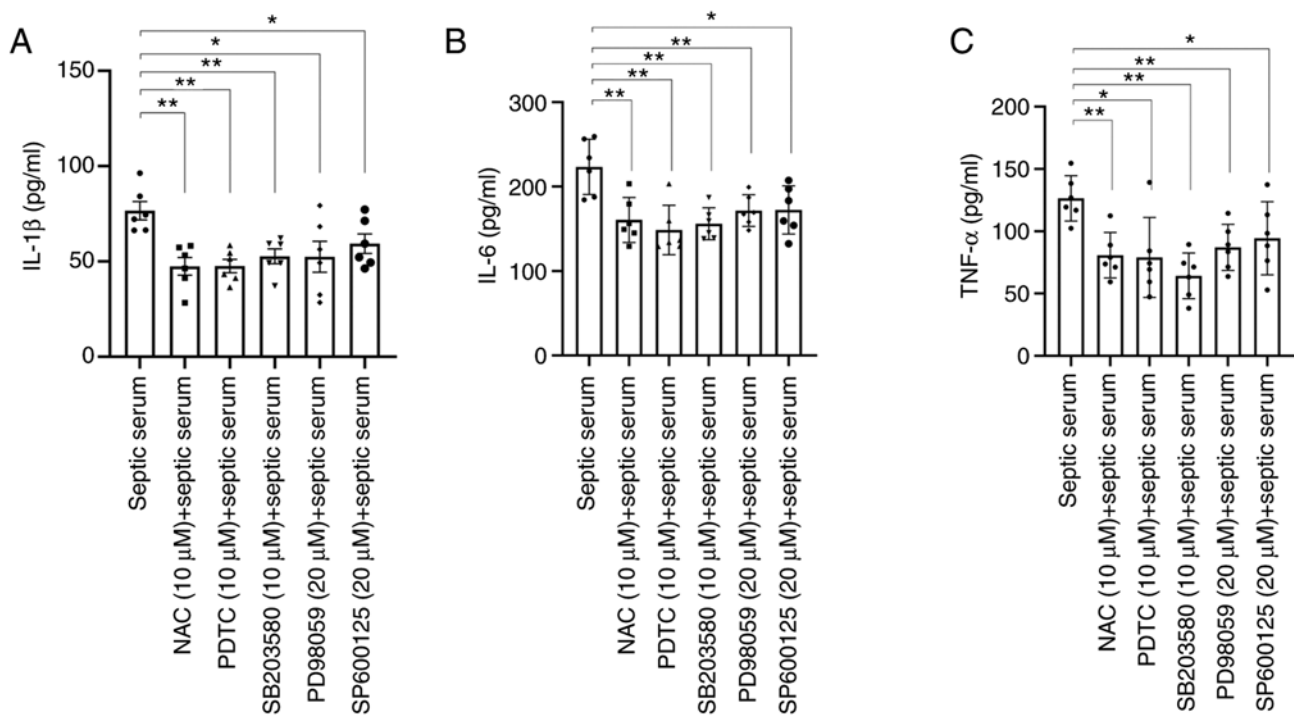


Figure 5. Inflammatory effects of septic serum are mediated by the reactive oxygen species/MAPK/NF- κ B signaling pathway in human umbilical vein endothelial cells. (A-C) The cells were treated with septic serum for 12 h with or without pretreatment with NAC, PD98059, SB203580 or SP600125 for 1 h, and the protein secretion levels of (A) IL-1 β , (B) IL-6 and (C) TNF- α were determined by ELISA. Data from six independent experiments are expressed as the mean \pm SEM. * P <0.05 and ** P <0.01. NAC, N-acetylcysteine.

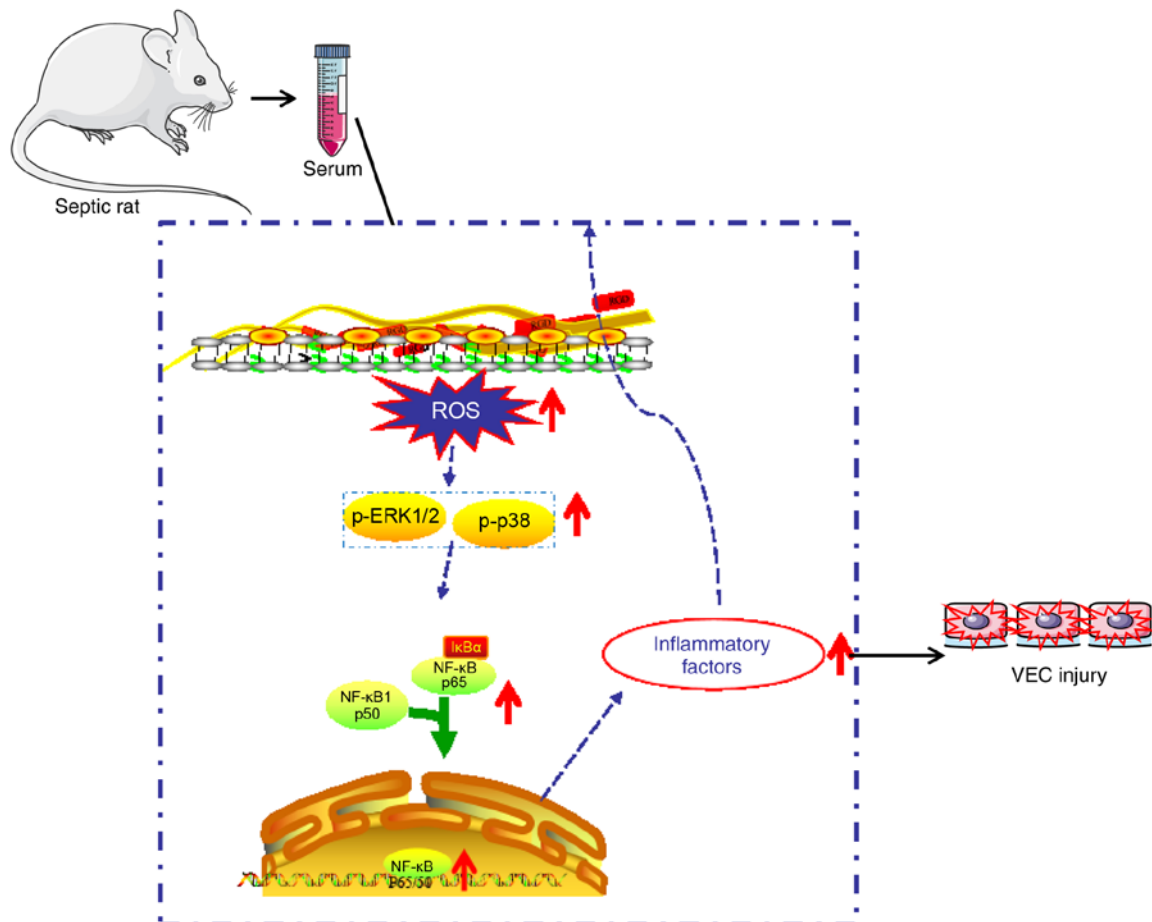


Figure 6. Septic inflammatory injury of serum-induced on human umbilical vein endothelial cells was mediated by increasing the generation of ROS, activating MAPKs and promoting NF- κ B transduction. ROS, reactive oxygen species; p-, phosphorylated; VECs, vascular endothelial cells.

novel strategy for vascular protection and the development of new types of antioxidants, as well as MAPK and NF- κ B inhibitors for the treatment of sepsis.

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Availability of data and materials

The data used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

SX and JZ made substantial contributions to the conception and design of the study. YY, ZY and JX acquired, analyzed and interpreted the data. BQ, JL, ZZ and YH interpreted the data, drafted the article and revised it critically for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The experimental protocol was approved by the Institutional Animal Care and Use Committee of the Shaanxi University of Chinese medicine (approval no. 201801115; Xianyang, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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