Original Paper

Continuous Blood Purification Ameliorates Multiple Organ Failure Through Inhibiting the Activation of the P38 MAPK Signaling Pathway in a Rat Model

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Key Words

Multiple organ failure • Continuous blood purification • P38 MAPK signaling pathway • INOS • Serum nitric oxide • MAPK

Abstract

Background/Aims: Multiple organ failure (MOF) is a primary threat to the survival of patients with systemic inflammation. Blood purification is employed in the treatment of MOF, as an artificial kidney or artificial liver. This study focuses on the effects of continuous blood purification (CBP) on ameliorating MOF through regulating the p38 mitogen-activated protein kinase (MAPK) signaling pathway in a rat model. **Methods:** A rat model of MOF was successfully established by endotoxin injection after hemorrhagic shock resuscitation. The mRNA expressions of inducible nitric oxide synthase (iNOS) and p38 MAPK of liver, kidney, and lung tissues in each group were measured by RT-qPCR at each measuring time point. To evaluate the activation of p38 MAPK signaling pathway, protein levels of phosphorylated p38 (p-p38) MAPK and p38 MAPK was measured by western blot analysis. The serum levels of nitric oxide and TNF- α were determined. **Results:** After CBP treatment, the levels of SGPT, SGOT, Cr, and BUN were significantly declined, while the PaO₂ value was increased. Expressions of p38 MAPK mRNA, iNOS mRNA, p-p38 MAPK protein and p38 MAPK protein, and nitric oxide and TNF- α levels were markedly elevated in MOF, an effect blunted by CPB. Meanwhile, pathological sections of liver, kidney, and lung tissues after CPB treatment ameliorated swelling and inflammation. **Conclusion:** Our study proved that CBP could downregulate the p38 MAPK signaling pathway, suppress iNOS expression, reduced the serum levels of nitric oxide and TNF- α , thus ameliorate symptom of MOF.

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Introduction

Multiple organ failure (MOF), a clinical syndrome of two or more organ failure (heart, liver, lung, and kidney) with high morbidity and mortality, is characterized as a sequela to ischemia/reperfusion (I/R) injury, hemorrhage shock, abdominal compartment syndrome (ACS) and infection [1-4]. Hemorrhage is a main cause of shock and preventable death in trauma [5]. During hemorrhagic shock, splanchnic hypoperfusion could result in cellular hypoxia and tissue damage, which further contributes to severe organ failure [6]. Previous studies well documented that I/R injury could promote the production of various reactive oxygen species (ROS), which caused lipid peroxidation, leading to cellular membrane instability and consequently, cell damage [7-9]. Cellular injury facilitated the expressions of tumor necrosis factor- α (TNF- α) and other inflammatory cytokines, which ultimately led to systemic inflammatory response syndrome (SIRS) and MOF [10]. Besides, endotoxemia has shown significant ability in regulating inducible nitric oxide synthase (iNOS) expression and serum nitric oxide levels [11]. And researchers have also found that MOF caused by endotoxemia seriously affected patients' survival rate [12]. Fortunately, previous studies have proved blood reperfusion might play an important role in restoring intravascular volume and improving the oxygen carrying capacity of blood cells [13, 14]. Moreover, blood purification has also shed lights on the treatment of MOF recently due to its capabilities of removing of toxic substances and inflammatory cytokines and maintaining of the stability of hemodynamics [15]. According to a previous study, continuous blood purification (CBP) could ameliorate the prognosis of patients who suffer from MOF, but the exact mechanisms were still unclear [16].

Mitogen-activated protein kinases (MAPKs), including p38 MAPK, c-Jun N-terminal kinases (JNKs) and extracellular signal-regulated kinases (ERKs), were critical components of the signaling transduction system, playing a key role in various cellular responses, such as proliferation, differentiation, and apoptosis [17, 18]. During hemorrhage shock, MAPKs showed remarkable ability to regulate vascular reactivity [19]. Besides, p38 MAPK signaling pathway, associated with I/R injury, was found to be a potential target for prevention and treatment of tissue injury [20, 21]. Therefore, the alleviation of circulatory shock and MOF induced by the inhibition of p38 MAPK signaling pathway might cause by the pathway downregulating the expressions of TNF- α and other inflammatory cytokines, as well as suppressing the inflammatory response to endotoxemia subsequently [22]. In our study, we focused on investigating the effects of CBP on ameliorating MOF via the p38 MAPK signaling pathway in a rat model.

Materials and Methods

Experimental animals and ethics statement

A total number of 90 Sprague Dawley (SD) rats of 6 weeks old (Shanghai SLAC Laboratory Animal Co., Ltd., Shanghai, China) were housed in separated cages under specific pathogen free (SPF) conditions, at room temperature ($25 \pm 3^\circ\text{C}$) and relative humidity (55~75%) with free access to sterile food and water. Their cages were cleaned every two days. All experiments were performed in accordance with the rules and regulations of the Animal Care and Use Committee and the Ethics Committee in China-Japan Friendship Hospital.

Animal grouping and model establishment

All rats were randomized into 3 groups: sham operation group ($n = 30$), MOF group ($n = 30$), and the CBP group ($n = 30$). MOF in rats were established by surgical trauma, hemorrhagic shock and reperfusion as the first hit and the endotoxin injection as the second hit. Rats were first intraperitoneally anesthetized with thiopental sodium (Sigma-Aldrich Chemical Company, St Louis MO, USA) at a dosage of 85 mg/kg according

to the body weight, then fixed and sterilized. Afterwards, common carotid artery, femoral artery, and femoral vein were separated and intubated respectively with the common carotid artery being connected to a digital PowerLab data acquisition system (PowerLab 8/30, AD Instruments, Castle Hill, Australia). Ten min after the surgery, mean arterial pressure (MAP) was reduced to 35~40 mmHg within 5 min and maintained for 1 h by withdrawing blood from the femoral artery, and the hemorrhagic shock was induced. Then, MAP of the rat was restored to over 80 % of the preoperative value by intravenously reinfusing with 60% lost blood and equilibrium liquid twice the volume of lost blood. After a recovery of 12 h, the rat was anesthetized with inhaled ethyl ether. Then *Salmonella abortus equi* endotoxin (Sigma-Aldrich Chemical Company, St Louis MO, USA) was injected at a dosage of 4.5 mg/Kg via femoral vein within 15 min. In the sham operation group, rats were only treated with surgical trauma.

CBP treatment

A Braun Dipact CRRT machine with an F60 channel and filter (B. Braun Melsungen AG, Melsungen, Germany) was applied on rats in the CBP group to give a CBP treatment 24 h after endotoxin injection. A two-way indwelling catheter was inserted through the right internal jugular vein to form an extracorporeal circulation system. Substitution fluid was infused at a flow rate of 200 mL/min with dilution method. Before connected with the catheter, the right internal jugular vein was circularly washed with heparin-saline solution (5000 U/L) for 30 min, and heparin of 1500 U/h was used for continuous anticoagulation during the treatment. The duration of CBP treatment lasted for 24 h to cover the whole experiments.

Measurements of the parameters related to MOF

Then 1 mL of peripheral blood were collected from saphenous vein of each rat and tested at 5 time points including T_0 (before withdrawing blood from rats), T_1 (24 h after endotoxin injection), T_2 (6 h after CBP), T_3 (12 h after CBP), and T_4 (24 h after CBP). After 1 h of standing, the blood samples were centrifuged at 3000 rpm for 10 min. Collected supernatant was then preserved at -80 °C for further use. Levels of serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), creatinine (Cr), blood urea nitrogen (BUN), and arterial oxygen partial pressure (PaO_2) of each blood sample were determined with an automatic biochemical analyzer (Olympus AU5400, Tokyo, Japan). According to the diagnostic index of MOF [23], rats with 2 or more than 2 organ failures 24 h after hits were considered as MOF rats. Otherwise, rats that died or exhibited MOF within 24 h after hits were considered as failed cases of hemorrhagic shock resuscitation (included in the total number). Diagnostic indexes of MOF were described as follows: respiratory failure: $PaO_2 < 8$ kPa (60 mmHg); liver failure: $SGOT/SGPT > \text{two times the control value}$; kidney failure: $Cr/BUN > \text{two times the control value}$; gastrointestinal failure: gastrointestinal tract showed mucosal bleeding, erosion and stress ulceration. Six rats in each group at each measuring time from T_0 to T_4 , was euthanized and liver, kidney, as well as lung tissues, were collected. Then part of the tissues were cut and fixed in formalin (Sigma-Aldrich Chemical Company, St Louis MO, USA), and the rest were preserved in liquid nitrogen at -80 °C.

Reverse-transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA from liver, kidney, and lung tissues were extracted using a RNeasy mini kit (Qiagen, GmbH, Hilden, Germany). Total RNA was regarded as a template for reverse transcription reaction using the TaqMan® MicroRNA Reverse Transcription kit (Applied Biosystems Inc., Foster City, CA, USA) with a total reaction volume of 15 µL under the following reaction condition: having been incubated for 30 min at 16 °C, the reaction system was then heated at 42 °C for 30 min, followed by 5-min incubation at 85 °C. The qRT-PCR was performed using TaqMan Universal PCR kit (Applied Biosystems Inc., Foster City, CA, USA) with the following reaction condition: having been initiated for 10 min at 95 °C, the reaction system was performed with 40 cycles of denaturation for 30 s at 95 °C and 1-min annealing at 60 °C. Afterwards, RT-PCR was conducted by using ABI 7500 Real-Time PCR System (Applied Biosystems Inc., Foster City, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was applied as an internal reference. We have exhibited the primers used in this experiment in Table 1. The relative expressions were calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

After being washed by phosphate-buffered saline (PBS), the tissues were ground into homogenate. Cell lysate containing protease inhibitor was supplemented into tissue homogenate, followed by shaking the mixture at 4°C for 5 min. After 10-min centrifugation at 14, 400 rpm, the supernatant was collected. The protein was extracted using the Qproteome Mammalian Protein Prer kit (Qiagen, GmbH, Hilden, Germany). After protein concentration having been measured and adjusted, 6 × loading buffer was added, and then samples were heated up to 100°C and preserved in liquid nitrogen at -20°C. 50 µg of protein sample was loaded

onto sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein was separated and transferred onto a nitrocellulose membrane by siphonage. The membranes were blocked with skim milk and then cultured with primary antibodies (p-p38 MAPK or p38 MAPK, 1:1000 dilution, mouse anti-human monoclonal antibodies) overnight at 4°C. The membranes were washed 4 times with Tris-buffered saline tween-20 (TBST) (each time for 10 min), and then incubated with diluted IRDye™ 800DX-conjugated goat anti-mouse immunoglobulin G (IgG, 1: 10, 000) for 1 h at room temperature. The protein bands were developed after washed by TBST for 4 times. GAPDH was conducted as an internal reference. Antibodies used in this experiment were all purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The intensity of protein band was determined quantitatively by LabWorks Image Acquisition and Analysis Software (UVP, Inc., Upland, CA, USA) to analyze the protein concentrations of p-p38 MAPK, p38 MAPK, and GAPDH.

Determination of serum nitric oxide levels

Serum nitric oxide levels in each sample were determined using nitrate reductase assay and Nitric Oxide Assay Kit (Shanghai Honsun Biological Technology Co., Ltd., Shanghai, China) at each measuring time point. An amount of 50 µL serum with 400 µL lysis buffer was incubated in water bath of 37°C for 1 h, and then 500 µL buffer was supplemented for 40 min standing at room temperature. The mixture was centrifuged for 10 min at 3500~4000 r/min. Collected supernatant was supplemented with chromogenic reagents for 10 min. The OD value of each sample was determined by a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 550 nm. On the basis of the standard curves with the OD values of the samples, serum nitric oxide levels were calculated and recorded.

Enzyme-linked immunosorbent assay (ELISA)

Serum level of TNF- α was detected in accordance with the instructions of ELISA kit (ml002859, Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China). A total of 100 µL from each standard fluid or sample was added to each well in duplicate, and 50 µL of the sample diluent was added to each well. Then 50 µL of the biotinylated antibody reagent was added to each well and incubated for 1 h at room temperature, at 20~25°C. Plates were then washed 4 times with a washing solution. Then, 100 µL of TMB substrate solution was added and incubated in the dark for 10 min at room temperature. Subsequently, 100 µL of stop solution was added to each well to stop the reaction. Absorbance was measured on an automated ELISA plate reader (Dynex, DSX, Chantilly, USA) set at the wavelength of 450 nm. The standard curve was generated by plotting the average absorbance obtained for each standard concentration on the vertical (Y) axis vs. the corresponding TNF- α level on the horizontal (X) axis. The amount of TNF- α in each sample was determined with this curve as pg/µL.

Table 1. Primer sequences for RT-qPCR. Note: RT-qPCR: reverse-transcription quantitative polymerase chain reaction; MAPK, mitogen-activated protein kinase; iNOS, inducible nitric oxide synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; F, forward; R, reverse

Gene	Sequence
p38 MAPK	F: 5'-TAGACGAATGGAAGAGCCTGA-3'
	R: 5'-GGCACTTGAATGGTATTTGGAGA-3'
iNOS	F: 5'-CAGCTGGGCTGTACAAACCTT-3'
	R: 5'-CATTGGAAGTGAAGCGTTTCG-3'
GAPDH	F: 5'-ACCACAGTCCATGCCATCAC-3'
	R: 5'-TCCACCACCCTGTTGCTGTA-3'

Hematoxylin-eosin (HE) staining

Fresh tissues were extracted and placed in 4% formalin overnight. On the following day, the tissues were dehydrated and embedded in paraffin. Then tissues were sliced into sections at a thickness of 4 μ m, followed by the staining with HE, observation and photograph under a fluorescence microscope. The detailed procedures were as follows: glass slide was dewaxed in xylene I and II and then in alcohol (100%, 90% and 70%), and washed with tap water for 2~3 min. After 5-min staining in hematoxylin, glass slide was washed again for 5~10 min. Next, glass slide was immersed in 1% hydrochloric acid for 5~10 s for color separation and then treated with 2-min washing with tap water, 2 min each time. Afterwards, glass slide was stained with eosin and incubated for 1~2 min, and washed with tap water for 1~2 s. After section, tissues were dehydrated with alcohol (80%, 90% and 100%) for 30 s. Finally, the sections were immersed in xylene I and II for 5 min and sealed with neutral balsam.

Statistical analysis

SPSS 19.0 software was applied for statistical analysis in this study. Data were expressed as mean $\pm$ standard deviation. Comparison of data between two groups was performed with *t*-test. Comparisons among multiple groups were assessed by mixed-design ANOVA. Pairwise comparison of data among groups was processed with LSD-*t* test. *P* < 0.05 was considered to be statistically significant.

Results

CBP treatment lowers the levels of SGPT, SGOT, Cr, and BUN in MOF rats

Initially, to manifest the effects of CBP treatment on organ functions related to MOF, we detected the levels of SGPT, SGOT, Cr, BUN and PaO₂ in all testing groups at each measuring time point, the results of which are shown in Table 2. Level of each parameter in the sham operation group remained almost unchanged at each time point (all *p* > 0.05). And no differences were observed among 3 groups at T₀ (all *p* > 0.05). However, the levels of SGPT, SGOT, Cr, and BUN in the MOF and CBP groups were increased at T₁, T₂, T₃ and T₄ than those in the sham operation group and at T₀, while the PaO₂ level was decreased (all *p* < 0.05) after the MOF model was established. Besides, the levels of SGPT, SGOT, Cr, and BUN in the MOF group were continually elevated at T₂, T₃, and T₄ whereas the level of PaO₂ value was significantly reduced (all *p* < 0.05). By contrast, the levels of SGPT, SGOT, Cr, and BUN in the CBP group were persistently declined at T₂, T₃, and T₄, with a significant increase of PaO₂ level (all *p* < 0.05). Moreover, no distinct differences indicated in each parameter between the

Table 2. CBP treatment decreases the levels of SGPT, SGOT, Cr, and BUN in MOF rats. Note: SGPT, serum glutamate pyruvate transaminase; SGOT, serum glutamate oxaloacetate transaminase; Cr, creatinine; BUN, blood urea nitrogen; T₀, before withdrawing blood from rats in the CBP group; T₁, 24 h after endotoxin injection; T₂, 6 h after CBP; T₃, 12 h after CBP; T₄, 24 h after CBP; ^a, vs. the sham operation group, *p* < 0.05; ^b, vs. the MOF group, *p* < 0.05; ^c, vs. the same group at T₀, *p* < 0.05; ^d, vs. the same group at T₁, *p* < 0.05; ^e, vs. the same group at T₂, *p* < 0.05; ^f, vs. the same group at T₃, *p* < 0.05; each group includes 6 rats; data were analyzed by mixed-design ANOVA

Time point	Group	SGPT (u/L)	SGOT (u/L)	Cr (umol/L)	BUN (mmol/L)	PaO ₂ (kPa)
T ₀	Sham operation group	37.29 $\pm$ 8.65	54.61 $\pm$ 12.13	15.35 $\pm$ 0.82	4.39 $\pm$ 0.21	14.16 $\pm$ 0.23
	MOF group	35.19 $\pm$ 3.25	55.43 $\pm$ 11.18	14.81 $\pm$ 1.40	4.19 $\pm$ 0.53	13.53 $\pm$ 0.35
	CBP group	35.49 $\pm$ 4.45	54.53 $\pm$ 5.38	13.23 $\pm$ 1.97	4.50 $\pm$ 0.31	14.01 $\pm$ 0.67
T ₁	Sham operation group	37.65 $\pm$ 6.10	53.78 $\pm$ 14.40	14.42 $\pm$ 1.22	4.31 $\pm$ 0.62	13.85 $\pm$ 0.59
	MOF group	154.06 $\pm$ 14.84 ^{ac}	181.60 $\pm$ 12.27 ^{ac}	26.96 $\pm$ 2.30 ^{ac}	7.49 $\pm$ 1.06 ^{ac}	14.48 $\pm$ 1.62 ^{ac}
	CBP group	148.48 $\pm$ 26.74 ^{ac}	198.57 $\pm$ 13.06 ^{ac}	26.94 $\pm$ 2.09 ^{ac}	7.55 $\pm$ 1.18 ^{ac}	14.48 $\pm$ 0.15 ^{ac}
T ₂	Sham operation group	37.30 $\pm$ 9.22	56.49 $\pm$ 6.62	14.45 $\pm$ 1.23	4.35 $\pm$ 0.21	14.00 $\pm$ 1.08
	MOF group	218.26 $\pm$ 21.61 ^{acdf}	390.13 $\pm$ 32.61 ^{acdf}	30.53 $\pm$ 3.96 ^{acdf}	8.10 $\pm$ 1.61 ^{acdf}	12.28 $\pm$ 0.30 ^{acdf}
	CBP group	128.77 $\pm$ 15.56 ^{abdef}	158.62 $\pm$ 19.30 ^{abdef}	23.16 $\pm$ 1.71 ^{abdef}	5.61 $\pm$ 0.84 ^{abdef}	14.97 $\pm$ 0.32 ^{abdef}
T ₃	Sham operation group	37.18 $\pm$ 2.43	55.01 $\pm$ 11.58	15.28 $\pm$ 0.78	4.31 $\pm$ 0.47	13.89 $\pm$ 0.65
	MOF group	352.09 $\pm$ 15.43 ^{acde}	628.74 $\pm$ 54.12 ^{acde}	34.79 $\pm$ 2.50 ^{acde}	9.83 $\pm$ 0.78 ^{acde}	11.52 $\pm$ 0.37 ^{acde}
	CBP group	116.55 $\pm$ 5.67 ^{abdef}	129.95 $\pm$ 10.26 ^{abdef}	20.67 $\pm$ 0.84 ^{abdef}	3.11 $\pm$ 0.26 ^{abdef}	15.96 $\pm$ 0.43 ^{abdef}
T ₄	Sham operation group	36.77 $\pm$ 9.68	55.39 $\pm$ 12.25	14.61 $\pm$ 2.32	4.43 $\pm$ 0.34	13.81 $\pm$ 0.68
	MOF group	564.98 $\pm$ 42.24 ^{acdef}	819.95 $\pm$ 53.85 ^{acdef}	37.76 $\pm$ 2.74 ^{acdef}	10.83 $\pm$ 0.64 ^{acdef}	10.20 $\pm$ 0.41 ^{acdef}
	CBP group	36.74 $\pm$ 18.48 ^{bdef}	54.41 $\pm$ 2.46 ^{bdef}	15.57 $\pm$ 1.65 ^{bdef}	4.68 $\pm$ 0.14 ^{bdef}	13.93 $\pm$ 0.61 ^{bdef}

Fig. 1. RT-qPCR demonstrates that CBP treatment down-regulates the mRNA expressions of iNOS and p38 MAPK in MOF rats. Panel A, relative mRNA expressions of p38 MAPK; Panel B, relative mRNA expressions of iNOS; MKPK, mitogen-activated protein kinase; iNOS, inducible nitric oxide synthase; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; T_0 , before hemorrhage in the CBP and MOF group; T_1 , 24 h after endotoxin injection; T_2 , 6 h after CBP treatment; T_3 , 12 h after CBP treatment; T_4 , 24 h after CBP treatment; *, vs. the sham operation group, $p < 0.05$; #, vs. the MOF group, $p < 0.05$; each group includes 6 rats; pairwise comparison of data among groups was processed with mixed-design ANOVA.

CBP and sham operation groups 24 h after CBP treatment (T_4) (all $p > 0.05$). These findings demonstrated that CBP treatment may exert protective role in organ functions in MOF rats.

CBP treatment down-regulates the mRNA expressions of iNOS and p38 MAPK in MOF rats

Subsequently, relative mRNA expressions of iNOS and p38 MAPK in liver, kidney, and lung tissues at each time point were determined by RT-qPCR in order to identify the effects of CBP treatment on the p38MAPK signaling pathway. As shown in Fig. 1, there were no remarkable differences shown in mRNA expressions of iNOS and p38 MAPK in the sham operation group at all testing time points (all $p > 0.05$). And no significant differences were showed in mRNA expressions of iNOS and p38 MAPK among 3 testing groups at T_0 (all $p > 0.05$). However, in contrast to the sham operation group, the mRNA expressions of iNOS and p38 MAPK in the MOF and CBP groups were markedly enhanced at T_1 , T_2 , T_3 , and T_4 (all $p < 0.05$) after the MOF model was established. Besides, mRNA expressions of iNOS and p38 MAPK in the MOF group were continually elevated at T_2 , T_3 , and T_4 , with a significant augment compared to the sham operation group (all $p < 0.05$). While in the CBP group, mRNA expressions of iNOS and p38 MAPK were persistently declined at T_2 , T_3 , and T_4 after CBP treatment. In the CBP group, the mRNA expressions of iNOS and p38 MAPK after CBP treatment were still elevated compared with those in the sham operation group, but markedly decreased than those in the MOF group (all $p < 0.05$). The above results showed that CBP treatment may inactivate the p38 MAPK signaling pathway in MOF rats.

CBP treatment declines the protein levels of p-p38 MAPK and p38 MAPK in MOF rats

Next, we performed western blot analysis to detect the influence of CBP treatment on the protein levels of p-p38 MAPK and p38 MAPK in liver, kidney, and lung tissues at each time point, the results of which are shown in Fig. 2. In the sham operation group at all testing time points, there were no significant differences in protein levels of p-p38 MAPK and p38 MAPK

Fig. 2. Western blot analysis manifests that CBP treatment down-regulates the protein levels of p-p38 MAPK and p38 MAPK in MOF rats. Panel A, protein levels of p-p38 MAPK; Panel B, protein levels of p38 MAPK; p-p38 MAPK, phosphorylated p38 mitogen-activated protein kinase; T₀, before hemorrhage in the CBP and MOF group; T₁, 24 h after endotoxin injection; T₂, 6 h after CBP treatment; T₃, 12 h after CBP treatment; T₄, 24 h after CBP treatment; *, vs. the sham operation group, $p < 0.05$; #, vs. the MOF group, $p < 0.05$; each group includes 6 rats; pairwise comparison of data among groups was processed with mixed-design ANOVA.

(all $p > 0.05$). Among 3 testing groups at T₀, no marked differences were exhibited in protein levels of p-p38 MAPK and p38 MAPK (all $p > 0.05$). However, in comparison with the sham operation group, protein levels of p-p38 MAPK and p38 MAPK in the MOF and CBP groups were markedly increased at T₁, T₂, T₃, and T₄ (all $p < 0.05$) after MOF model establishment. Besides, protein levels of p-p38 MAPK and p38 MAPK in the MOF group were continually elevated at T₂, T₃, and T₄, which was evidently enhanced than those in the sham operation group (all $p < 0.05$). By contrast, protein levels of p-p38 MAPK and p38 MAPK in the CBP group were continuously declined at T₂, T₃, and T₄ after CBP treatment. The expressions of p38 MAPK and iNOS in CBP group after treatment were still elevated compared with those in the sham operation group, but markedly decreased compared with those in the MOF group (all $p < 0.05$). It can be concluded that CBP treatment might prevent the p38MAPK signaling pathway from activation in MOF rats.

CBP treatment reduces serum levels of nitric oxide and TNF- α in MOF rats

In the following experiment, ELISA was conducted to evaluate the effects of CBP treatment on serum levels TNF- α in all testing groups at each time point and the content of nitric oxide was determined by nitric oxide assay kit. No marked differences were observed

in serum levels of nitric oxide and TNF- α in 3 testing groups before hemorrhage (T_0) (all $p > 0.05$). And there were no remarkable differences in serum levels of nitric oxide and TNF- α in the sham operation group among all testing time points (all $p > 0.05$). Compared with the sham operation group, serum levels of nitric oxide and TNF- α in the MOF and CBP groups were markedly elevated at T_1 , T_2 , T_3 , and T_4 (all $p < 0.05$) after the establishment of MOF model. Besides, serum levels of nitric oxide and TNF- α in the MOF group were continually elevated at T_2 , T_3 , and T_4 , which were remarkably increased than those in the sham operation group (all $p < 0.05$). However, In the CBP group, serum levels of nitric oxide and TNF- α in the CBP group were persistently declined at T_2 , T_3 , and T_4 after CBP treatment. The serum levels of nitric oxide and TNF- α in the CBP group after treatment were still facilitated compared with those in the sham operation group, but markedly lower than those in the MOF group (all $p < 0.05$) (Fig. 3). In that case, we can conclude that CBP treatment may decrease the serum levels of nitric oxide and TNF- α in MOF rats.

Histological analysis of liver, kidney, and lung tissues during CBP treatment

In the following experiment, in order to determine the effects of CBP treatment on the histological changes of liver, kidney, and lung tissues in MOF rats, we adapted HE staining. No marked differences showed in each tissue among 3 testing groups at T_0 . And in the sham operation group, each tissue showed no obvious differences among all testing time points. Nevertheless, tissue histopathological changes of different degrees were observed in the MOF and CBP group after the MOF model was established in liver, kidney, and lung of rats. Swelling and deformation of renal tubular epithelial cells, as well as cell detachment and thrombus deposition could be observed in rats in the MOF and CBP groups. As for the liver tissues in rats in the MOF and CBP groups, central vein of liver indicated narrowing, congestion, and swelling, associated with ballooning degeneration or other degenerative changes in hepatocytes. Besides, lung tissues in rats with MOF exhibited edema, swelling, alveolous atrophy, and inflammatory cell infiltration. However, these symptoms mentioned above were all gradually relieved after CBP treatment, and the tissues in the CBP group gradually returned to normal status 24 h after the treatment (T_4) (Fig. 4). We can reach a conclusion that CBP treatment could relieve the symptoms of MOF.

Fig. 3. CBP treatment decreases serum levels of nitric oxide and TNF- α in MOF rats. Panel A, serum level of nitric oxide; Panel B, serum level of TNF- α ; T_0 , before hemorrhage in the MOF and CBP group; T_1 , 24 h after endotoxin injection; T_2 , 6 h after CBP treatment; T_3 , 12 h after CBP treatment; T_4 , 24 h after CBP treatment; *, vs. the sham operation group, $p < 0.05$; #, vs. the MOF group, $p < 0.05$; NO, nitric oxide; each group includes 6 rats; pairwise comparison of data among groups was processed with mixed-design ANOVA.

Discussion

MOF, known as a severe syndrome with no effective pharmacologic therapy, has been a major health burden worldwide and an outstanding pathogenesis of morbidity and mortality among critically ill patients [24]. Fortunately, CBP treatment has shown significant therapeutic effect in ameliorating the prognosis of MOF, but the exact mechanisms are still unclear [16]. We aimed to explore novel treatments for MOF by investigating the modulatory effects of CBP on ameliorating MOF via p38 MAPK signaling pathway in a rat model. Identification of the potential function of the p38 MAPK signaling pathway in MOF may shed lights on the novel therapeutic strategies for MOF and the functional repair of tissue injury.

MOF model in rats was established through 2 steps, with hemorrhagic shock as the first hit and endotoxemia as the second hit. Levels of SGPT, SGOT, Cr, BUN, and PaO_2 value were chosen to evaluate the progress of MOF in liver, kidney, and lung tissues. The results revealed that the levels of SGPT, SGOT, Cr, and BUN in the CBP group after treatment were significantly declined compared with those in the MOF group, while that the PaO_2 value was increased, indicating

Fig. 4. CBP treatment affects the pathological changes of kidney, liver and lung in rats. Panel A, images of rat liver (200 ×); Panel B, images of rat kidney (200 ×); Panel C, images of rat lung (200 ×); HE, hematoxylin and eosin; T_0 , before hemorrhage in the CBP and MOF group; T_1 , 24 h after endotoxin injection; T_2 , 6 h after CBP treatment; T_3 , 12 h after CBP treatment; T_4 , 24 h after CBP treatment.

that damage caused by MOF was greatly alleviated by CBP treatment. In addition, results of HE staining also confirmed that the swelling and inflammatory infiltration of liver, kidney, and lung tissues in the CBP group was gradually relieved after treatment. A former study has proved that CBP could ameliorate the prognosis of patients suffering from MOF via unknown mechanisms, which was in accordance with our results [16]. CBP can not only improve the general conditions but also effectively improve gut barrier dysfunction, whose beneficial effect on gut barrier dysfunction is associated with the improvement of cytoskeletal instability, by down-regulating iNOS through the removal of excess proinflammatory factors [25]. Another data showed that after CBP treatment, endothelial hyperpermeability induced by serum from severe acute pancreatitis (SAP) patients with lung injury was ameliorated [26]. Moreover, CBP treatment could improve myocardial function, shorten the oliguria period, decrease the fatality rate, and improve the prognosis of cardiorenal syndrome (CRS) patients [27].

p38 MAPK signaling pathway was a prominent factor on regulating cell apoptosis, immune, and inflammatory responses through its positive regulation on the post-transcriptional control of TNF- α and the cytokine expression under stress conditions, including I/R injury [28-30]. I/R injury is a common pathological condition that could cause acute inflammatory reaction, severe MOF, and even mortality [31]. After transferred to the nucleus under stress conditions, p38 MAPK could activate the phosphorylation of the downstream genes, facilitating the expression of genes relevant to the regulation of multiple transcription factors [32, 33]. Besides, due to target genes of the p38 MAPK signaling pathway participating in the regulation of various cellular responses, p38 MAPK signaling pathway might play a dominant role in multiple cellular regulations [34]. See that the p38 MAPK expression was elevated in tissues undergoing MOF, we confirmed that the p38 MAPK pathway was activated in such pathological condition. With comprehensive consideration of our results and previous research, we speculate that the suppression of p38 MAPK signaling pathway could alleviate tissue damage in MOF through molecular mechanisms [35], which contributed to the p38 MAPK signaling pathway being a potential novel target for the treatment of MOF.

The overproduction of various cytokines has been reported to participate in the aggravation of acute disorder, among which endotoxemia was demonstrated to contribute to the development and progression of MOF [12, 36]. Thus the restore of cytokine balance by inhibiting the overproduction of both pro- and anti-inflammatory cytokines is considered important in treatment of MOF [37]. Recently, blood purifying technique has shed lights on the treatments of MOF and other critical illnesses due to its critical capabilities of removing toxic substances and inflammatory cytokines as well as maintaining the stability of hemodynamics [15]. As data above, cytokine signaling, cellular stress response, and inflammatory cytokine production might result from the activation of the p38 MAPK signaling pathway [38]. Therefore, we resumed that CBP treatment might regulate the expression of the p38 MAPK signaling pathway via some specific cytokines, and finally ameliorate the symptoms of MOF. Our results have proved our hypothesis, where the p38 MAPK signaling pathway was suppressed during CBP treatment in the MOF rats. However, precise mechanisms still need to be further clarified in the future.

Previous studies have also documented that the p38 MAPK signaling pathway participates in acute disorder through the regulation of inflammatory cytokines [29, 39]. To further explore related mechanisms, iNOS expression and serum nitric oxide levels were analyzed in our experiment. The expression of iNOS and serum nitric oxide levels were initially elevated by endotoxin injection during the establishment of MOF model [11]. The inhibition of nNOS activity at resuscitation protected rats against the MOF and vascular dysfunction through the reduction of the levels of pro-inflammatory cytokines TNF- α and IL-6 in hemorrhagic shock rats [40]. Similarly, we found that the serum nitric oxide levels and iNOS expression in liver, kidney, and lung tissues of rats were attenuated after CBP treatment, consequently leading to the amelioration of MOF. However, little is known about the potential roles of iNOS

in vascular decompensation and inflammation associated with MOF, which still needs more investigation in further study.

Conclusion

Our study provides evidences that CBP might ameliorate MOF through the suppression of the expressions of p38 MAPK signaling pathway and iNOS, as well as the downregulation of serum nitric oxide levels. Our findings revealed that CBP confers potential protective effect on MOF. Besides, these results provided further rational basics for the promotion of MOF therapies in some clinical conditions. However, results of this study were still limited due to the complicated relationship between the regulation of MOF and cytokine signaling, cellular stress response, as well as inflammatory cytokine production. Further investigations are needed to support the findings as well as to explore other underlying mechanisms.

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Disclosure Statement

We declare that we have no conflicts of interest.

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