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Original Article

Effects of methylene blue on the nitric oxide-soluble guanylate cyclase-cyclic guanylyl monophosphate pathway and cytokine levels in rats with sepsis

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Abstract: Background: Methylene blue (MB) inhibits the production of nitric oxide (NO) and cyclic guanylyl monophosphate (cGMP) and thus reverses septic hypotension, but the specific effects of MB on the NO-soluble guanylate cyclase (sGC)-cGMP pathway in different organs of septic rats are unclear. The present study aimed to elucidate the effects of MB on the NO-sGC-cGMP pathway and inflammatory reactions in rats with sepsis. Methods: A Wistar rat model of sepsis was established by cecal ligation and puncture (CLP). The rats were given an injection of 15 mg/kg of MB via the caudal vein at 6, 12, or 18 h after CLP. The rats were sacrificed at 6 h after the injection. Then, mRNA and protein expressions of sGC alpha 1 (sGCα1) in lung, liver, and kidney tissues were measured by real-time polymerase chain reaction and western blotting, respectively. Levels of cGMP in the lung, liver, and kidney tissues were assessed by enzyme-linked immunosorbent assays (ELISAs). Levels of serum lactic acid, procalcitonin (PCT), interleukin-6 (IL-6), IL-10, and tumor necrosis factor alpha (TNF-α) were also detected by ELISAs. A NO reduction method was used to detect the level of serum NO. Results: The levels of sGCα1 and cGMP in lung, liver, and kidney tissues were upregulated in the septic rats and were downregulated after MB administration ($P < 0.01$ or $P < 0.05$). Additionally, the levels of serum NO were increased in septic rats and were decreased after MB administration ($P < 0.01$ or $P < 0.05$). In a separate set of experiments, the levels of PCT, IL-6, IL-10, and TNF-α were increased ($P < 0.01$) in septic rats but were unchanged after MB administration. Conclusion: The NO-sGC-cGMP pathway is activated in different organs of septic rats. Moreover, MB inhibits the NO-sGC-cGMP pathway, but does not affect systemic inflammation.

Keywords: Sepsis, nitric oxide, soluble guanylate cyclase alpha 1, cyclic guanylyl monophosphate, methylene blue, cytokine

Introduction

Sepsis, a life-threatening organ dysfunction, results from a dysregulated host response to invading pathogens [1]. As a current major cause of death, sepsis represents a substantial health care burden [2]. Previous research has shown that the nitric oxide (NO)-soluble guanylate cyclase (sGC)-cyclic guanylyl monophosphate (cGMP) pathway plays significant roles in the process of refractory hypotension and organ failure in sepsis. Lipopolysaccharides (LPSs) induced by pathogenic microorganisms prompt the host to produce a large number of inducible NO synthases (iNOSs), which results in the mass production of NO. The major target of NO is sGC, which is a heterodimer of α (1 and 2) and heme-containing β (1 and 2) subunits [3]. Excessive NO leads to increased expression of sGC in vascular smooth muscle cells, and aggregation of intracellular cGMP, which results in decreased vascular tension, decreased sensitivity to catecholamines, decreased blood pressure, and even recalcitrant hypotension. Thus, the inhibition of the NO-sGC-cGMP pathway may constitute a way to control the occurrence and development of sepsis and septic shock.

Methylene blue (MB), which has antioxidant, anti-inflammatory, neuroprotective, and mitochondrial-protective effects, has been widely used as a dye and medication [4]. MB inhibits both iNOS and sGC. By competitively blocking

the target enzyme of NO, MB inhibits the responsiveness of vessels to cGMP-dependent vasodilators and restores vascular tone [5]. In 1992, Schneider [6] first applied MB in the treatment of septic patients and found that, in septic shock patients, an intravenous injection of MB (2 mg/kg) could improve low vascular resistance and increase mean arterial pressure (MAP). A study by Kirov et al. [7] also showed that, in septic patients, MB could significantly increase MAP, maintain systemic hemodynamic stability, improve tissue perfusion, increase cardiovascular responsiveness to vasopressin, and reduce the use of vasopressin.

MB has been shown to increases MAP and maintain hemodynamic stability in animal experiments and clinical studies. However, the specific effects of MB on the NO-sGC-cGMP pathway in multiple organs of septic rats remain unclear. In addition, a previous study has suggested that MB may not reduce inflammatory responses in septic patients [8]. Thus, in order to explore the value of MB in the treatment of sepsis, we observed the effects of MB on the NO-sGC-cGMP pathway in different organs and on indices of systemic inflammatory responses in septic rats.

Materials and methods

Animals

Adult female Wistar rats (180-220 g) were used for all experiments in the present study. The animals were housed in individual cages under controlled temperature (23°C ± 2°C) and a 12-h light-dark cycle, with free access to food and distilled water. All of the experimental procedures were approved by the ethics committee of the First Affiliated Hospital of Xinjiang Medical University. This study was conducted in accordance with the guidelines for the care and use of experimental animals, and the procedure or animal disposal was completed in accordance with the relevant animal ethical requirements.

Experimental design

A total of 136 rats were randomly separated into a sham group, a sepsis (cecal ligation and puncture, CLP) group, and a CLP+MB group, according to a random-number table. Each group was further subdivided into 6, 12, and 18-h subgroups according to the time after operation, with six rats included in each subgroup. Rats in the CLP+MB group were given a 15 mg/kg injection of MB (Jumpcan, Inc., Taizhou, Zhejiang, China) via the caudal vein at the aforementioned time points after CLP, while the remaining rats were given the same dose of normal saline at the corresponding time points.

Preparation of the sepsis model

The CLP approach, as modified by Rittirsch et al. [9], was used to establish the sepsis model used in the present study. In the sham group, only laparotomy and isolation of the caecum mesentery were performed without ligation or perforation. All of the rats were injected subcutaneously with normal saline (30 mL/kg) after the operation. The rats were sacrificed at 6 h after the intravenous injection of MB or normal saline. The blood, lung, liver, and kidney tissues were collected, immediately placed in liquid nitrogen, and were then stored at -80°C until future use.

RNA isolation and real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted from lung, liver, and kidney tissues with TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA), and the total RNA level was measured by a nucleic-acid quantometer. RNA was used to synthesize complementary DNA with a reverse transcription kit (Thermo Fisher Scientific, Waltham, MA, USA). Real-time polymerase chain reaction (RT-PCR) was performed using an Applied Biosystems 7500 Fast Real-Time PCR system with a QuantiNova SYBR Green PCR Kit (KIAQEN, Hamburg, Germany). The conditions of RT-PCR were as follows: 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 s, and annealing and extension at 60°C for 30 s. The primers for sGCα1 were designed and synthesized by Sango Biotech (Shanghai, China). The sequences were as follows: forward 5'-CGCTTTGACCAACAGTGTGGA-3' and reverse 5'-GGGCCATCAGTGCTATCTGGA-3', and the expected size of the PCR amplification product was 127 bp. Then, 3-phosphate glyceraldehyde dehydrogenase (GAPDH) was employed as an internal control, and the primers of GAPDH were produced by AST (Holly, MI, USA). The sequences were as follows: accession number NM_

017008.4, forward 5'-GACATGGCCGCCTGGAGAAAC-3' and reverse 5'-AGCCCAGGATGCCCTTTAGT-3', and the expected size of the PCR amplification product was 92 bp. The expression level of sGCα1 was calculated by the ΔΔCT calculation.

Western blotting

After being fully ground in liquid nitrogen, sample tissues (i.e., lung, liver, and kidney) were fully lysed via a protein lysate. The extracted proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membranes, and were blocked with 5% skimmed milk. Subsequently, the membranes were incubated overnight at 4°C with primary antibodies against sGCα1 (1:1000; Abcam, Cambridge, UK) and GAPDH (1:1000; CST, MA, USA). The membranes were incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at 37°C. The protein bands were visualized, and the gray values were scanned. The ratio of gray values of sGCα1 to GAPDH was used to indicate the relative expression level of sGCα1 protein in each tissue sample.

Enzyme-linked immunosorbent assay (ELISA)

Levels of cGMP in the lung, liver, and kidney tissues were assessed using the Cyclic GMP EIA Kit (581021, Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's instructions. Levels of serum lactic acid, procalcitonin (PCT), interleukin-6 (IL-6), IL-10, and tumor necrosis factor alpha (TNF-α) were detected using commercially available ELISA kits (Elabscience, Wuhan, Hubei, China) in accordance with the manufacturer's instructions.

Serum nitric-oxide detection

A NO reduction method was used to detect the level of serum NO. NO_3^- is specifically reduced to NO_2^- by nitrate reductase, which can be used to measure NO levels by determining the chromogenic depth. NO assay kits (A012-1; Nanjing Jiangcheng Bioengineering Institute, Nanjing, Jiangsu, China) were used for the detection.

Statistical analysis

The statistical package for the social sciences version 17.0 software program (IBM Corp., Armonk, NY, USA) was used for data analysis. The Kolmogorov-Smirnov method was used to test the normality of each sample distribution. Normally distribution data are expressed as the mean ± standard deviation (SD), and one-way analysis of variance was used for comparisons of multiple groups. The least significant difference test was used to evaluate pairwise comparisons if the variance was homogeneous, while the TamhaneT2 test was adopted when the variance was not homogeneous. Non-normal distributed data are expressed as the median (interquartile range), with the lower quartile (QL) and upper quartile (QU) being reported as follows: [M (QL, QU)]. The Kruskal-Wallis H test was used for comparisons of multiple groups of non-normally distributed data, whereas the Mann-Whitney U test was used for such comparisons between only two groups. $P < 0.05$ was considered to be statistically significant.

Results

Effects of MB on sGCα1 mRNA levels and protein levels in lung, liver, and kidney tissues in septic rats

The expression of sGCα1 mRNA in lung tissues was upregulated for all of the subgroups in the CLP group compared to that in the sham group and for all of the subgroups in the CLP+MB group as compared with that of the CLP group (**Figure 1A**). In a separate set of experiments, the expression of sGCα1 in lung tissues was downregulated for all subgroups in the CLP group versus that in the sham group and was downregulated for all subgroups in the CLP+MB group in comparison with that in the CLP group (**Figure 1B** and **1C**).

Additionally, the expression of sGCα1 mRNA in liver tissues was upregulated for subgroups (except for the 12-h group) in the CLP group as compared with that in the sham group and for all subgroups in the CLP+MB group versus that in the CLP group (**Figure 2A**). The expression of sGCα1 in liver tissues was downregulated for all subgroups (except for the 12-h group) in the CLP group in comparison with that in the sham group and was downregulated for all of the subgroups in the CLP+MB group as compared with that in the CLP group (**Figure 2B** and **2C**).

The expression of sGCα1 mRNA in kidney tissues was upregulated for all of the subgroups

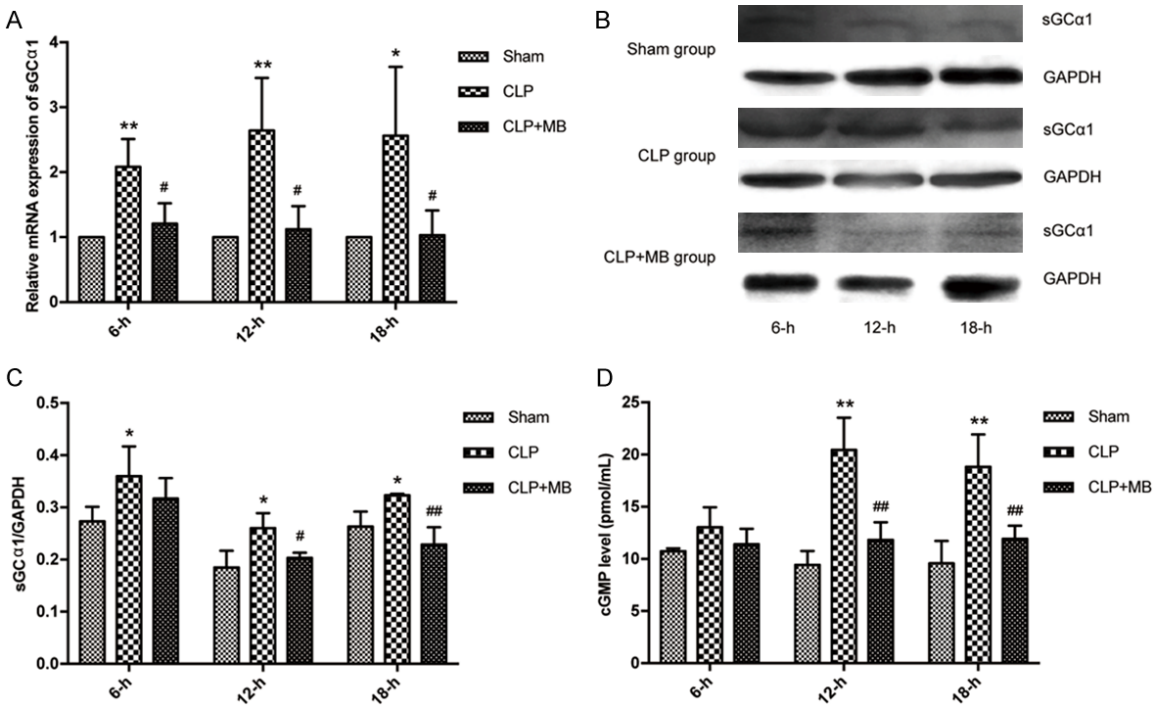

Figure 1. sGCα1 expressions and cGMP concentrations in the lung tissues of septic rats. A. The expressions of sGCα1 mRNA; sGCα1 mRNA in the lung was measured via RT-PCR. B and C. Protein expression levels of sGCα1; protein levels of sGCα1 in the lung were measured via western blotting. D. The cGMP concentration; cGMP in the lung was measured using ELISAs. Data are expressed as the mean ± SD; n = 6. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. Sham group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. CLP group.

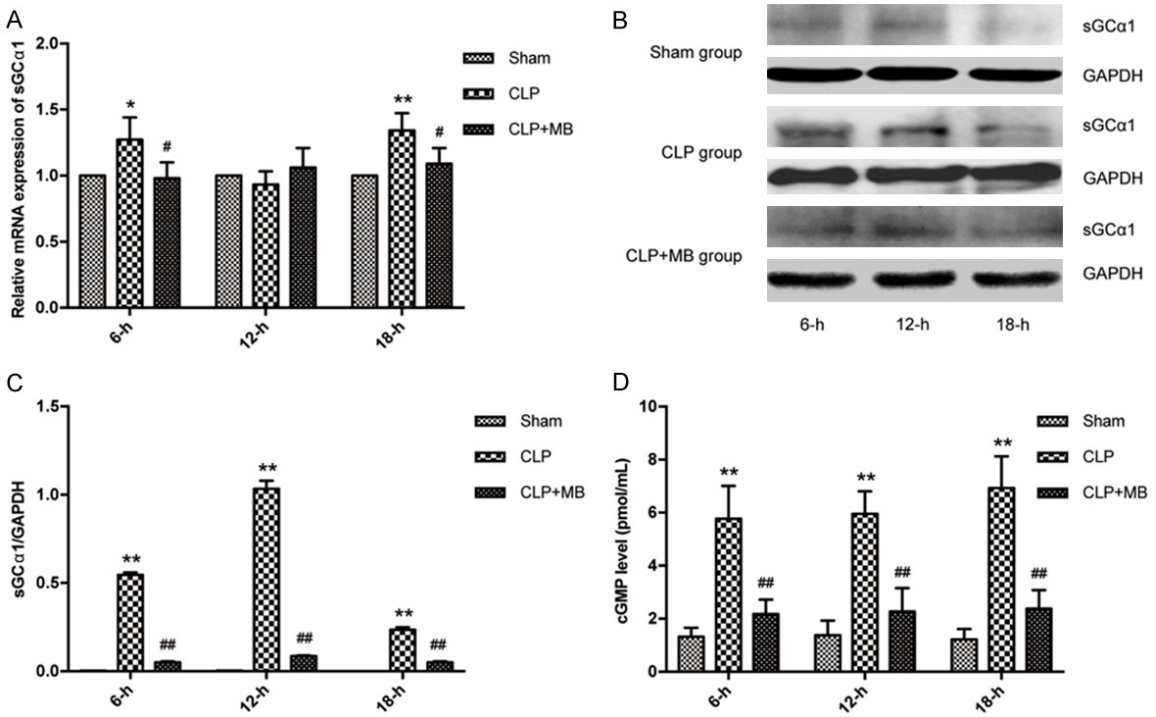

Figure 2. sGCα1 expressions and cGMP concentrations in the liver tissues of septic rats. A. The expressions of sGCα1 mRNA; sGCα1 mRNA in the lung was measured using RT-PCR. B and C. Protein expression levels of sGCα1; protein levels of sGCα1 in the lung were measured via western blotting. D. The cGMP concentration; cGMP in the lung was measured using ELISAs. Data are expressed as the mean ± SD; n = 6. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. Sham group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. CLP group.

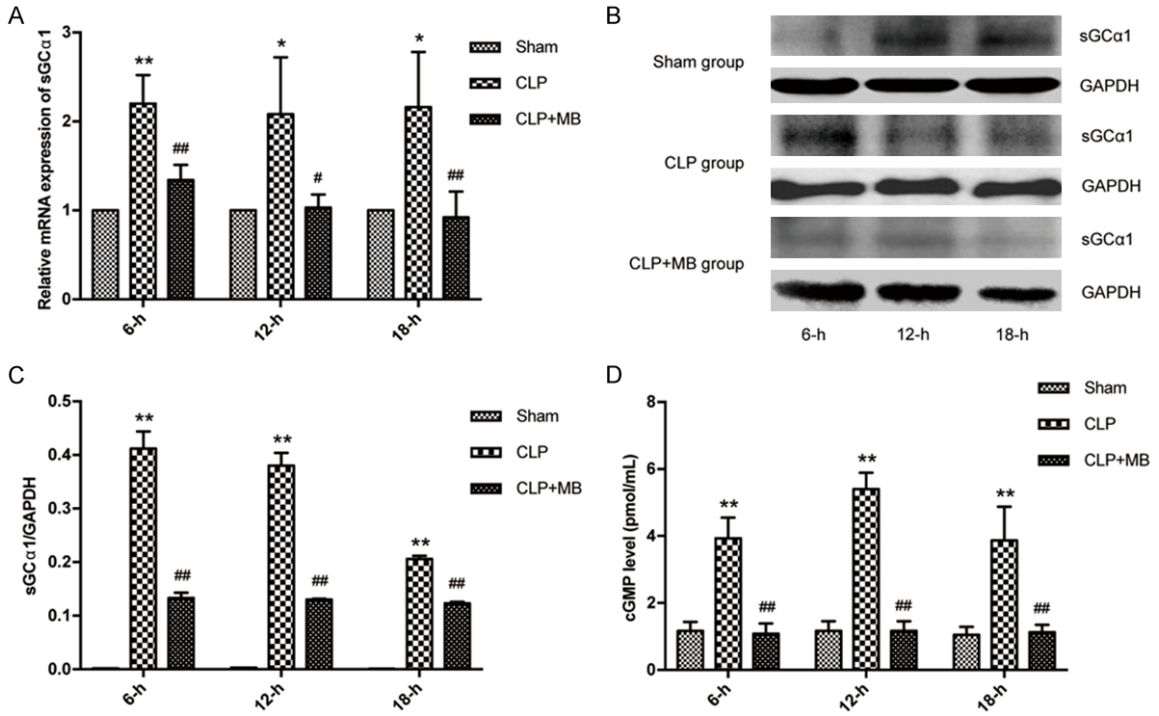

Figure 3. sGCα1 expressions and cGMP concentrations in the kidney tissues of septic rats. A. The expressions of sGCα1 mRNA; sGCα1 mRNA in the lung was measured using RT-PCR. B and C. Protein expression levels of sGCα1; protein levels of sGCα1 in the lung were measured via western blotting. D. The cGMP concentration; cGMP in the lung was measured using ELISAs. Data are expressed as the mean ± SD; n = 6. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. Sham group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. CLP group.

in the CLP group in comparison with that in the sham group and was similarly upregulated for all of the subgroups in the CLP+MB group versus that in the CLP group (**Figure 3A**). The expression of sGCα1 in kidney tissues was downregulated for all of the subgroups in the CLP group as compared with that in the sham group and was downregulated for all of the subgroups in the CLP+MB group as compared with that in the CLP group (**Figure 3B** and **3C**).

Effects of MB on cGMP concentrations in lung, liver, and kidney tissues in septic rats

The cGMP concentrations in lung tissues were upregulated for all subgroups (except for the 6-h group) in the CLP group as compared with those in the sham group, and were upregulated for all subgroups (except for the 6-h group) in the CLP+MB group as compared with those in the CLP group (**Figure 1D**).

Furthermore, the cGMP concentrations in liver tissues were upregulated for all of the subgroups in the CLP group versus those in the sham group and were upregulated for all of the subgroups in the CLP+MB group versus those in the CLP group (**Figure 2D**).

The cGMP concentrations in kidney tissues were upregulated for all of the subgroups in the CLP group as compared with those in the sham group and were upregulated for all of the subgroups in the CLP+MB group in comparison with those in the CLP group (**Figure 3D**).

Effects of MB on serum NO levels in sepsis rats

The levels of serum NO were increased for all of the subgroups in the CLP group as compared with those in the sham group and were decreased for all of the subgroups in the CLP+MB group as compared with those in the CLP group (**Figure 4A**).

Effects of MB on serum lactic acid, PCT, IL-6, IL-10 and TNF-α levels in septic rats

The levels of serum lactic acid were increased for all of the subgroups in the CLP group versus those in the sham group and were decreased

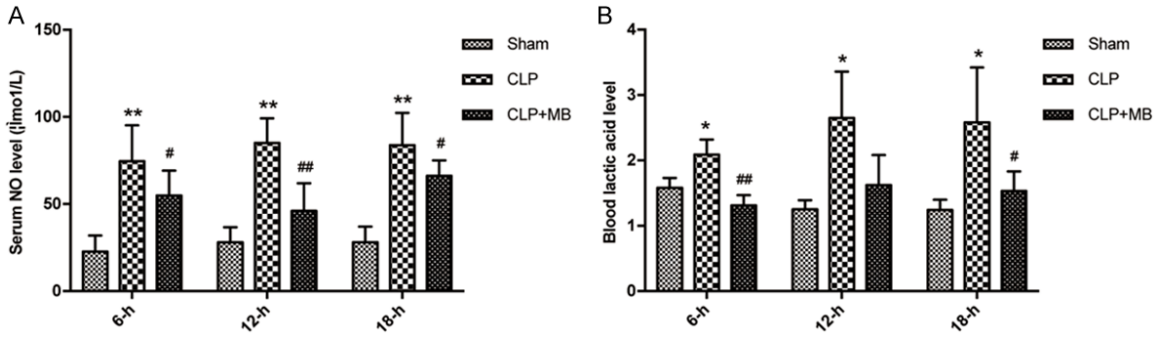

Figure 4. Serum NO levels and blood lactic acid levels in septic rats. A. Serum NO levels; the NO level in serum was measured by the NO reduction method. B. Blood lactic acid level; the lactic acid level in the blood was measured via ELISAs. Data are expressed as the mean ± SD; n = 6. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. Sham group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. CLP group.

Table 1. Comparison of PCT in serum [M (QL, QU)]

Group	PCT (pg/mL)		
	Sham (n = 6)	CLP (n = 6)	CLP+MB (n = 6)
6-h (n = 6)	39.25 (24.07)	166.13 (87.07)	137.98 (92.64)
12-h (n = 6)	21.15 (17.71)**	201.02 (133.91)**	228.96 (53.21)**
18-h (n = 6)	11.49 (8.70)	152.57 (141.57)	208.83 (132.17)

**$P < 0.01$ vs. Sham group.

Table 2. Comparison of IL-6 in serum [M (QL, QU)]

Group	IL-6 (pg/mL)		
	Sham (n = 6)	CLP (n = 6)	CLP+MB(n = 6)
6-h (n = 6)	13.55 (9.28)	80.94 (70.78)	96.66 (57.05)
12-h (n = 6)	22.56 (21.06)**	39.70 (16.90)**	58.35 (17.31)**
18-h (n = 6)	12.92 (9.38)	61.40 (51.00)	63.77 (52.75)

**$P < 0.01$ vs. Sham group.

Table 3. Comparison of IL-10 in serum [M (QL, QU)]

Group	IL-10 (pg/mL)		
	Sham (n = 6)	CLP (n = 6)	CLP+MB (n = 6)
6-hour (n = 6)	13.42 (14.93)	386.79 (273.08)	302.32 (78.44)
12-hour (n = 6)	16.37 (11.28)**	209.31 (64.89)**	272.3 (161.80)**
18-hour (n = 6)	10.57 (6.35)	192.53 (78.68)	183.19 (79.42)

**$P < 0.01$ vs. Sham group.

Table 4. Comparison of TNF-α in serum [M (QL, QU)]

Group	TNF-α (pg/mL)		
	Sham (n = 6)	CLP (n = 6)	CLP+MB (n = 6)
6-h (n = 6)	9.93 (5.65)	70.14 (25.77)	71.10 (16.32)
12-h (n = 6)	11.97 (8.92)**	60.81 (25.37)**	67.45 (9.76)**
18-h (n = 6)	10.70 (7.38)	58.06 (27.71)	54.72 (19.39)

**$P < 0.01$ vs. Sham group.

for all of the subgroups in the CLP+MB group versus those in the CLP group (**Figure 4B**).

Additionally, the levels of serum PCT, IL-6, IL-10, and TNF-α were increased for all of the subgroups in the CLP group as compared with those in the sham group, but remained unchanged for all of the subgroups in the CLP+MB group versus those in the CLP group (**Tables 1**-**4**).

Discussion

Systemically, large amounts of NO lead to hypotension, microcirculatory dysfunction, and refractoriness to vasopressor catecholamines [10]. Additionally, excessive NO levels and activation of the sGC-cGMP pathway are important in sepsis-induced organ damage. One study of a rat CLP model of sepsis showed that sGC α1 and β1 subunit mRNA and protein levels were increased in the lungs and that sodium nitroprusside-stimulated cGMP accumulation was higher in the lungs at 48 h after CLP [11]. Gill et al. [12] found that septic apoptosis of pulmonary microvascular endothelial cells is a result of leukocyte activation and iNOS-dependent signaling andwhich, in turn,

may contribute to pulmonary microvascular barrier dysfunction as well as albumin hyperpermeability in sepsis-induced lung injury. It was also revealed that iNOS upregulation contributes to liver microvascular dysfunction and liver damage induced by LPSs in a murine model of endotoxemia [13]. LPSs impair NO-dependent modulation of intrahepatic resistance, which increases vascular inflammation and hepatic oxidative stress [14]. A study of endotoxemia in mice showed that serum NO increased in a time-dependent manner, reaching the highest levels at 24 h, and that sGC and cGMP increased in renal cortical slices [15]. In a study of human endotoxemia and sepsis, the upregulation of renal iNOS and subsequent higher urinary excretion of NO metabolites were associated with renal proximal tubule damage [16]. A study by Oliveira-Pelegrin et al. [17] suggested a role of the NO-cGMP pathway in hormonal synthesis in the supraoptic and paraventricular nuclei during polymicrobial sepsis.

Targeting the NO-sGC-cGMP pathway is accepted as a way to treat sepsis. MB, a United States Food and Drug Administration approved pharmaceutical drug, has been safely used to treat methemoglobinemia and cyanide poisoning [18]. Unlike L-arginine analogs that non-selectively inhibit NOS, MB is a selective iNOS inhibitor. MB attenuates transcription-factor binding to iNOS promoters amid iNOS mRNA transcription [19]. MB also directly inhibits the activity of iNOS and reduces the synthesis of NO [20, 21]. As for sGC, MB binds to its iron heme moiety of sGC and prevents the accumulation of cGMP [5]. Some evidence has suggested that MB exerts its acute vasopressor effect mainly via sGC inhibition over NOS inhibition [8].

By inhibiting the excessive production of iNOS and cGC, MB blocks the acute hemodynamic effect and other effects of NO, which reverses sepsis-associated vasoplegia and possibly alleviates organ injury. The administration of MB clearly increased MAP and systemic vascular resistance in patients with septic shock, although the effects of MB on mortality are still unknown [22]. MB can also increase myocardial contractility depressed by cGMP. Evgenov et al. [23] found that MB decreased the enhanced lung fluid filtration by reducing pulmonary capillary pressure and permeability during the early phase of endotoxemia in sheep. A separate clinical study of sepsis showed that MB increased MAP but had no effect on oxygen delivery [7]. Another clinical study of sepsis suggests that a short-term infusion of MB increases MAP, decreases NO production, and attenuates the urinary excretion of renal tubular-injury markers [24]. However, the effects of MB on different organs in sepsis have remained unclear.

In the present study of a rat model after CLP, an intravenous injection of MB (15 mg/kg) inhibited the expression of sGCα1 mRNA and protein in lung, liver, and kidney tissues and also downregulated the expression of cGMP protein in these same tissues. The inhibition of the sGC-cGMP pathway may partially account for the MB effects on various tissues and organs. Our present study also found that the administration of MB induced an increase in serum NO levels but failed to reduce pro/anti-cytokine levels. Endotoxin and pro-inflammatory cytokines increase iNOS expression and NO production [25]. In turn, NO stimulates the synthesis of pro-inflammatory cytokines via the activation of the nuclear factor kappa B pathway. Therefore, MB may affect the release of cytokines. Currently, there are only two known randomized controlled trials that have evaluated the use of MB in patients with sepsis [7, 26]. Neither of these two studies showed a difference in cytokine levels between the MB group and the control group. Although MB could restore vascular tone, it did not change serum cytokine levels, which may account for why MB does not have an effect on mortality. In a consecutive study of 20 patients with refractory septic shock, Park et al. [8] reported that MB elevated the MAP without altering the productions of NO, IL-1, IL-10, or TNF-α. In our present study, the decrease of NO levels was not accompanied by a decrease of serum cytokine levels. It is likely that a relatively large dose of MB was administered in the present study. Moreover, the present study still did not identify a difference in PCT level with MB administration. A limitation of the present study is that we only explored the NO level and the expression of sGCα1/cGMP in three organs. In addition, we did not monitor any hemodynamic parameters.

In conclusion, we found that the NO-sGC-cGMP pathway was activated in a rat CLP model of sepsis and that MB decreased serum NO lev-

els, inhibited the sGC-cGMP pathway in different organs, and alleviated lactic acidosis, but did not lower serum levels of PCT or pro-inflammatory cytokines. Future investigations are required to further elucidate the mechanisms and clinical application of MB in the treatment of sepsis.

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Disclosure of conflict of interest

None.

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