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Vascular Reactivity and Membrane Potential in Survivors and Non-survivors with Peritonitis-induced Septic Shock

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Background: Vascular hyporeactivity and hyperpolarization are important causes of circulatory failure in septic shock. However, there is no data on the differences in vascular reactivity and resting membrane potential for survivors and non-survivors of sepsis. Thus, the aim of this study was to examine and compare the changes in vascular reactivity and resting membrane potential for survivors and non-survivors following cecal ligation and puncture (CLP)-induced sepsis. **Methods:** Wistar rats were subjected to CLP or a sham operation after the carotid artery and vein were cannulated. The changes in hemodynamics, biochemical variables, aortic isometric tension, smooth muscle membrane potential, and aortic superoxide levels were monitored during the experimental period. **Results:** The CLP surgery caused circulatory failure, multiple organ dysfunction syndrome (MODS), vascular hyporeactivity to norepinephrine (NE), and vascular hyperpolarization. Compared with survivors, non-survivors showed more severe organ dysfunction and a lower increase in aortic superoxide levels at 9 h. In addition, non-survivors displayed lower decreases in vascular reactivity to NE and resting membrane potential in the aortas compared to those of the survivors. **Conclusions:** These results demonstrate significant differences in vascular reactivity and resting membrane potential for survivors and non-survivors after CLP-induced sepsis. However, vascular hyporeactivity and hyperpolarization only partially contributed to the early death of septic animals, while early death in our study was most likely due to organ dysfunction (i.e. MODS) in this CLP-induced sepsis model.

Key words: sepsis, multiple organ dysfunction syndrome, vascular hyporeactivity, resting membrane potential, superoxide

INTRODUCTION

Vascular hyporeactivity to vasoconstrictors is an important cause of circulatory failure in septic shock. Systemic hypotension and decreased organ perfusion lead to multiple organ dysfunction syndrome (MODS) and death.[1,2] Overproduction of nitric oxide (NO) through the inducible NO synthase (iNOS) pathway has been proposed to contribute to vascular hyporeactivity to clinically used vasoconstrictor agents, such as norepinephrine (NE).[3-5] After the administration of NO synthesis inhibitors, the vascular hyporeactivity to catecholamines is significantly improved in septic shock.[6-8] Further, vascular catecholamine responsiveness and survival were improved in iNOS-deficient mice in a clinically relevant model of sepsis.[9] In addition, there is an imbalance between the formation of superoxide anion and the ability of superoxide dismutase (SOD) in septic shock.[10,11] Overproduction of superoxide anion reacts with catecholamines and inactivates them, resulting in vascular hyporeactivity to exogenous NE. Administration of an SOD mimetic, M40403, can restore this decreased vasopressor response to NE and reverse the hypotension in septic animals.[12]

The vasodilating action of NO is mediated through activation of soluble guanylyl cyclase and myosin light-chain phosphatase. In addition, NO causes vasodilatation by activating potassium channels in vascular smooth muscle cells.[13-15] Increased opening of the potassium channels in the vascular smooth muscle results in membrane hyperpolarization, which causes arterial dilation by inhibiting the voltage-dependent calcium channel.[16] Abnormal activation of potassium channels is involved in the hyporesponsiveness to vasoconstrictors and hyperpolarization in isolated thoracic aortas obtained from endo-

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toxemic rats. This vascular hyporeactivity to NE induced by endotoxin can be partially reversed by large conductance calcium-activated potassium channel inhibitors.[17,18]

Different animal models are used to study the pathophysiology and treatment of sepsis. A realistic sepsis model, cecal ligation and puncture (CLP), is similar to the progression of human sepsis to cause an early hyperdynamic phase and a late hypodynamic phase in animals.[19-22] Over the last two decades, our laboratory has used this model to study new therapeutic drugs in sepsis, and showed CLP rats revealed circulatory failure and MODS, as seen in clinical patients with septic shock.[23-27] However, we found some of the CLP-induced septic animals will survive less than 18 h despite being under the same experimental procedure. It seems certain factors may contribute to the early death of animals in this model. Although a correlation between serum interleukin-6 levels and survival time has been reported in experimental sepsis,[28] there is no information regarding the differences in vascular reactivity and resting membrane potential between survivors (at 18 h) and non-survivors (died about at 10 h) of sepsis. Thus, we examined and compared the changes in vascular reactivity and membrane potential for survivors and non-survivors following CLP-induced sepsis.

MATERIALS AND METHODS

Animal experiments

Ten-week-old male Wistar rats weighing 280-350 g were purchased from BioLASCO Taiwan Co (Taipei, Taiwan). Rats were guaranteed to be free of particular pathogens. The Institutional Committee on Care and Use of Animals (National Defense Medical Center, Taipei, R.O.C., Taiwan) approved this study, and the experiments were performed in adherence to the National Institutes of Health guidelines for the treatment of animals and ethical animal research. Rats were bred and maintained under a 12-h light/dark cycle at a controlled temperature (21±2 ^{o}C) with free access to food and tap water.

Surgical procedures and experimental protocol

Rats were anesthetized by injection with sodium pentobarbital (40-50 mg/kg intraperitoneally). The left carotid artery was cannulated and exteriorized to the back of the neck and connected to a pressure transducer (P23ID, Statham, Oxnard, CA, USA) for measuring mean arterial blood pressure (MAP) and heart rate (HR). The right jugular vein was cannulated and exteriorized to the back of the neck for the administration of drugs. After the rats recovered to a normal condition overnight, intraperitoneal sepsis was induced by CLP as described by Wichterman *et al*.[19] The rats were anesthetized with sodium pentobarbital (30-40 mg/kg intravenously), a small midabdominal incision was performed, and the cecum was exposed. The cecum was then isolated and ligated with a 3-0 silk ligature just distal to the ileocecal valve, punctured twice at opposite ends with an 18-gauge needle, and returned into the abdominal cavity. Sham-operated (SOP) rats underwent the same surgical procedure, except the cecum was neither ligated nor punctured.

Our previous studies showed the mortality rate of rats receiving CLP surgery (18-gauge needle) was approximately 62% at 18 h after CLP.[23-27] Due to this high mortality of CLP rats at 18 h after this surgery, we chose 18 h as the cut-off time. Animals were divided into three groups: (1) sham-operated (SOP); (2) survivors (at 18 h) of CLP; and (3) non-survivors (died at about 10 h) of CLP. During the experimental period, we examined changes in hemodynamics (i.e., MAP, HR, and pressor responses to 1 μg/kg NE), hepatic function index (i.e., alanine aminotransferase [ALT], aspartate aminotransferase [AST]), renal function index (i.e., creatinine [CRE], blood urea nitrogen [BUN]), and cell injury index (i.e., lactate dehydrogenase [LDH]). To normalize the baseline value of pressor responses to NE in all groups, we calculated the value of pressor responses to NE (by area under curve) in the resting state (i.e., time 0) of each group as 100%. Blood samples (0.8 mL at each time point) were obtained at the baseline (i.e., time 0) and specified times (i.e., at 9 h and 18 h) throughout all procedures. Each volume of blood removed was immediately replaced by the injection of an equal volume of sterile saline. At the end of the *in vivo* experiments, animals were sacrificed and thoracic aortas were obtained and cleared of adhering periadventitial fat for isometric tension and membrane potential recording experiments. In addition, the aortas of animals were immediately excised to analyze superoxide levels.

Quantification of organ function/injury

Blood samples were collected from a catheter placed in the carotid artery, and were then immediately centrifuged at 16,000 g for 2 min to obtain serum for measuring biochemical variables. Eighty microliters of serum was used to analyze liver or kidney functions at the baseline (i.e., time 0) and at 9 and 18 h. The following enzymes measured in the serum were regarded as biochemical indicators of MODS. For instance, serum ALT and AST levels were measured to evaluate liver dysfunction and serum CRE and BUN levels were measured to

evaluate renal dysfunction. In addition, LDH was measured to evaluate the extent of organ injury. All of these biochemical variables were analyzed by Fuji DRI-CHEM 3030 (Fuji Photo Film, Tokyo, Japan).

Determination of vascular reactivity

At the end of the *in vivo* experiments, the rats were euthanized, and thoracic aortas were quickly removed and placed in Krebs' solution, the composition of which was (in millimoles per liter) NaCl, 118; $NaHCO_3$, 25; glucose, 11; $MgSO_4$, 1.17; KH_2PO_4, 1.2; KCl, 4.7; $CaCl_2$, 2.5. The thoracic aortas were cleared of adhering periadventitial fat and were cut into segments 2.5 mm in length. The aortic rings were mounted in 20-mL organ baths filled with warmed (37 ^oC), oxygenated (95 % O_2/5 % CO_2) Krebs' solution. Isometric force was measured with Grass FT03 type transducers (Grass Instruments, Quincy, MA, U.S.A.) and recorded on a MacLab Recording and Analysis System (AD Instruments Pty Ltd., Castle Hill, Australia). The rings were allowed to equilibrate for 60 min under an optimal resting tension of 2 g and the experimental protocols begun once the aortas had reached a steady basal resting tension. NE (10^{-6} M) and acetylcholine (ACh, 10^{-6} M) were applied to establish to establish baseline responsiveness. Then, concentration-response curves to NE (10^{-9} - 10^{-5} M) were obtained to evaluate the vascular activity to NE.

Recording of membrane potential

The rats were euthanized, and the thoracic aortas were quickly removed and placed in Krebs' solution at the end of the *in vivo* experiments. The thoracic aortas were cleared of adhering periadventitial fat, with part of them being cut into lengths of 3 mm and opened longitudinally. The vessel was pinned down, intimal side upward, on the bottom of an organ chamber and infused at a constant flow rate of 3 mL/min with warmed (37 ^oC), oxygenated (95 % O_2/5 % CO_2) Krebs' solution. One end of the segment was fixed to the organ bath chamber, whereas the other end was connected to a Grass FT03 transducer (Grass Instrument Co., Quincy, MA, U.S.A.). After the preparations had equilibrated for at least 60 min, glass microelectrodes filled with 3 M KCl (tip resistance, 10 - 30 MΩ) were inserted into the aortic smooth muscle from the intimal side. Electrical signals were detected by an electrometer (Duo 773; World Precision Instruments Inc., Sarasota, FL, U.S.A.), monitored, and recorded continuously on a computer monitor oscilloscope (with Clampex 7 software).

Measurement of superoxide production in the aorta

At the end of the *in vivo* experiments, thoracic aortas were obtained from the SOP and CLP rats and cleared of adhering periadventitial fat. Part of them were cut into rings of 4-6 mm width and incubated with warmed (37 ^oC), oxygenated (95 % O_2/5 % CO_2) Krebs-HEPES buffer for 5-10 min. Then, they were transferred to 96-well microplates containing 100 μL of Krebs-HEPES buffer with 50 μL of lucigenin (1.25 mM) and were placed into a microplate luminometer (Hidex Microplate Luminometer, Finland). Luminescence counts were obtained in duplicate at 10-sec intervals. Then, the vessels were dried in a 95 ^oC oven for 24 h. Superoxide levels were expressed as count per second per milligram of organ dry weight.

Determination of plasma NO levels

Thirty μL of plasma obtained at 9 h was deproteinized by incubating with 95% ethanol (4 ^oC) for 30 min. The samples were subsequently centrifuged for 6 min at 16,000 g. The nitrate concentration depicted in the study is actually the total nitrite and nitrate concentration in plasma. With this method, nitrate is reduced to NO via nitrite. The amounts of nitrate in the plasma (6 μL) were measured by adding a reducing agent (0.8% VCl3 in 1 N HCl) to the purge vessel to convert nitrate to NO., which was stripped from the plasma using a helium purge gas. The NO was then drawn into a Sievers NO analyzer (Sievers 280 NOA, Sievers, Boulder, CO, USA). Nitrate concentrations were determined from a curve constructed from standard solutions of sodium nitrate (Sigma Chemical, St. Louis, MO, USA).

Statistical analysis

The data are presented as mean$\pm$SEM of n determinations, where n represents the number of animals studied. Statistical evaluation was performed by analysis of variance (ANOVA) followed by a multiple comparison test (Student-Newman-Keuls test). A P value of less than 0.05 was considered to be statistically significant.

RESULTS

Hemodynamic variables in survivors and non-survivors

Non-survivors and survivors showed significant (i) decreases in MAP, (ii) decreases in pressor response to NE, and (iii) increases in HR when compared with the SOP group at 9 h and 18 h (Fig. 1, A-C). Non-survivors further showed a significant decrease in MAP at 9 h when compared with survivors. However, in the SOP group,

none of the hemodynamics were significantly changed during the experimental period.

Organ function in survivors and non-survivors

At 18 h, the CLP surgery caused significant increases of serum ALT, AST, BUN, and LDH levels in survivors when compared with the SOP group (Fig. 2, A-E). In addition, non-survivors showed significant increases in ALT, AST, CRE, BUN, and LDH levels at 9 h when compared with the SOP or survivors group. However, none of these functional indexes were significantly altered during the experimental period in the SOP group.

Vascular reactivity to NE in survivors and non-survivors

NE (10^{-9}-10^{-5} M)-induced contraction occurred in a concentration-dependent manner in aortas from the SOP group (Fig. 3). The concentration-response to NE in aortas from survivors and non-survivors of CLP was reduced when compared with the SOP group. This reveals vascular hyporeactivity to NE exists in aortas obtained from CLP rats. However, compared with survivors, non-survivors further a lower decrease in NE-induced contraction (Fig. 3).

Aortic membrane potential in survivors and non-survivors

The resting membrane potential of the aorta in the SOP group was -43.1±0.7 mV (Fig. 4). The membrane potential recording showed thoracic aortas obtained from CLP rats were more hyperpolarized than the SOP group. In addition, the resting membrane potential in the aorta of non-survivors was more positive than that in the survivors (Fig. 4).

Aortic superoxide levels in survivors and non-survivors

The basal production of superoxide was detectable in aortas obtained from the SOP group (Fig. 5). Survivors showed a significant increase in aortic superoxide levels when compared with the SOP group. However, the superoxide levels in aortas obtained from non-survivors was much lower than that in the survivors and similar to the SOP group (Fig. 5).

Plasma NO levels in survivors and non-survivors

The basal NO level was detectable in plasma obtained from the SOP group (Fig. 6). Survivors showed a significant increase in plasma NO levels when compared with the SOP group. However, the plasma NO level in

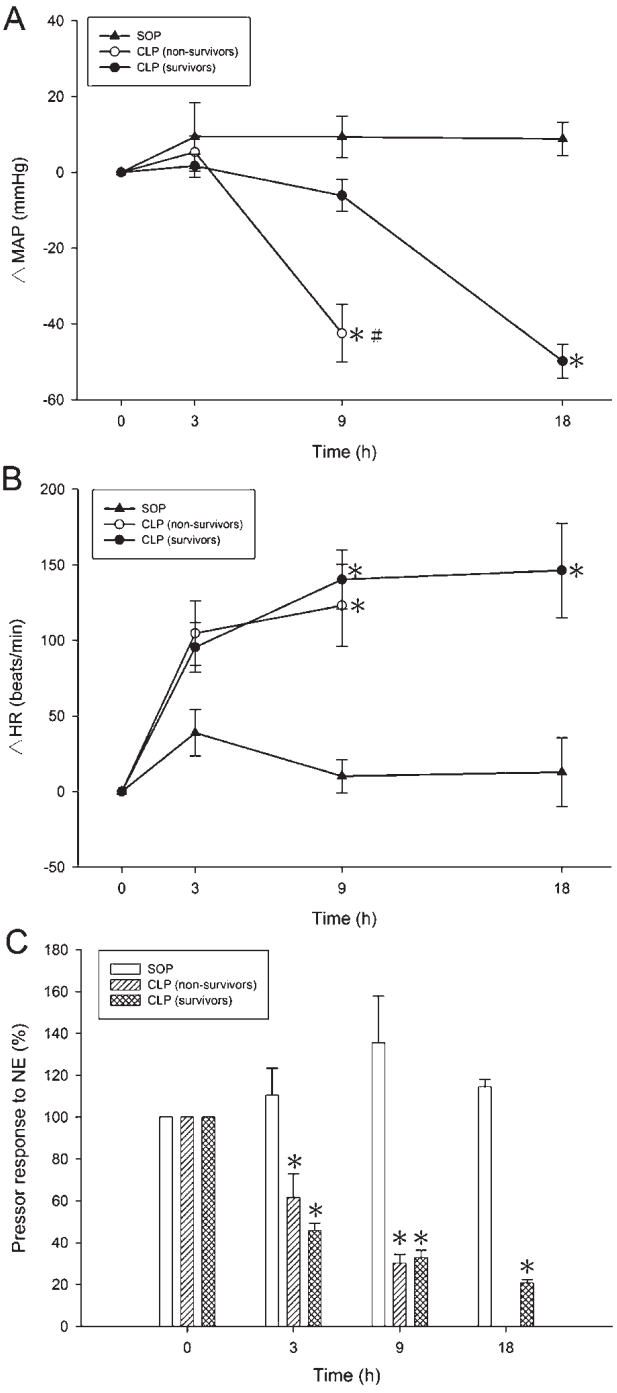

Fig. 1 Changes in (A) mean arterial blood pressure (MAP), (B) heart rate (HR), and (C) pressor response to NE during the experimental period in the SOP and CLP groups. Depicted are animals that received SOP (n=4) and CLP (n=13 in survivors, n=8 in non-survivors) surgery. The values of all groups are normalized with MAP and HR at 0 h as zero. Data are expressed as mean±SEM. $^{*}P < 0.05$, all versus SOP rats; $^{\#}P < 0.05$, non-survivors versus survivors.

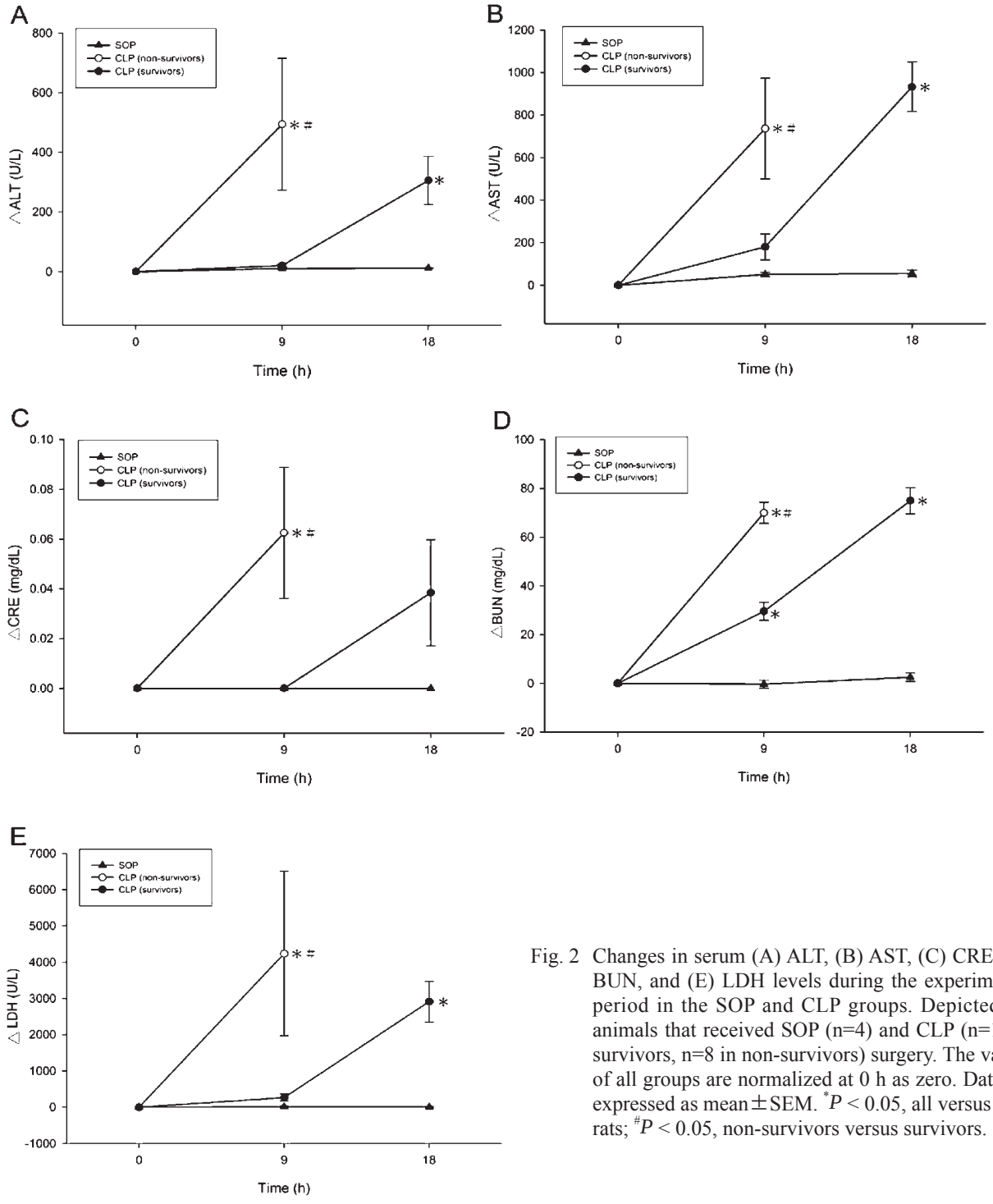

Fig. 2 Changes in serum (A) ALT, (B) AST, (C) CRE, (D) BUN, and (E) LDH levels during the experimental period in the SOP and CLP groups. Depicted are animals that received SOP (n=4) and CLP (n=13 in survivors, n=8 in non-survivors) surgery. The values of all groups are normalized at 0 h as zero. Data are expressed as mean±SEM. $^{*}P < 0.05$, all versus SOP rats; $^{\#}P < 0.05$, non-survivors versus survivors.

non-survivors was less than that in survivors (Fig. 6). Although non-survivors had higher NO levels than the SOP group, it was not significant (p = 0.127). This could be due to the small sample size.

DISCUSSION

Although previous studies have indicated vascular hyporeactivity to vasoconstrictors and vascular hyper-

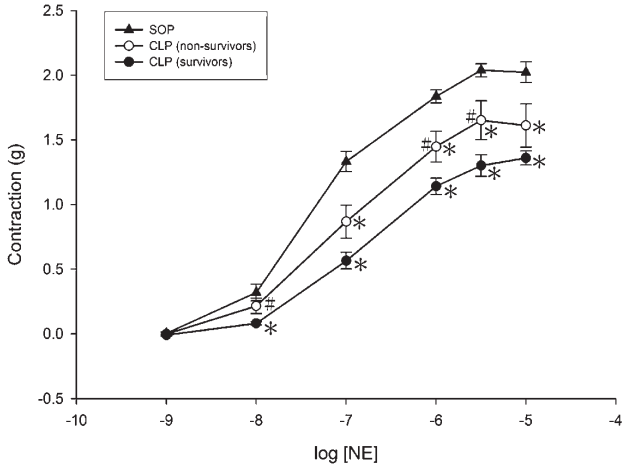

Fig. 3 Concentration-response curves of NE in aortic rings obtained from the SOP and CLP groups. Depicted are animals that received SOP (n=4) and CLP (n=13 in survivors, n=8 in non-survivors) surgery. Data are expressed as mean±SEM. $^{*}P < 0.05$, all versus SOP rats; $^{\#}P < 0.05$, non-survivors versus survivors.

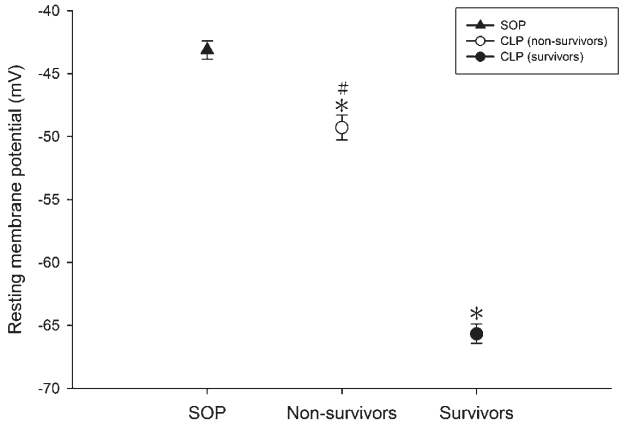

Fig. 4 The resting membrane potential in aortic strips obtained from the SOP and CLP groups. Depicted are animals that received SOP (n=4) and CLP (n=13 in survivors, n=8 in non-survivors) surgery. Data are expressed as mean±SEM. $^{*}P < 0.05$, all versus SOP rats; $^{\#}P < 0.05$, non-survivors versus survivors.

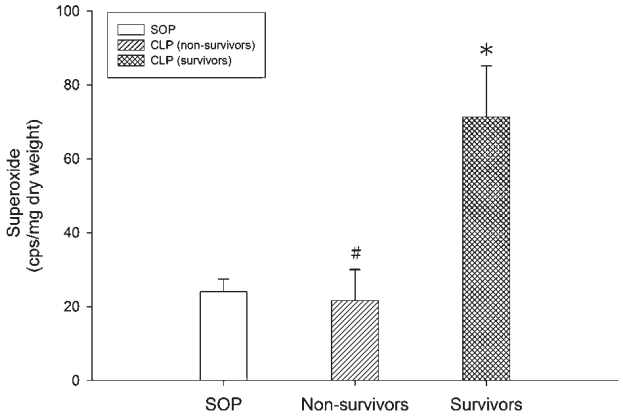

Fig. 5 Changes of aortic superoxide anion during the experimental period in the SOP and CLP groups. Depicted are animals that received SOP (n=4) and CLP (n=8 in survivors, n=4 in non-survivors) surgery. Data are expressed as mean± SEM. $^{*}P < 0.05$, all versus SOP rats; $^{\#}P < 0.05$, non-survivors versus survivors.

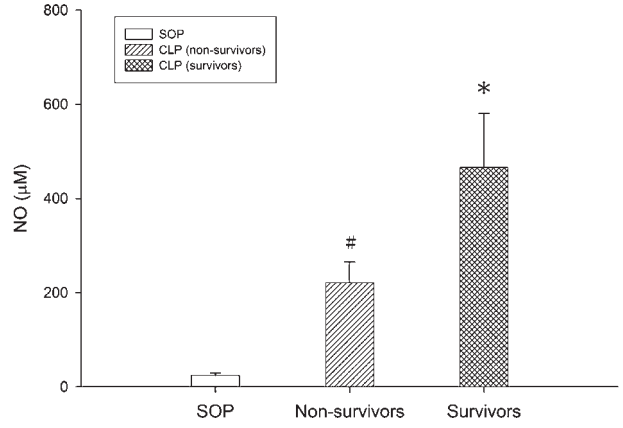

Fig. 6 Changes of plasma NO levels during the experimental period in the SOP and CLP groups. Depicted are animals that received SOP (n=4) and CLP (n=6 in survivors, n=6 in non-survivors) surgery. Data are expressed as mean±SEM. $^{*}P < 0.05$, all versus SOP rats; $^{\#}P < 0.05$, non-survivors versus survivors.

polarization are crucial factors of circulatory failure in septic shock,[2,29] no direct evidence has shown differences in vascular reactivity and membrane potential for survivors and non-survivors following CLP-induced sepsis. In this study, both survivors and non-survivors of CLP manifested circulatory failure, MODS, vascular hyporeactivity to NE, and vascular hyperpolarization. However, compared with survivors, non-survivors showed significant increases in (i) degree of MODS at 9 h *in vivo*, (ii) vascular reactivity to NE, and (iii) resting membrane potential in aortas *ex vivo*. These results demonstrate significant differences in vascular reactivity and membrane potential for survivors and non-survivors, and the early MODS could contribute to the early death of animals in this model.

Excessive inflammatory response and cytokine release occur in sepsis, leading to increased superoxide anion and NO production.[30,31] Overproduction of superoxide anion causes the inflammatory cascade at the vessel wall, resulting in overexpression of P-selectin and intracellular adhesion molecule-1.[32] Strong interaction between leukocytes and endothelial cells is implicated in vascular dysfunction and tissue injury.[33] In addition, superoxide anion reacts rapidly with NO to form the peroxynitrite.[34] Peroxynitrite formation in tissues has been demonstrated from different animals challenged with lipopolysaccharide, zymosan, and CLP.[35-37] The most important effect of peroxynitrite in sepsis is exerted on mitochondria to cause single-stranded DNA breaks. DNA injury activates poly (ADP-ribose) polymerase (PARP) to destroy NAD^+, causing energy depletion and cellular injury.[38,39] One crucial factor underlying the pathogenesis of MODS in sepsis may be due to the derangements in cellular energy metabolism by peroxynitrite.[40] PARP activation causes organ damage in CLP-induced septic shock. However, PARP-deficient mice or PARP inhibitor-treated porcine show significant improvement in survival of sepsis.[41,42] In this study, superoxide levels in the aortas of non-survivors were much lower than those in survivors. We suggest this reduced superoxide anion bioavailability may be due to an overproduction of peroxynitrite in non-survivors, leading to MODS and death occurring early. Despite peroxynitrite being suggested as a potential cause of early death in this study, the role of peroxynitrite needs to be further clarified.

Vascular hyporeactivity is regarded as a decreased vascular smooth muscle contraction to a vasopressor when compared to the normal response. It can be observed experimentally in the organ chamber or in clinical practice by establishing a dose–response curve to a vasoconstrictor. Patients with septic shock show a decreased response to phenylephrine stimulation, indicating vascular hyporeactivity exists in septic shock.[43,44] Several mechanisms could participate in the vascular hyporeactivity to vasopressor in sepsis. Excess production of NO by iNOS seems to be a major possibility in sepsis-induced vascular hyporeactivity.[29] In the present study, non-survivors showed a significant increase in NE-induced contraction compared with survivors. We suggest overproduction of peroxynitrite in non-survivors decreases NO bioavailability, resulting in this increased vasoconstriction. In addition, peroxynitrite can oxidize tetrahydrobiopterin to reduce endothelial NO production and inactivate prostacyclin synthase to decrease prostacyclin production.[45,46] These could explain why NE-induced contraction in non-survivors is higher than that in survivors.

NO diffuses into the vascular smooth muscle and induces vasorelaxation via cyclic guanosine monophosphate (cGMP), resulting in a decreased contractile response. In addition, NO activates potassium channels and causes vascular smooth muscle cells hyperpolarization to blunt the vasoconstrictor response of NE during sepsis.[2,47] In this study, the membrane potential of the aorta from CLP-induced septic animal was more hyperpolarized than that in the SOP group. These observations indicate hyperpolarization of the vascular smooth muscle is involved in this vascular hyporeactivity induced by CLP. However, the resting membrane potential in the aorta of non-survivors was more positive than that in the survivors. It is possible non-survivors produce excess peroxynitrite, and hence, this decreases the effects of NO on the potassium channels in vascular smooth muscle.

Despite the clinical relevance and widespread use of CLP in experimental sepsis research, one of the major concerns is its consistency of survival time. There was varying mortality in this model, despite using the same experimental procedure. Indeed, the failure rate was 20% (10/50) to induce peritonitis and septic shock after cecal ligation in this study. Thus, we excluded these data according to the septic shock definition (e.g. blood pressure lower than 70 mmHg and blood glucose lower than 40 mg/dL at the end point). Therefore, this model needs to find key biomarkers and establish a standardized manner to enhance its reproducibility, in particular, for new investigators and a series of CLP model sepsis studies.

In conclusion, this study demonstrated non-survivors (i) had a marked MODS early and (ii) increased vascular reactivity and resting membrane potential when compared with survivors. These findings indicate vascular hyporeactivity and hyperpolarization only partially contribute to early death, while the early occurrence of MODS (possibly caused by overproduction of peroxynitrite) most likely leads to the early death of animals in this model. Therefore, the improvement of peroxynitrite levels in the earlier phase may prevent early MODS and death in peritonitis-induced sepsis.

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DISCLOSURE

The authors declare they have no conflict of interest.

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