Shock: Injury, Inflammation, and Sepsis: Laboratory and Clinical Approaches

Low-dose sodium nitrite fluid resuscitation prevents lethality from crush syndrome by improving nitric oxide consumption and preventing myoglobin cytotoxicity in kidney in a rat model

--Manuscript Draft--

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Abstract:	Objective: Crush syndrome (CS) is a serious medical condition characterized by muscle cell damage resulting from pressure. CS has a high mortality, even when patients receive fluid therapy. We examined whether administration of $NaNO_2$-containing fluid can improve survival in a rat model of CS. Design: The CS model was generated by subjecting anesthetized rats to bilateral hind limb compression with a rubber tourniquet for 5 h. Rats were then randomly divided into six groups: (i) sham; (ii) CS with no treatment; (iii) CS with normal saline treatment; (iv) CS with normal saline + 25 mEq/L bicarbonate treatment; and (v and vi) CS with normal saline + 200 or 500 μmol/kg $NaNO_2$. Measurements and Main Results: Blood and tissue samples were collected for histological and biochemical analyses at predetermined time points before and after reperfusion. Ischemic compression of rat hind limbs reduced nitrite content in the crushed muscle, and subsequent reperfusion resulted in reactive oxygen species-

	induced circulatory dysfunction and systemic inflammation. Rats treated with 200 μmol/kg $NaNO_2$ showed increased nitric oxide (NO) levels, blood circulation, and neoangiogenesis, decreased generation of reactive oxygen species, and suppression of the inflammatory response, leading to complete recovery. Conclusions: Treatment with 200 μmol/kg $NaNO_2$ prevents muscle damage induced by ischemia reperfusion via the protective effects of NO and suppression of systemic inflammation, thereby increasing survival rates in CS.
Response to Reviewers:	Response to Reviewer #1 1.Introduction in its current form is very naïve. I think the authors should expand the introduction especially they should include some rationale why various parameters (MPO, lung and kidney etc.) were assessed in this study. → Thank you for your suggestion. We agree, and have revised the text accordingly. 2.The study is based on n=3 and it is not clear how the PI used ANOVA with tukeys to establish the significance among various experimental groups? → Thank you for your question. The determination of statistical significance using ANOVA and Tukey's test, as well as the P level that was used to define statistical significance, is already described in both the 'Statistical analysis' subsection of Materials and methods and in figure legends. With the exception of the determination of the most appropriate dose of sodium nitrite for use in resuscitation, we have not reported the P values of the differences between the sham groups and the S, SB, and S-200N groups, because we judged that these results detracted from the main narrative of the paper. Moreover, we found statistically significant improvements using even the most stringent parameters. 3.The authors should properly label each of the figure to make more reader friendly. For example, labels for each bar should be presented within the figure itself and not in the figure legged. Similarly, the western blot data is not labeled properly. Furthermore, Figure 5 is also not properly labelled to establish the symbols and their assigned groups. → Thank you for your advice. For clarity, we have revised the figures and figure legends in accordance with your suggestions. 4.NO_2^-, TBARS are shown in Figure 2 and this should be corrected on page 11 as in its current form the text says Figure 1. → Thank you for pointing out this error. We have corrected this in the revised manuscript. 5.How muscles were removed to measure MPO activity? Was the passenger blood removed from the muscle pieces? → Thank you for your questions. In our experiments, the crush injury was applied to the gastrocnemius muscle, as described in the subsection of 'Material and methods' titled "Determination of reactive oxygen species production and MPO activity". We have revised this section to include a description of the removal of passenger blood from the muscle pieces. 6.More details on Figure 2 in supplement section are needed. How did the authors establish neoangiogenesis? Moreover, the presence of neutrophils as depicted by black arrowheads is difficult to establish so it would be good if authors replace these with better images. → Thank you for your question and advice. vWF is produced within endothelial cells, and during stanching, activated vWF causes platelets to interact with the injured vascular endothelial tissue. While angiogenic mechanisms were not considered here, we used immunostaining of vWF to visualize the vascular endothelial tissue and thus evaluate neoangiogenesis following the crush injury. We observed less prominent vascular endothelial damage in the S-200N group than in the CS-only group (Supplemental Digital Content Figure 2, D). For clarity, we have revised the figure. The tissue microphotographs were evaluated by a pathologist at the New Histo Science Laboratory (test number: 14N0523), although a scoring system was not used. Unfortunately, these are the only microphotographs that were provided and so clearer images are not available. 7.How the current study in review is different from the one published earlier by these authors in J Trauma Acute Care Surg 72(6):1548-1554, 2012?

→ Thank you for the question. In the previous article, we reported results following the bolus administration (single injection) of sodium nitrite to rats with crush syndrome. In contrast, in the present article, we describe the continuous administration of fluid containing sodium nitrite to rats with crush syndrome.
Response to Reviewer #2
1.The graphs in all figures are missing labels to indicate the different experimental groups.
→ Thank you for your comment. We have revised the figures and figure legends to improve labeling and clarify this issue.

2.Whenever possible, please provide a complete description of what was actually measured on the y-axes labels. The acronyms that are currently shown may not be known by all readers. Alternatively, these acronyms and abbreviations should be defined in each figure legend.
→ Thank you for the advice. We have revised the figure legends to fully explain all acronyms and abbreviations used.

3.The authors should also re-define the different experimental groups in the legends.
→ Thank you for the advice. The figure legends have been revised as suggested.

4.In some cases (Fig. 5), it is difficult or even impossible to distinguish the different lines and symbols from each another.
→Thank you for your comment. In this experiment, the sham and S-200N groups both had a survival rate of 100%, and so the overlapping symbols cannot be avoided. However, we hope that the enlarged open circle makes it clear that there are two symbols overlaid at this point. Additionally, the results section entitled “NaNO2 treatment increases survival rates after CS” clarifies that the survival rate in both cases was 100%.

5.The microphotographs of tissue sections are too small and their resolution is too low to determine the extents of organ damage. In addition to showing these images, a scoring system to estimate lung and kidney damage would be helpful.
→ Thank you for your comments and advice. We have enlarged the images in the figure as suggested. While a scoring system was not used to estimate the extent of tissue damage, the tissue microphotographs were evaluated by a pathologist at the New Histo Science Laboratory (test number: 14N0523), and we have included information in the manuscript about the relative levels of tissue damage between groups.

Response to Reviewer #3
1.According to authors’ previous studies (Ref. 5), the survival rates of the CS-model groups remained at 100% until 3 hours, however, dropped to 25% at 24 hours after reperfusion. In the present study, the survival rate of rats in the CS-only group decreased over time to 85%, 50%, 35%, and 35% at 3, 6, 24, and 48 h after reperfusion. The survival rate is different. The explanation is suggested to be added. In addition, there are 15 animals in CS group. How to obtain the data (i.e. 85%, 50%, 35%, and 35% at 3, 6, 24, and 48 h after reperfusion)?
→ Thank you for your questions. The article referred to above described a single time point (24 hours). As explained in Materials and methods, the present article describes the continuous administration of fluid containing sodium nitrite, with samples taken at various time points. This is the same method that is described in Refs. 6 and 7, and, indeed, the survival rate of the CS group in our study is consistent with those reported in these studies. We therefore think that our observations are consistent with the reported literature and that there is no conflict.
The survival rate for the CS-only group, as well as the number of animals in this group, were stated incorrectly in the manuscript and have been revised. Survival rates were calculated using the Kaplan-Meier method.

2.Hemeoxygenase (HO)-1 is reported to be protective following injury. In the present study, the levels of HO-1 is higher in CS group compared with S-200N group. The additional infoamtion is suggested to be added.
→ Thank you for raising an important discussion point for this study. Generally, HO-1 is a protective agent in ischemia-reperfusion injury. However, in our particular case, expression of both HO-1 and TBARS was not induced because fluid resuscitation

	prevented oxidative damage, as can be seen by the similar TBARS level in the S-200N and sham groups. This suggests that the protection offered by S-200N treatment is independent of HO-1 expression. We have revised the discussion accordingly. 3.Muscle TBARS concentration is higher in CS group compared with S-200N group. How about the levels of superoxide and anti-oxidative stress activity (GSH,…)? The additional experiments are suggested to be added. → Given that TBARS levels are significantly lower in the S-200N group than in the CS-only group, we agree that the investigation of markers of anti-oxidative activity such as GSH would be interesting, particularly as this expression is unaffected by HO-1. However, this study focuses on the anti-oxidative activity of nitrite, and the implications of this for mitochondrial function.

Nov. 7, 2016

Dr. Irshad Chaudry
Editor
Shock: Injury, Inflammation, and Sepsis: Laboratory and Clinical

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"Low-dose sodium nitrite fluid resuscitation prevents lethality from crush syndrome by improving nitric oxide consumption and preventing myoglobin cytotoxicity in kidney in a rat model"

Dear Dr. Irshad Chaudry,

We are submitting a revised version of our manuscript. We are grateful for the opportunity to resubmit our manuscript, and appreciate the interest shown by you and the reviewers, as well as the helpful comments you have provided.

In the revised version of the manuscript, we have incorporated the following corrections (marked in yellow highlight in the manuscript).

(1) We modified the Introduction to add a more detailed discussion of why parameters such as MPO and lung and kidney function were assessed in this study, as suggested by Reviewer #1.
(2) In the Materials and methods section, we have clarified how passenger blood was removed from the muscle samples, as suggested by Reviewer #1.
(3) We modified the figures to include larger microphotographs of tissue sections in response to comments by Reviewers #2 and 3 that these were too small.
(4) The figures and figure legends have been modified in accordance with the suggestions made by all reviewers.
(5) We have addressed other possible antioxidative effects and HO-1 expression in the Discussion, as suggested by Reviewers #2 and 3.

A "Response to Reviewers", addressing the issues raised by all reviewers in detail, is included below.

We thank you and the reviewers again for considering our work, and hope that this revision adequately addresses all of your concerns and questions. We look forward to hearing from you.

Yours sincerely,

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Low-dose sodium nitrite fluid resuscitation prevents lethality from crush syndrome by improving nitric oxide consumption and preventing myoglobin cytotoxicity in kidney in a rat model

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Conflicts of Interest and Source of Funding

The authors declare no conflict of interest.

Running head

The effects of low-dose sodium nitrite fluid resuscitation on crush syndrome

Abstract

Objective: Crush syndrome (CS) is a serious medical condition characterized by muscle cell damage resulting from pressure. CS has a high mortality, even when patients receive fluid therapy. We examined whether administration of $NaNO_2$-containing fluid can improve survival in a rat model of CS.

Design: The CS model was generated by subjecting anesthetized rats to bilateral hind limb compression with a rubber tourniquet for 5 h. Rats were then randomly divided into six groups: (i) sham; (ii) CS with no treatment; (iii) CS with normal saline treatment; (iv) CS with normal saline + 25 mEq/L bicarbonate treatment; and (v and vi) CS with normal saline + 200 or 500 μmol/kg $NaNO_2$.

Measurements and Main Results: Blood and tissue samples were collected for histological and biochemical analyses at predetermined time points before and after reperfusion. Ischemic compression of rat hind limbs reduced nitrite content in the crushed muscle, and subsequent reperfusion resulted in reactive oxygen species-induced circulatory dysfunction and systemic inflammation. Rats treated with 200 μmol/kg $NaNO_2$ showed increased nitric oxide (NO) levels, blood circulation, and neoangiogenesis, decreased generation of reactive oxygen species, and suppression of the inflammatory response, leading to complete recovery.

Conclusions: Treatment with 200 μmol/kg $NaNO_2$ prevents muscle damage induced by ischemia reperfusion via the protective effects of NO and suppression of systemic inflammation, thereby increasing survival rates in CS.

Key words

crush syndrome, nitric oxide, sodium nitrite, fluid resuscitation, anti-inflammatory

Introduction

Crush syndrome (CS) is a serious medical condition resulting from physical trauma obtained during events such as earthquakes, landslides, and traffic accidents, that is characterized by circulatory shock, kidney failure, and systemic inflammation (1, 2), and is associated with high mortality rates, especially in the acute phase. CS develops after decompression of limb muscles (reperfusion) following prolonged compression (ischemia) of a large proportion of skeletal muscle, resulting in rhabdomyolysis (muscle cell breakdown). However, CS has not only local effects but can also cause systemic failure resulting from acute respiratory distress and systemic inflammatory response syndromes, multiple organ failure, and confounding late-phase symptoms (3).

Fluid therapy is the first-line treatment for CS; shock and acute kidney failure can be prevented by early provision of fluid. Electrolyte abnormalities are common in patients with crush-related acute kidney failure, with fatal hyperkalemia being the most severe case for which treatment with normal saline containing sodium bicarbonate is recommended (4).

In addition to studies and clinical reports describing renal dysfunction caused by crushed muscle, CS can also cause a systemic inflammatory response that can lead to damage in more distant organs such as the lung (3,5). We previously developed a rat model of severe CS (6) and reported that effective rapid phleboclysis consisting of a single injection of sodium nitrite ($NaNO_2$) improved survival by enhancing the recovery of muscle tissue nitrite (NO_2^-) levels and increasing myeloperoxidase (MPO) activity in both the muscle and the lung (7). $NaNO_2$

is thought to enhance kidney function and reduce systemic inflammation by increasing nitric oxide (NO) recovery. However, doses of 200 and 500 μmol/kg can also induce vasodilation and methemoglobinemia. We hypothesized that side effects could be avoided by continuous infusion of $NaNO_2$-containing fluid. Our results demonstrate that intravenous administration of NO_2^- is an effective first-line fluid therapy for treating CS.

Materials and methods

Animal model of CS

Male Wistar rats weighing 250–300 g were obtained from Japan SLC (Shizuoka, Japan) and housed in a room maintained at a temperature of 23°C ± 3°C and a relative humidity of 55% ± 15% on a 12:12-h light/dark cycle, with free access to food and water. Animal experiments were carried out according to the guidelines for animal use, and were approved by the Life Science Research Center of Josai University (approval nos. H24065 and H25012). Anesthesia was induced by intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). Body temperature was maintained throughout the experiment using a heating pad. The CS model was established as previously reported (6). Briefly, a rubber tourniquet was applied to the bilateral hindlimb of each rat and wrapped five times around a 2-kg metal cylinder; the end of the band was glued. After a given period of time, compression was released by cutting the band and removing the tourniquet.

Experimental design

Animals were randomly divided into six groups: (i) sham; (ii) CS with no treatment (CS only group); (iii) CS with normal saline treatment (S group); (iv) CS with normal saline + 25 mEq/l bicarbonate treatment (SB group); and (v and vi) CS with normal saline + different doses of $NaNO_2$ salt (200 or 500 μmol/kg $NaNO_2$; Wako Pure Chemical Industries, Osaka, Japan) (S-200N and S-500N groups, respectively). Groups iii–vi were subjected to

compression with the rubber tourniquet, followed by 3 h of reperfusion by massive fluid resuscitation at a rate of 30 mL/kg/h.

Analysis of mean arterial pressure, blood gas levels, biochemical parameters, coagulation, and nitrogen oxide and interleukin levels

Mean arterial pressure (MAP) was determined using a Softron device (Softron Co., Tokyo, Japan). Blood samples from each group were obtained 1, 3, 6, and 24 h after reperfusion. The pH, partial pressure of oxygen (O_2) and carbon dioxide (CO_2), bicarbonate (HCO_3^-) concentration, and base excess of arterial blood drawn from the femoral artery were analyzed using an i-STAT300F blood gas analyzer (FUSO Pharmaceutical Industries, Osaka, Japan). Venous blood from the postcaval vein was collected and centrifuged to measure plasma levels of potassium (K^+), blood urea nitrogen (BUN), creatinine (Cre), and creatine phosphokinase (CPK) (measurements were carried out by SRL Inc., Tokyo, Japan). Methemoglobin (Met-Hb) was measured as previously described (7). Platelets were measured using a Celltac hematology analyzer (Nihon Kohden Co., Tokyo, Japan), and the levels of von Willebrand factor (vWF) were measured using a rat vWF enzyme-linked immunosorbent assay (ELISA) kit (USCN, Houston, TX, USA). Nitrite (NO_2^-) concentrations in muscle and plasma were measured with CII and FX NO_2^-/nitrate (NO_3^-) assay kits (Dojindo Laboratories, Tokyo, Japan) according to the manufacturer's instructions. Plasma levels of interleukin (IL)-6 and -10 were measured using ELISA kits (Pierce Biotechnology, Rockford, IL, USA) according

to the manufacturer's instructions.

Assessment of kidney function

The bladder was cannulated in parallel to the jugular vein with PE-50 tubing. Urine samples were obtained from 1 h prior to decompression until immediately after decompression (0 h). Urine samples were collected every hour for 24 h and centrifuged at 1500 × *g* for 5 min at room temperature. Kidney function was determined based on glomerular filtration rate (GFR), urine volume, urine osmotic pressure (Osmomat 030-D; Gonotec GmbH, Berlin, Germany), N-acetyl-β-D-glucosaminidase (NAG) level (Shionogi & Co., Osaka, Japan), and urine pH (Pretest 5bII; Wako Pure Chemical Industries).

Determination of reactive oxygen species production and MPO activity

Reactive oxygen species (ROS) production in the injured gastrocnemius muscle was determined by measuring the concentration of thiobarbituric acid-reactive substances (TBARS). MPO activity in the muscle tissue was measured as previously described (6). For histological evaluations, tissue samples were fixed in 10% formalin and embedded in paraffin, and sections were cut and stained with hematoxylin and eosin (H&E), elastic Van Gieson (EVG), and anti-vWF antibody (Agilent Technologies, CA, USA). Microphotographs of the tissue sections were then evaluated by a pathologist (New Histo Science Laboratory, Tokyo, Japan). Blood was removed from the sampled tissues by the continuous infusion of phosphate

buffer solution through the heart.

Western blotting

Western blotting was carried out as previously described (8). Briefly, rat muscle tissue was homogenized and centrifuged, and proteins in the lysate were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis using antibodies against inducible NO synthase (iNOS; Cell Signaling Technology, Tokyo, Japan), heme oxygenase (HO)-1 (Thermo Fisher Scientific K.K., Kanagawa, Japan), and α-tubulin (Cell Signaling Technology). Protein bands were visualized with an enhanced chemiluminescence detection system (SuperSignal West Dura Extended Duration Substrate; Pierce Biotechnology) with horseradish peroxidase-conjugated secondary antibodies (Pierce Biotechnology). Band intensity was quantified using ChemiDoc XRS+ Molecular Imager with Image Lab software (Bio-Rad Laboratories, Hercules, CA, USA), with α-tubulin used as a loading control.

Statistical analysis

Results are the average of three independent experiments and are expressed as mean ± SEM. Differences between groups were assessed by analysis of variance (ANOVA), with post-hoc analysis using Tukey's Honest Significant Difference test (also known as Tukey's test). Survival curves were generated using the Kaplan-Meier method, and survival was compared with the log-rank test. Differences were considered significant for P values < 0.05.

Results

$NaNO_2$ treatment improves Met-Hb and blood gas levels and MAP

NO_2^- is reduced to NO in vivo; NO accumulation can lead to methemoglobinemia and reduced blood pressure. We measured the effect of $NaNO_2$ concentration (200 and 500 μmol/kg) on Met-Hb and blood gas levels and MAP in CS rats until reperfusion at 24 h.

During fluid resuscitation, MAP was similar between the S-500N and CS only groups; however, there was no change in MAP in the S-200N group throughout the experiment (Supplemental Digital Content Table 1). Blood gas parameters relative to the CS only group were improved in the S-200N and S-500N groups, but were largely unchanged in the S group (Supplemental Digital Content Tables 2 and 3). Oxygen saturation was within the normal range in all experimental groups (data not shown). Thus, treatment with 500 μmol/kg $NaNO_2$ increased Met-Hb, decreased MAP, and negatively affected blood gas parameters. We therefore excluded the S-500N group in subsequent experiments.

$NaNO_2$ treatment improves kidney function in CS

Plasma Cre and BUN levels were higher in the CS only as compared to the sham group, but were lower in the S-200N than in the CS only group after 3 and 24 h of reperfusion (Figure 1A, B). GFR as well as urine volume and osmolality were lower in the CS only than in the sham group, but were higher in the SB and S-200N groups than in the CS only and S groups (Figure 1C–E). NAG level in the distal convoluted tubule was higher in the CS only group

than in the sham group, and was reduced by S-200N treatment relative to the CS only group (Figure 1F).

Acute kidney injury in CS is characterized by acute tubular necrosis, formation of a myoglobin cast, and dilation of distal convoluted tubules followed by myoglobinuric nephropathy. The CS only and S groups showed moderate pathological dilation of distal convoluted tubules; this was improved in the SB and S-200N groups after 24 h of reperfusion (Figure 1G). Myoglobinuric kidney failure in CS develops as a result of myoglobinuria as well as low urine pH. Here we found that urine pH was lower in the CS only than in the sham group after 24 h of reperfusion (6.0 ± 0.0 vs. 5.0 ± 0.0; $P < 0.05$), whereas the S and CS only groups showed similar values (5.0 ± 0.0 vs. 5.0 ± 0.0). However, pH was increased in the SB and S-200N groups relative to the CS only group (6.0 ± 0.0 and 6.5 ± 0.2 vs. 5.0 ± 0.0, respectively; $P < 0.05$).

Muscle tissue injury is reversed by $NaNO_2$ treatment

NO_2^- concentrations in injured muscle were lower in the CS only, S, and SB groups after 3 h of reperfusion, but higher after 24 h of reperfusion, than in the sham group (Figure 2A). Conversely, NO_3^- concentrations in injured muscle were higher in the CS only and S-200N groups after 3 h of reperfusion, but lower after 24 h of reperfusion, than in the sham group (Figure 2B). Moreover, TBARS, MPO activity, and iNOS and HO-1 expression were increased in the CS only and S groups relative to the sham group (Figure 2C–F). The CS only

group had higher plasma CPK, K^+, NO_2^-, and NO_3^- levels than the sham group, indicating hyperkalemia leading to muscle cell destruction (Supplemental Digital Content Figure 1A, B). The CS only group showed moderate edema and neutrophil infiltration into injured muscle as compared to the sham group (data not shown).

NO_2^- concentrations in injured muscles were similar in the S-200N and sham groups (Figure 2A). However, plasma NO_2^- concentrations were significantly higher in the S-200N group than in the CS only, S, SB, and sham groups at 3 h, but by 24 h the S-200N group concentration had reduced to the level of the sham group. Conversly, plasma NO_3^- concentrations in the S-200N group were substantially higher than in the sham group at both 3 and 24 h, with no reduction over time (Supplemental Digital Content Figure 1C, D). TBARS, MPO activity, and iNOS and HO-1 expression were lower in the sham and S-200N groups than in the CS only group, but comparable levels were seen in the CS only group and the S and SB groups (Figure 2). Fluid resuscitation (i.e., S, SB, and S-200N groups) worsened edema relative to the sham and CS only groups after 3 h of reperfusion, resulting in moderate edema; however, the degree of edema was similar in the S-200N and sham groups at 24 h (Supplemental Digital Content Figure 2A, B). Moreover, neutrophil infiltration was reduced in the S-200N as compared to the CS only, S, and SB groups at 24 h (Supplemental Digital Content Figure 2, B). We observed that vascular endothelial cell staining in the S-200N group was more prominent than in the CS only group (Supplemental Digital Content Figure 2D).

Lung tissue injury in CS is mitigated by $NaNO_2$ treatment

There were no pathological changes in any of the experimental groups after reperfusion for 3 h; however, the CS only group showed moderate alveolar edema and neutrophil infiltration at 24 h (Figure 3A), which was associated with an increase in lung tissue MPO activity (Figure 3B). S-200N treatment reduced the area of damage and degree of injury to the alveolar lumen structure as well as alveolar hemorrhage compared to the CS only group after 24 h of reperfusion (Figure 3A), with a corresponding reduction in lung tissue MPO activity (Figure 3B). Additionally, plasma concentrations of the proinflammatory cytokines IL-6 and -10 were higher in the CS only as compared to the sham group at 3 and 24 h of reperfusion (Figure 3C-D).

$NaNO_2$ treatment mitigates endothelial damage and coagulation

Levels of plasma vWF, platelets, and fibrinogen were measured in order to assess endothelial damage and coagulation resulting from crush injury (Figure 4). Plasma vWF level was higher in the CS only as compared to the sham group, whereas levels were lower in the S-200N than in the CS only, S, and SB groups (Figure 4A). Plasma platelet and fibrinogen levels were lower after 24 h in the CS only group relative to the sham group; however, this trend was reversed by fluid resuscitation (Figure 4B, C).

$NaNO_2$ treatment increases survival rates after CS

As expected, 100% of the mice in the sham group survived for at least 48 h after reperfusion. The survival rate of rats in the CS only group decreased over time to 85%, 55%, 35%, and 35% at 3, 6, 24, and 48 h after reperfusion, respectively. A similar trend was observed in the S group. The SB group showed a higher survival rate than the CS and S groups up to 48 h after reperfusion. Unexpectedly, $NaNO_2$ treatment resulted in a 100% survival rate over the 48 h experimental period in the S-200N group (Figure 5).

Discussion

Up to 20% of deaths after major earthquakes occur shortly after extrication (9) and are usually due to crush injury; this can be aggravated by extra-renal complications (infections or pulmonary, cardiovascular, hematological, gastrointestinal, neurological, or psychiatric problems), which can increase morbidity and mortality (10–11). On-site CS therapy can improve the probability of survival; it is recommended that normal saline (1000 mL/h for adults) be administered to prevent acute kidney malfunction. In this study, we administered fluid at a rate of 30 mL/kg/h to examine the effect of $NaNO_2$ on survival rate, followed by rates of 10, 20, and 30 mL/kg/h.

The optimal dosage of NO_2^- required for a protective effect was 48–480 μmol (in tissue) or 10–200 nM (in blood) (12). Previous studies reported dosages of 10 or 50 μM and 0.2 μmol/min/kg (13, 14). We found that 500 μmol $NaNO_2$ could negatively affect CS symptoms. In contrast, a dosage of 200 μmol $NaNO_2$ (i.e. S-200N group) was within the NO_2^- concentration range in injured muscle tissue (47±21 to 56±21 nmol/g tissue, Figure 2A) and plasma (5.1±5.4 to 29.2±30.9 μM, Supplemental Digital Content Figure 1C) at 3 and 24 h, and did not have adverse effects.

Renal failure is a serious complication of CS that can result from circulatory shock, renal afferent arteriolar vasoconstriction (urinary concentration), increased urinary myoglobin levels, or metabolic acidosis (urinary acidity) (15–17), all of which can cause precipitation in distal convoluted tubules and tubular cast formation with subsequent tubular obstruction.

Myoglobin accumulation has also been shown to cause oxidant injury (18, 19). In these cases, renal replacement therapy can improve patient survival.

Normal and bicarbonate-containing saline are recommended as fluids for preventing hyperkalemia and circulatory shock associated with CS. Moreover, maintaining urinary pH above 6.5 can prevent intratubular deposition of myoglobin (20). NO has been shown to increase urinary flow (21). In the present study, the S-200N group showed an improvement in GFR and urinary pH and volume relative to the CS only group, indicating that treatment with 200 μmol/kg $NaNO_2$ protected against renal failure in CS. Interestingly, NAG level in renal tubules, a marker of cytotoxicity, was normalized in the S-200N as compared to the CS only group. NO is a free radical scavenger that binds strongly to iron in hemoglobin and myoglobin. Moreover, Kanner et al. reported than NO prevented the initiation of lipid peroxidation by ROS and myoglobin (22) The NO_2^- concentration administered in this study was higher than endogenous levels; it is possible that NO_2^--derived NO suppressed free radicals bound to myoglobin in urine.

The principal findings of this study are that resuscitation from CS using $NaNO_2$-containing fluid can improve systemic and microvascular hemodynamics. The importance of exogenous NO_2^- administration under anemic, hypoxic, and hypovolemic conditions is evidenced by the resultant decrease in vascular resistance and increase in perivascular perfusion. Restoration of blood pressure with incomplete recovery of perfusion after resuscitation prevents microvascular collapse. Low-dose NO_2^- supplementation after fluid resuscitation improved

microvascular perfusion via peripheral vasodilation without decreasing blood pressure. NO production was demonstrated by the decrease in NO_2^- concentration in the early phase of reperfusion in injured muscle. Under ischemic (low-oxygen) conditions, mitochondrial dysfunction results in ROS generation. NO protects mitochondria by acting as an oxygen radical scavenger (23). Furthermore, NO promotes antioxidation through the induction of superoxide dismutase (24). HO-1 induction is known to be protective following injury, however in this study, while HO-1 expression was higher in the CS only group than in the sham control group; it was significantly reduced by S-200N treatment. This suggests that nitrite-containing fluid resuscitation prevented oxidative damage and thus the induction of HO-1, and therefore offers protection independently from HO-1 expression. The overall protection provided by NO_2^- (i.e., hypoxia-induced mitochondrial ROS production and blood coagulation) depends on soluble guanylyl cyclase signaling (25, 26). NO is an important regulatory factor that inhibits leukocyte adhesion (27), expression of intercellular adhesion molecule and p-selectin (28), and plasma vWF levels (29). We propose that NO may prevent excessive damage resulting from inflammation and promote the recovery of muscle cells following crush injury.

Interestingly, fluid containing $NaNO_2$ induced neoangiogenesis and does not increase red blood cell (RBC) counts after crush injury. The decrease in blood circulation stimulated RBC production, as indicated by hematocrit level in the CS only group, which increased for 24 h of reperfusion. However, administration of $NaNO_2$-containing fluid produced similar results to

those observed in the sham group. $NaNO_2$ is a highly stable compound that releases NO only under conditions of ischemia, hypoxia, or low pH; as such, cardiovascular therapy with NO_2^- could be targeted to ischemic or hypoxic tissues, thereby reducing the risk of systemic hypotension (30). Exogenous NO_2^- can increase perfusion when administered after fluid resuscitation following hemorrhagic shock, underscoring its potential as a therapeutic agent (31, 32). During the stanching process, the injured vascular endothelial cells produce vWF, causing them to interact with platelets. Using immunostaining to detect and visualize vWF, and thus the vascular endothelial cell network, the degree of neoangiogenesis can be evaluated. We observed that the vascular endothelial damage seen in the S-200N group was less pronounced than in the CS only group (Supplemental Digital Content Figure 2D). We suggest that increasing endogenous NO enhances not only oxygen delivery but also the induction of neoangiogenesis, while decreasing shear stress via a modest vasodilatory effect (33–35) (Supplemental Digital Content Table 5 and Figure 2).

In conclusion, administration of $NaNO_2$ can lead to a dramatic improvement in survival following CS owing to the anti-oxidative, anti-coagulant, and anti-inflammatory effects of NO_2^- acting as a NO donor in hypoxic/acidified organs.

Author contributions

IM led the project and designed and performed most of the experiments; RS assisted with the survival and biochemical marker analyses. YM, MH, and TF carried out pharmacokinetic analyses of blood and tissue samples as well as statistical analyses. YS performed NO_2^- and NO_3^- analyses. JK, YI, and IK conceived the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

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Figure legends

Figure 1. Effect of fluid resuscitation on kidney function in CS rats.

(A) BUN concentration, (B) Cre concentration, (C) GFR, (D) urine volume, (E) urine osmotic pressure, (F) NAG concentration, and (G) hematoxylin and eosin-stained kidney sections following reperfusion for 3 and 24 h. White bar, sham; black bar, CS only; gray bar, S; diagonal bar, SB; shaded bar, S-200N. Values represent mean ± SEM (n = 3). $^{\#}P < 0.05$ vs. sham group, $^{*}P < 0.05$ vs. CS only group; $^{\dagger}P < 0.05$ vs. S group, $^{\ddagger}P < 0.05$ vs. SB group (Tukey's test). Micrographs are representative of three independent experiments (200× magnification; scale bars = 100 μm). Black arrowhead: dilated kidney tubule.

Figure 2. Effect of fluid resuscitation on NO_2^-, NO_3^-, and TBARS levels, MPO activity, and iNOS and HO-1 expression in muscle tissue of CS rats.

(A) muscle NO_2^- level, (B) muscle NO_3^- level, (C) muscle TBARS, (D) muscle MPO activity, (E) iNOS/α-tubulin ratio, and (F) HO-1/α-tubulin ratio following 3 and 24 h of reperfusion. White bar, sham; black bar, CS only; gray bar, S; diagonal bar, SB; shaded bar, S-200N. Values represent mean ± SEM (n = 3). $^{\#}P < 0.05$ vs. sham group, $^{*}P < 0.05$ vs. CS only group; $^{\dagger}P < 0.05$ vs. S group, $^{\ddagger}P < 0.05$ vs. SB group (Tukey's test).

iNOS and HO-1 expression is shown relative to that of the sham group.

Figure 3. Effect of fluid resuscitation on lung injury and distant organs in CS rats.

(A) Hematoxylin and eosin-stained lung tissue sections. (B) lung MPO activity, (C) plasma IL-6 concentration, and (D) plasma IL-10 concentration following 3 and 24 h of reperfusion. White bar, sham; black bar, CS only; gray bar, S; diagonal bar, SB; shaded bar, S-200N. Values represent mean ± SEM (n = 3). $^{\#}P < 0.05$ vs. sham group, $^{*}P < 0.05$ vs. CS only group; $^{\dagger}P < 0.05$ vs. S group, $^{\ddagger}P < 0.05$ vs. SB group (Tukey's test). Micrographs are representative of three independent experiments (400× magnification; scale bars = 200 μm). Black arrowhead: neutrophil.

Figure 4. Effect of fluid resuscitation on blood coagulation, vWF level, platelet count, and fibrinogen expression in CS rats.

(A) Plasma vWF, (B) platelet, and (C) fibrinogen levels following 3 and 24 h of reperfusion. White bar, sham; black bar, CS only; gray bar, S; diagonal bar, SB; shaded bar, S-200N. Values represent mean ± SEM (n = 3). $^{\#}P < 0.05$ vs. sham group, $^{*}P < 0.05$ vs. CS only group; $^{\dagger}P < 0.05$ vs. S group, $^{\ddagger}P < 0.05$ vs. SB group (Tukey's test).

Fibrinogen levels are shown relative to those of the sham group.

Figure 5. Cumulative survival in CS rats in the 48 h after reperfusion.

Survival rates from 0 to 48 h after reperfusion. Open circle, sham (n = 10); closed circle, CS only (n = 20); open square, S (n = 10); open diamond, SB (n = 10); closed square, S-200N (n

= 10). $^{\#}P < 0.05$ vs. sham group; $^{*}P < 0.05$ vs. CS only group; $^{\dagger}P < 0.05$ vs. S group. Survival curves were generated by the Kaplan-Meier method and compared using the log-rank test.

Supplemental Digital Content Figure 1. Effect of fluid resuscitation on plasma NO_2^-, NO_3^-, CPK, and K^+ levels in CS rats.

(A) plasma CPK, (B) plasma K^+, (C) plasma NO_2^-, and (D) plasma NO_3^- levels from after 3 and 24 h of reperfusion. White bar, sham; black bar, CS only; gray bar, S; diagonal bar, SB; shaded bar, S-200N. Values represent mean ± SEM (n = 3). $^{\#}P < 0.05$ vs. sham group; $^{*}P < 0.05$ vs. CS only group; $^{\dagger}P < 0.05$ vs. S group, $^{\ddagger}P < 0.05$ vs. SB group (Tukey's test).

Supplemental Digital Content Figure 2. Effect of fluid resuscitation on muscle tissue architecture in crush-injured muscle, as determined by H&E, EVG, and vWF staining.

(A) H&E-stained section of muscle reperfused for 3 h. (B) H&E-, (C) EVG-, and (D) vWF-stained muscle tissue sections after 24 h of reperfusion. Micrographs are representative of three independent experiments (400× magnification; scale bars = 200 μm). Black arrowhead: neutrophil; white arrowhead: hemorrhage; gray arrowhead: neoangiogenesis.

Figure 1

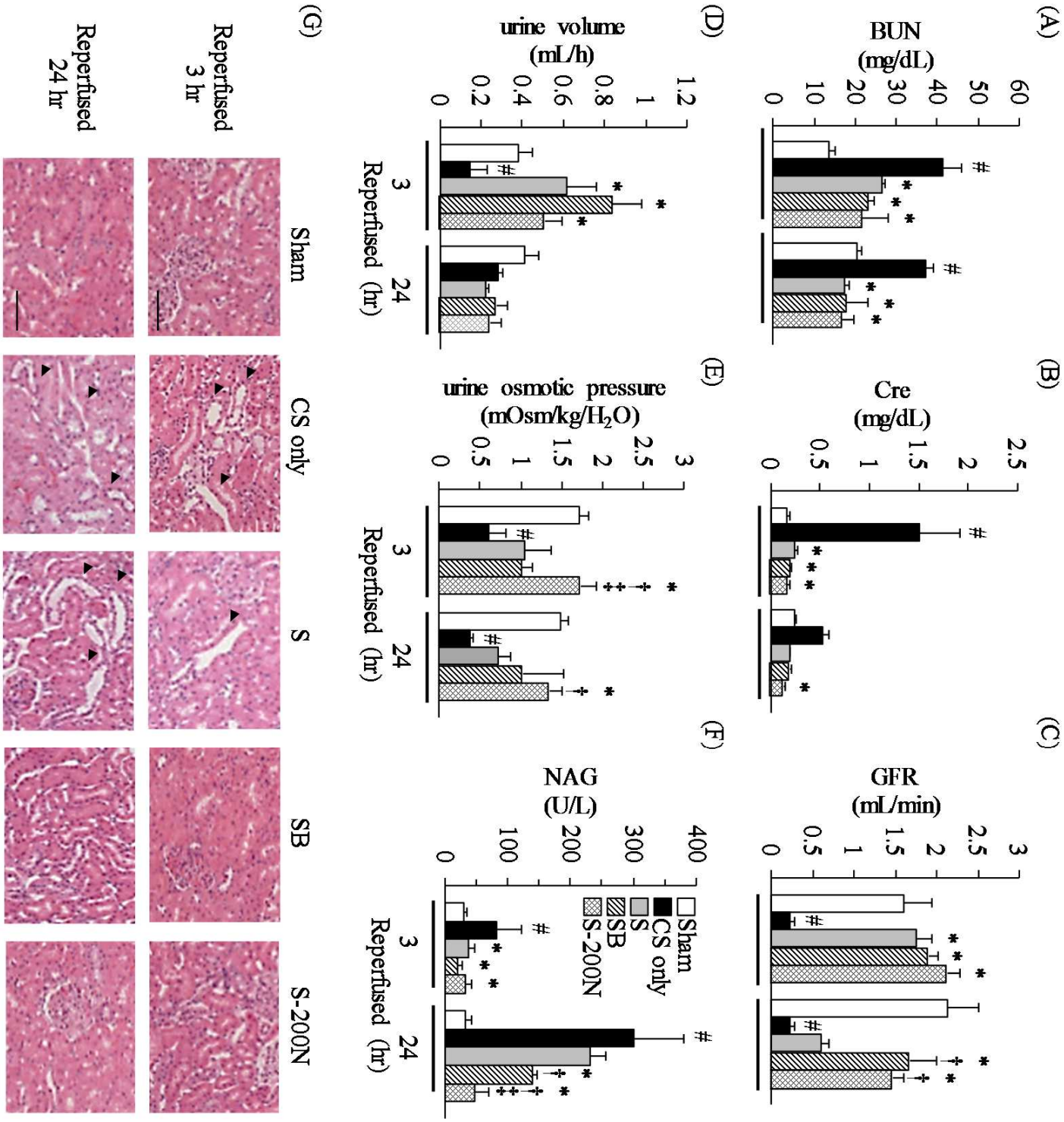
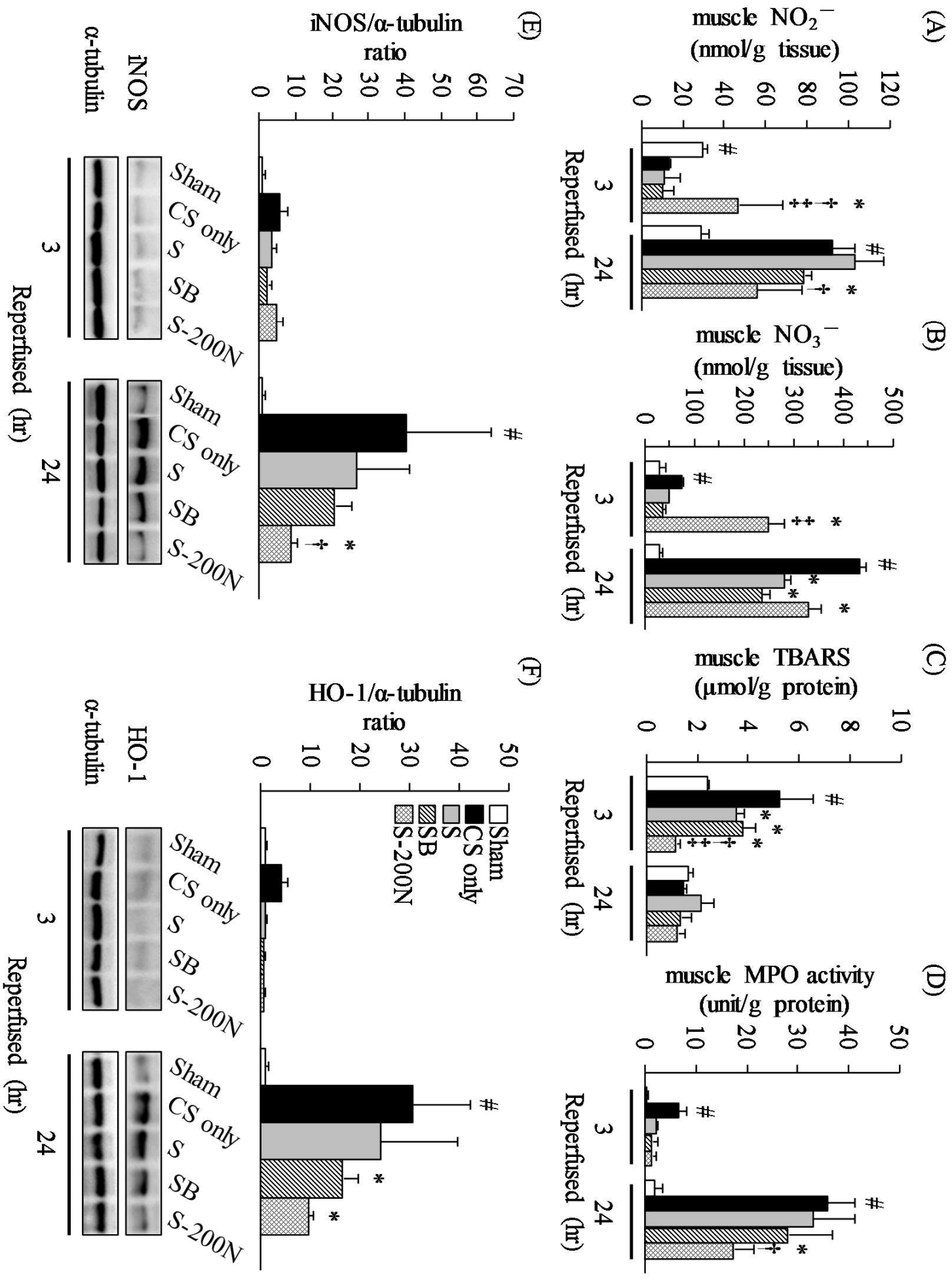

(A)
BUN (mg/dL)
(B)
Cre (mg/dL)
(C)
GFR (mL/min)
(D)
urine volume (mL/h)
(E)
urine osmotic pressure (mOsm/kg/H2O)
(F)
NAG (U/L)
Sham
CS only
S
SB
S-200N
3
24
Reperfused (hr)
(G)
Reperfused 3 hr
Reperfused 24 hr
Sham
CS only
S
SB
S-200N

Figure 2


(A)
muscle NO_2^- (nmol/g tissue)
(B)
muscle NO_3^- (nmol/g tissue)
(C)
muscle TBARS (μmol/g protein)
(D)
muscle MPO activity (unit/g protein)
Reperfused (hr)
3
24
(E)
iNOS/α-tubulin ratio
iNOS
α-tubulin
(F)
HO-1/α-tubulin ratio
HO-1
Sham
CS only
S
SB
S-200N

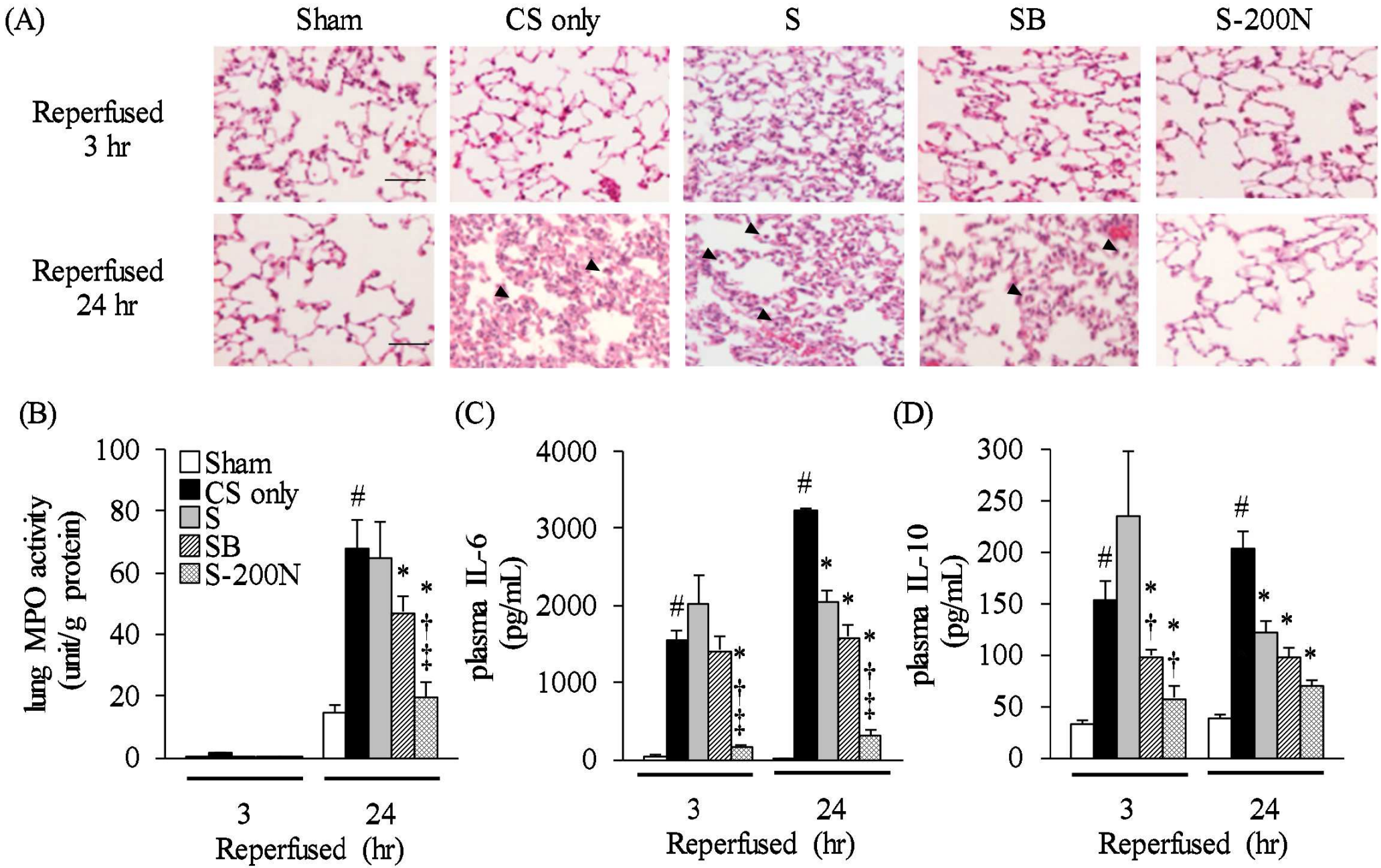

(A)
Sham
CS only
S
SB
S-200N
Reperfused 3 hr
Reperfused 24 hr
(B)
lung MPO activity (unit/g protein)
Sham
CS only
S
SB
S-200N
Reperfused (hr)
(C)
plasma IL-6 (pg/mL)
Reperfused (hr)
(D)
plasma IL-10 (pg/mL)
Reperfused (hr)

Figure 3

Figure 4

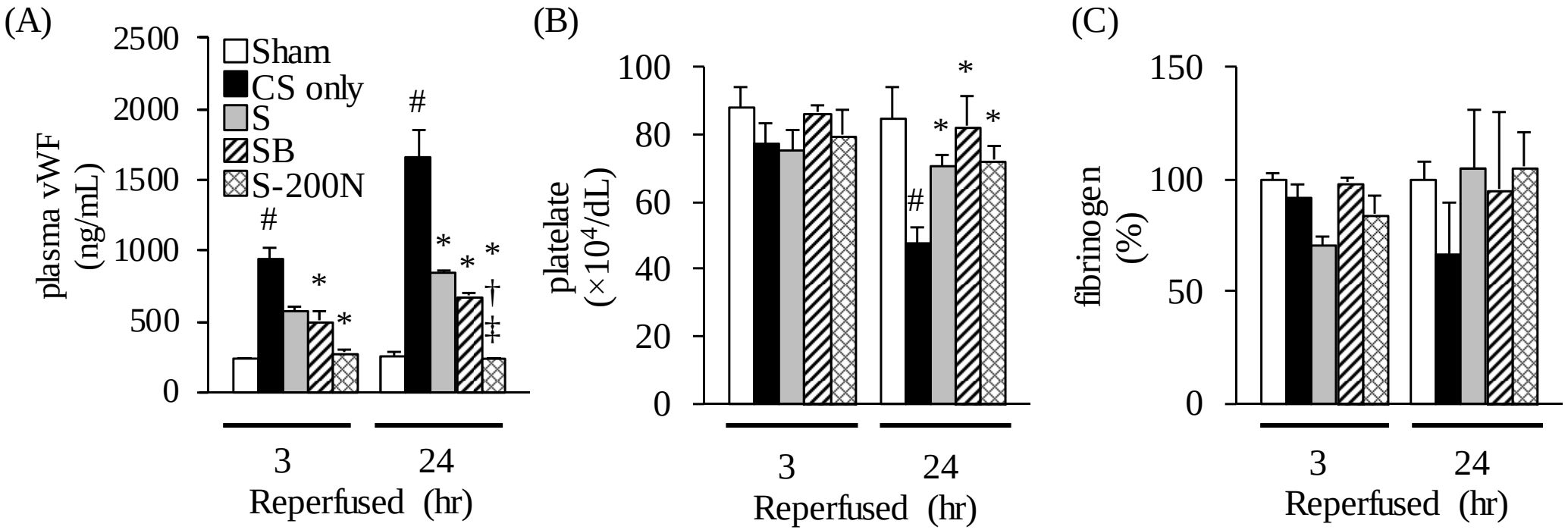

(A)
plasma vWF (ng/mL)
2500
2000
1500
1000
500
0
Sham
CS only
S
SB
S-200N
#
*
*
#
*
*
*
†
‡
3
24
Reperfused (hr)
(B)
platelate (×10⁴/dL)
100
80
60
40
20
0
#
*
*
*
3
24
Reperfused (hr)
(C)
fibrinogen (%)
150
100
50
0
3
24
Reperfused (hr)

Figure 5

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SDC Figure 1

SDC Figure 2