Salvianolic acid B improves the survival rate, acute kidney dysfunction, inflammation and NETosis-mediated antibacterial action in a crush syndrome rat model

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Abstract. Crush syndrome (CS) is a potentially lethal condition characterized by muscle cell damage resulting from decompression following compression. Patients with CS can develop cardiac failure, kidney dysfunction, shock, systemic inflammation and sepsis. Salvianolic acid B (SalB) has cardiac and kidney protective effects and anti-oxidative, anti-inflammatory, anti-apoptotic and anti-bacterial properties. The present study aimed to demonstrate the survival benefit of SalB in the CS rat model, which comprised anesthetized rats with bilateral hindlimb compression by a rubber tourniquet for 5 h. The rats examined were randomly divided into four groups: i) Sham; ii) sham treated with SalB; iii) CS rat model without treatment; and iv) CS rat model treated with SalB. Under continuous monitoring and recording of arterial blood pressures, blood and tissue samples were collected for biochemical analyses at designated timepoints before and after reperfusion. SalB administration improved the survival rate, kidney function (by treating shock and metabolic acidosis) and inflammation (by reducing mitochondrial dysfunction and endothelial damage). Reduced incidence of cardiac failure due to hyperkalemia was associated with reduced muscle injury via the prevention of mitochondrial dysfunction. Additionally, indirect antibacterial action by the neutrophil extracellular trap system (NETs) was observed. SalB administration to the CS rat model led to a substantial improvement in survival following CS by decreasing kidney and cardiac dysfunctions, inflammation, and endothelial dysfunction by improving the mitochondrial function and through antibacterial effects via NETs.

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Abbreviations: CS, crush syndrome; SalB, salvianolic acid B; NETs, neutrophil extracellular trap system; MODS, multiple organ dysfunction syndrome; NEU, neutrophil; s-SalB, sham with 20 mg/kg of SalB; SalB10, CS with 10 mg/kg of SalB; SalB20, CS with 20 mg/kg of SalB; SalB50, CS with 50 mg/kg of SalB; CS-SalB, CS with 20 mg/kg of SalB; NOx, nitrogen oxide; IL, interleukin; MAP, mean arterial pressure; HR, heart rate; Temp, rectal temperature; Hct, hematocrit; K^+, potassium; BUN, blood urea nitrogen; BE, base excess; TBARS, thiobarbituric acid reactive substance; MPO, myeloperoxidase; SOD, superoxide dismutase; RBC, red blood cell; WBC, white blood cell; PLT, platelet; APTT, activated partial thromboplastin time; PT, prothrombin time; FIB, fibrinogen; vWF, von Willebrand factor; CPK, creatine phosphokinase; NO_2^-, nitrite; NO_3^-, nitrate; iNOS, inducible nitric oxide synthase; HMGB1, high mobility group box 1; TNF, tumor necrosis factor; PAI-1, plasminogen activator inhibitor-1; NAG, N-acetyl-β-D-glucosaminidase; KIM-1, kidney injury marker-1; NGAL, neutrophil gelatinase-associated lipocalin; Cre, creatinine; GFR, glomerular filtration rate; ROS, reactive oxygen species; Cyt *c*, cytochrome *c*; DPPH, 1,1 diphenil-2-picryl-hydrazal; AA, ascorbic acid; MIC, minimum inhibitory concentration; CAMHB, cation-adjusted Mueller-Hinton broth

Key words: CS, SalB, kidney, NETs

Introduction

Crush syndrome (CS) occurs as a result of physical trauma sustained during events such as earthquakes and is associated with high mortality due to circulatory shock, kidney failure, and systemic inflammation (1,2). CS not only has localized effects but is also associated with systemic failure resulting from acute respiratory distress syndrome, systemic inflammatory response syndrome, multiple organ dysfunction syndrome (MODS), and sepsis, following ischemia-reperfusion and breakdown of muscle cells. The prevention of kidney failure is an important part of treatment strategies for CS because it contributes to worsening acute symptoms (3).

In general, kidney dysfunction is prevented by hemodialysis and fluid therapy (i.e., kidney replacement therapy) (4-7). Fluid therapy is the first-line treatment for CS. Acute kidney failure can be prevented by early fluid resuscitation using normal saline solution containing sodium bicarbonate (8). However, the

mortality rate of patients is high despite treatment, as experienced during the Hanshin Awaji, Marmara, and Wenchuan earthquakes (i.e., 13.4, 15.2, and 11%, respectively) (9-11). The high mortality rate results from the risk of inflammatory response and infection even after treatment; that is, injury caused by unusual and several complex pathological mechanisms, MODS, and sepsis. Moreover, approximately 20% of patients with CS die from heart disease-related symptoms, and over 50% die from sepsis and systemic disease (12,13). Consequently, studies on therapeutic effects focus on not only acute kidney and cardiac failure that lead to death, but also inflammation and infection, highlighting the importance of developing a therapeutic strategy for all pathological condition phases of CS.

Salvianolic acid B (4-[(1E)-3-[(1R)-1-carboxy-2-(3,4-dihydroxyphenyl)ethoxy]-3-oxo-1-propen-1-yl]-2-(3,4-dihydroxyphenyl)-2,3-dihydro-7-hydroxy-3-benzofurancarboxylic acid (2S,3S)-3-[(1R)-1-carboxy-2-(3,4-dihydroxyphenyl) ethyl] ester: SalB) is one of the components of *Radix Salvia miltiorrhiza*. SalB exerts cardiac and kidney protective effects (14-17) in an ischemia-reperfusion model by suppressing oxidative stress, inflammation, and apoptosis (18-21) and shows antibiotic properties (22,23). In our previous study, we focused on not only prevent death with cardiac failure and acute kidney failure, but also anti-inflammatory effect associated with the endothelial damage via ischemia-reperfusion injury, oxidative damage via mitochondrial dysfunction, and the activities of neutrophil (NEU) during inflammation (24-27). The inflammation and infection often observed in patients with CS may be related to neutrophil extracellular trapping (NET) mechanisms mediated by the NETosis process of the NEU at the site of injury. NETosis is neutrophil-related cell death characterized by the secretion of large web-like structures (28). With this, the treatment of infections prevents not only systemic dissemination of pathogens, but also blood coagulation and endothelial damage (29). However, only a few studies have focused on these mechanisms. In this study, we aimed to demonstrate the survival benefit of SalB in the CS rat model.

Materials and methods

Animal CS model. 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 relative humidity of 55±15% with a 12/12-h light/dark cycle and free access to food and water. All animal experiments were approved by the Institutional Animal Care and Use Committee of Josai University (approval no. JU18030). Anesthesia induction and maintenance was performed using inhaled 2% isoflurane. Body temperature was maintained throughout the experiment using a heating pad. The CS model was established as previously reported (24). Briefly, a rubber tourniquet was applied to both the hindlimbs of each rat, which was wrapped five times around a 2.0 kg-metal cylinder, and the end of the band was glued. Just before compression for 5 h, the tourniquet was removed from the limbs by cutting the band (i.e., reperfusion 0 h).

SalB preparation. SalB (Carbosynth) dosages were determined based on previous reports (30-32) and were 10, 20, and 50 mg/kg. SalB was dissolved in 1% dimethyl sulfoxide saline solution (vehicle): 100 μl.

Experimental design. The experimental design is shown in Fig. 1. Experiment 1 (survival): The anesthetized (2% isoflurane inhalation) rats were randomly divided into six groups: i) sham with vehicle (sham groups serving as controls, which were subjected to the same treatments as the CS-model rats except for compression or decompression with rubber tourniquets), ii) sham with 20 mg/kg SalB (s-SalB), iii) CS with vehicle (CS group), and iv) CS with 10 mg/kg SalB (SalB10), v) CS with 20 mg/kg SalB (SalB20) and vi) CS with 50 mg/kg SalB (SalB50). Vehicle and SalB were administered via a tail vein single injection after maintaining anesthesia for 5 h or decompression from a rubber tourniquet. Emerge from anesthesia, rats are replaced in the cage (free access to food and water). Survival measurement points were 0, 1, 3, 6, 24, and 48 h after reperfusion (each group; n=15).

Experiment 2 (observation of vital signs): Among the tested SalB dosages (10, 20, and 50 mg/kg), 20 mg/kg was chosen for this experiment as maximum survival was obtained at this dosage. To examine the effects of SalB in CS, the animals were randomly divided into four groups: vii) sham with vehicle, viii) s-SalB, ix) CS group, and x) CS with 20 mg/kg SalB (CS-SalB). The anesthetized (2% isoflurane inhalation) rats were subdivided above groups, and cannulated a polyethylene catheter (PE-50 tubing) from a carotid artery at 3, 6, 24 and 48 h after reperfusion for sequential sampling (each group; n=6).

Experiment 3 (assessment of therapeutic effects): To examine the effects of SalB in CS, the animals were randomly divided into four groups: xi) sham with vehicle, xii) s-SalB, xiii) CS group, and xiv) CS-SalB. Rats were subdivided at 3, 6, 24, and 48 h after reperfusion for these sampling points (each point; n=6).

The experimental rats (n=210) were monitored for health and behavior every hour until 6 h and every 12 h thereafter. The rats were euthanized (confirmation by pupillary reflex to light) when a no food and/or water intake state, dyspnea (i.e. mouth breathing) state, and autotomy (i.e. bite) occurred in the pressure area. All the rats used in the experiments were euthanized (confirmation by pupillary reflex to light) by administering an overdose of sodium pentobarbital (100 mg/kg body weight, intravenously); Experimental 1: 48 h after reperfusion; Experimental 2: 48 h after reperfusion; Experimental 3: just each sampling time.

Analysis of mean arterial pressure and blood gas levels. Experimental 2: Mean arterial pressure (MAP), heart rate (HR), and rectal temperature (Temp) were recorded using a PowerLab data acquisition system (AD Instruments). A carotid artery was cannulated with PE-50 tubing connected to a pressure transducer (AD Instruments). Arterial blood samples from each mouse were obtained at 3, 6, 24, and 48 h after reperfusion using a carotid artery catheter over time (24). The arterial levels of hematocrit (Hct), potassium (K^+), blood urea nitrogen (BUN), pH, base excess (BE), anion gap, and lactate were analyzed using the i-STAT300F blood gas analyzer $CG4^+$ and $EC8^+$ cartridges (FUSO Pharmaceutical Industries). These measurements were performed under maintained anesthesia (2% isoflurane inhalation) for up to 6 h, after which only the catheter was attached to the back of the rat, and then

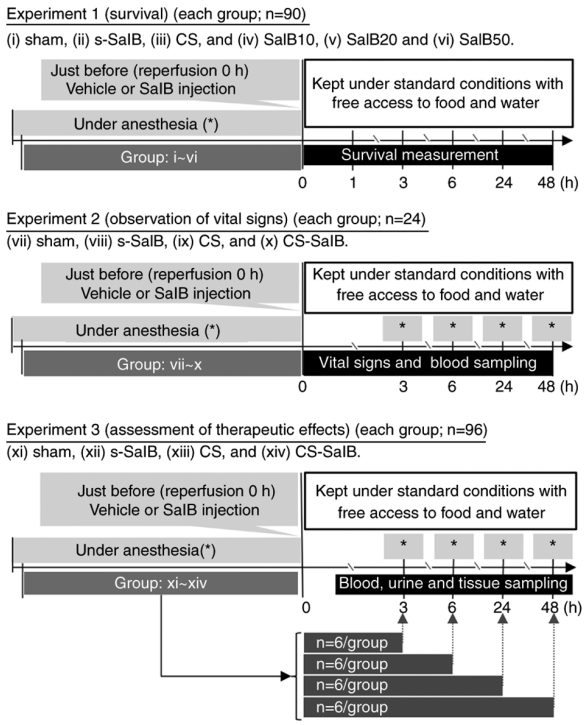

Figure 1. Experimental design. CS, crush syndrome; SalB, salvianolic acid B; s-SalB, sham with 20 mg/kg of SalB; SalB10, CS with 10 mg/kg of SalB; SalB20, CS with 20 mg/kg of SalB; SalB50, CS with 50 mg/kg of SalB.

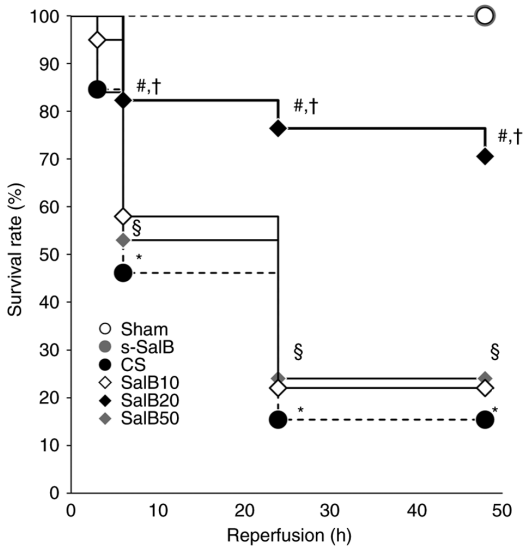

Figure 2. Effect of SalB treatment for varying durations on CS rat viability. Survival curves were obtained using the Kaplan-Meier method (n=15). *P<0.05 vs. sham group, #P<0.05 vs. CS group, †P<0.05 vs. SalB10 group, §P<0.05 vs. SalB20 group (log-rank test). CS, crush syndrome; SalB, salvianolic acid B; s-SalB, sham with 20 mg/kg of SalB; SalB10, CS with 10 mg/kg of SalB; SalB20, CS with 20 mg/kg of SalB; SalB50, CS with 50 mg/kg of SalB.

anesthetized and measured again 1 h before 24 and 48 h (i.e., after 23 and 47 h of Reperfusion).

Analysis of biochemical parameters and coagulation levels. Experimental 3: In each experimental group (3, 6, 24, and 48 h after reperfusion, respectively n=6), venous blood and tissue samples from the gastrocnemius muscles were subjected to inflammation, and tissue thiobarbituric acid reactive substance (TBARS), myeloperoxidase (MPO) activity, an index of mitochondrial permeability transition, and superoxide dismutase (SOD) activity (27) were measured. The rats used in the experiments were euthanized by administering an overdose of sodium pentobarbital and then venous blood (from the inferior vena cava) and tissue (kidney and muscle) was collected. Venous blood separated into whole blood, serum, and plasma. Red blood cell (RBC), white blood cell (WBC), NEU, and platelet (PLT) counts in whole blood were measured using Vet Scan HM5 (ABAXIS Inc.). Activated partial thromboplastin time (APTT) and prothrombin time (PT) were measured using Sysmex CA-100 (Sysmex Co.). The plasma level of creatine phosphokinase was measured using the Creatine Kinase Assay kit, EnzyChrom (BioAssay Systems Co.), fibrinogen (FIB) was measured using the Rat FIB ELISA kit (ASSAYPRO), von Willebrand factor (vWF) was measured using the rat vWF ELISA kit (USCN), and creatine phosphokinase (CPK) was measured using the Creatine Kinase Assay kit, EnzyChrom (BioAssay Systems Co.).

Analysis of interleukin (IL) levels. Experimental 3: The serum levels of high mobility group box 1 (HMGB1), IL-6, IL-8, IL-10, IL-1β, and tumor necrosis factor (TNF)-α were measured using HMGB1 ELISA kit II (Shino-Test Co.). Rat IL-6, IL-8, IL-10, L-1β/IL-1F2, and TNF-α levels were measured using the Quantikine® ELISA kit (RandD Systems, Inc.), and plasminogen activator inhibitor-1 (PAI-1) was measured using the Rat PAI1 ELISA kit (Abcam) according to the manufacturer's instructions.

Analysis of kidney dysfunction. Experimental 3: In the blood sample, the effect of N-acetyl-β-D-glucosaminidase (NAG) on kidney function was determined using the β-N-acetylglucosaminidase assay kit QuantiChrom (BioAssay Systems Co.), kidney injury marker-1 (KIM-1) using Rat TIM-1/KIM-1/HAVCR Immunoassay (RandD Systems, Inc.), neutrophil gelatinase-associated lipocalin (NGAL) using the Rat Lipocalin-2 ELISA kit (Abcam), and creatinine (Cre) using the Creatinine Assay kit, QuantiChrom (BioAssay Systems Co.). Urine samples were then collected by bladder and centrifuged at 1,500 x g for 5 min at 20-25˚C. Cre using the Creatinine Assay kit, QuantiChrom (BioAssay Systems Co.), urine osmotic pressure (Osmomat 030-D; Gonotec GmbH), urine volume and glomerular filtration rate (GFR). For histological evaluations, tissue samples were fixed in 10% formalin and embedded in paraffin, and sections were cut and stained with hematoxylin and eosin. Microphotographs of the tissue sections were then evaluated by a pathologist (New Histo Science Laboratory). Renal injuries were scored by calculating the percentage of tubules that displayed tubular dilation, cast formation, and tubular necrosis according to a previously described method (33). Specifically, for each kidney, 12 cortical tubules from at least 4 different areas (i.e., 3 cortical tubules/area) were scored, and care was taken to avoid repeated scoring of different convolutions of the same tubule. Higher scores represented more severe damage (the maximum score per tubule was 7), and points were given for the presence and extent of tubular

Table I. Effect of SalB on kidney function parameters in blood and urine sample in the CS rats.

Parameter	Group	Reperfusion, h			
		3	6	24	48
BUN, mg/dl	sham	15.3±1.1	12.7±1.2	14.6±1.1	19.5±1.8
	s-SalB	14.9±1.3	25.7±0.6[a]	24.2±10.0	17.3±0.2
	CS	29.3±5.6[a]	41.3±5.5[a]	98.5±3.5[a]	88.3±2.9[a]
	C-SalB	16.2±2.0[b]	21.0±1.8[b]	33.3±2.3[b]	35.1±4.4[b]
Cre, mg/dl	sham	0.3±0.3	0.2±0.0	0.2±0.0	0.2±0.0
	s-SalB	0.3±0.1	0.2±0.0	0.2±0.1	0.2±0.0
	CS	0.9±0.4	1.2±0.3[a]	1.5±0.1[a]	1.5±0.3[a]
	C-SalB	0.4±0.2	0.5±0.3[b]	0.5±0.2[b]	0.6±0.3[b]
Urine osmotic pressure, mOsm/kg • H_20	sham	1.47±0.02	1.37±0.09	1.38±0.06	1.39±0.07
	s-SalB	1.58±0.02	1.38±0.35	1.31±0.12	1.25±0.17
	CS	1.12±0.02[a]	0.85±0.22[a]	0.66±0.15[a]	0.54±0.04[a]
	C-SalB	1.53±0.01[b]	1.51±0.08[b]	1.81±0.03[b]	1.40±0.11[b]
Urine volume, ml/l	sham	0.42±0.02	0.40±0.05	0.35±0.05	0.50±0.06
	s-SalB	0.46±0.25	0.50±0.06	0.60±0.02	0.45±0.03
	CS	0.30±0.01	0.21±0.02	0.16±0.00	0.23±0.02
	C-SalB	0.39±0.12	0.56±0.02[b]	0.54±0.07[b]	0.45±0.07[b]
GFR, ml/min	sham	1.69±0.22	1.50±0.19	1.45±0.10	1.36±0.23
	s-SalB	1.98±0.45	1.75±0.24	1.35±0.65	0.98±0.11
	CS	0.98±0.14[a]	0.78±0.24[a]	0.55±0.33[a]	0.58±0.19[a]
	C-SalB	1.78±0.45[b]	1.53±0.33[b]	1.75±0.57[b]	1.78±0.22[b]

[a]P<0.05 vs. sham group; [b]P<0.05 vs. CS group (Tukey-Kramer test). Values are presented as the mean ± SEM (n=6 each). CS, crush syndrome; SalB, salvianolic acid B; s-SalB, sham with 20 mg/kg of SalB; CS-SalB, CS with 20 mg/kg of SalB; BUN, blood urea nitrogen; Cre, creatinine; GFR, glomerular filtration rate.

epithelial cell flattening (1 point), brush border loss (1 point), cell membrane bleb formation (1 point), interstitial edema (1 point), cytoplasmic vacuolization (1 point), cell necrosis (1 point), and tubular lumen obstruction (1 points).

Determination of reactive oxygen species (ROS) production, MPO activity, and mitochondrial function. ROS production in the injured gastrocnemius muscle was determined by measuring the concentration of TBARS, MPO activity in the blood and muscle tissue, and mitochondrial function by cytochrome *c* (Cyt *c*) leakage into the cytoplasm. JC-1 fluorescence intensity was used to determine mitochondrial permeability transition (i.e., mitochondrial inner membrane function) using the method described by Murata *et al* (27). Briefly, for the JC-1 method, mitochondrial fraction was isolated using a mitochondrion isolation kit (Sigma). Muscle tissue was homogenized and then centrifuged at 600 x g for 5 min. The supernatant was centrifuged at 11,000 x g for 10 min and then spun at 16,000 x g for 20 min at 4˚C to remove any residual mitochondria. The pellet (mitochondrial fraction) was suspended with storage buffer, and then total protein concentration was measured. In short, 90 μl of 0.2 μg/ml JC-1 staining solution was added into the wells of a 96-well plate and then 10 μl of the isolated mitochondrial sample (0.2 μg protein) was added. The plate was incubated at room temperature in the dark for 7 min for uptake saturation. The absorbance was then measured with a microplate spectrophotometer at emission and excitation wavelengths of 540 and 570 nm (red), respectively, for apoptotic mitochondria, and 485 and 535 nm, respectively, for healthy mitochondria (green).

SOD activity was determined using the SOD Assay kit-WST (Dojindo Laboratories). 1,1-diphenil-2-picryl-hydrazal (DPPH) antioxidant assay of SalB was performed as described by Sharma and Bhat (34). Briefly, 20 μM DPPH methanol solution and 5, 10, 25, 50, 100, 250, 500, 750, and 1,000 μM SalB methanol solutions were mixed in a microplate at a 1:1 volume ratio. The mixture was then incubated at 30˚C for 30 min (light shielded), and the absorbance was measured at 280 nm ($SalB_{abs}$). The absorbance of the mixture of DPPH methanol solution/methanol (1:1) was used as DM_{abs}, and that of methanol as M_{abs}. Ascorbic acid (AA) has a high antioxidant capacity, and it was used as an antioxidant reference (0.018, 0.039, 0.156, 0.313, 0.625, 1.25, and 2.5 μM) for comparison with SalB. The DPPH radical scavenging activity ratio (AU) was calculated using the following equation: $AU=[1-(SalB_{abs}-M_{abs})/(DM_{abs}-M_{abs})]$ x100, and 50% AU was calculated from the linear equation of AU and SalB or AA.

Analysis of nitrogen oxide (NOx)and inducible nitric oxide synthase (iNOS) levels. NOx [(total nitrite (NO_2^-) and nitrate

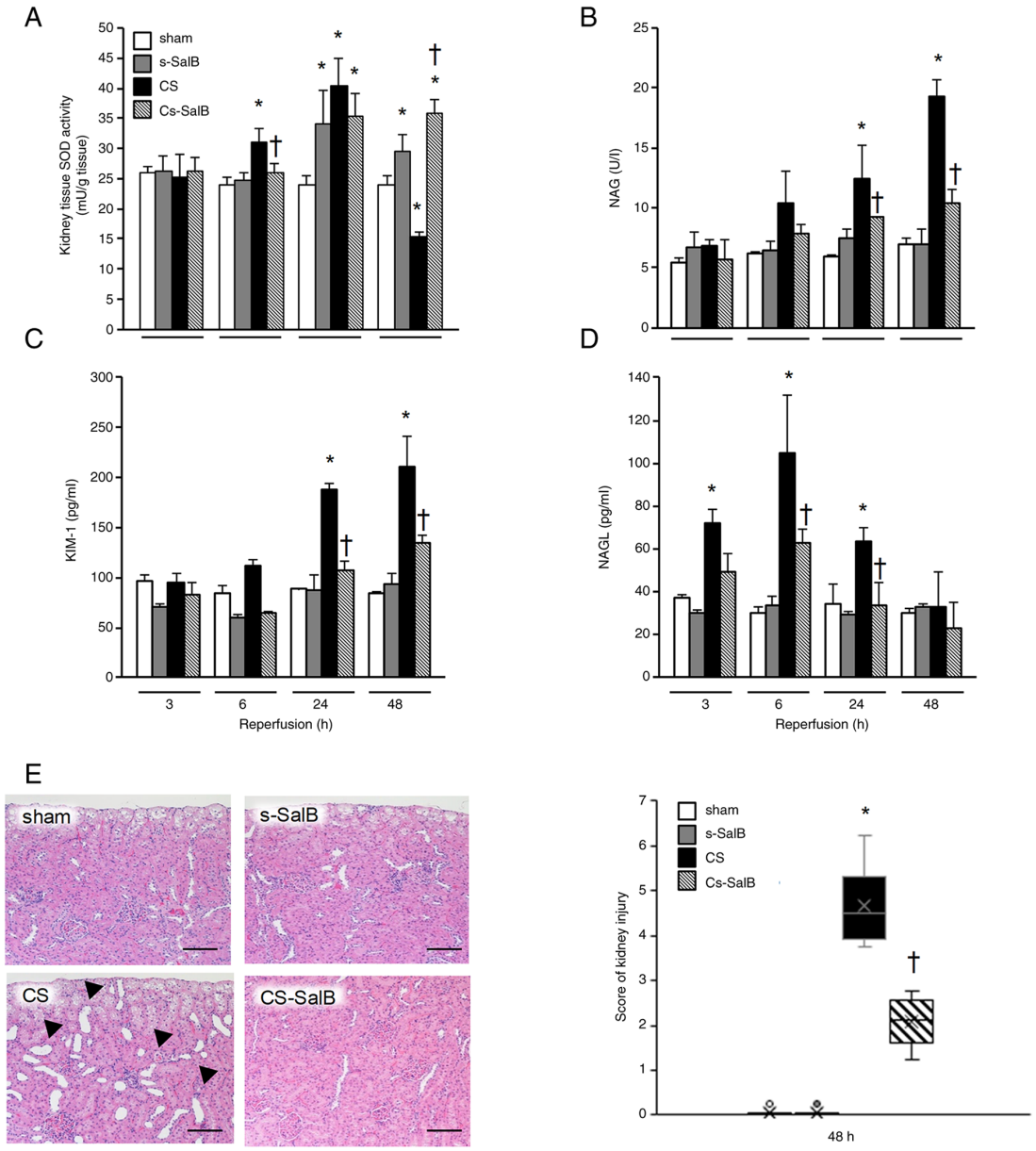

Figure 3. Effect of SalB on kidney function in the CS rats. (A) Kidney SOD activity, (B) serum NAG level, (C) serum KIM-1 level, (D) serum NGAL level and (E) hematoxylin and eosin-stained kidney sections and kidney injury score following reperfusion for 48 h. (A-D) Bar graph values are presented as the mean ± SEM (n=6). *P<0.05 vs. sham group, †P<0.05 vs. CS group (Tukey-Kramer test). Micrographs are representative of three independent experiments (magnification, x200; scale bar, 100 μm). Black arrowhead, dilated kidney tubule. (E) Box plot for kidney injury score, *P<0.05 vs. sham group, †P<0.05 vs. CS group (Kruskal-Wallis test). CS, crush syndrome; SalB, salvianolic acid B; s-SalB, sham with 20 mg/kg of SalB; CS-SalB, CS with 20 mg/kg of SalB; SOD, superoxide dismutase; NAG, N-acetyl-β-D-glucosaminidase; KIM-1, kidney injury marker-1; NGAL, neutrophil gelatinase-associated lipocalin.

(NO_3^-)] concentrations in the serum were measured using the CII and FX NO_2^-/NO_3^- assay kits (Dojindo Laboratories) according to the manufacturer's instructions. Western blotting for inducible nitric oxide synthase (iNOS) and β-actin was carried out as previously described (26). 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 iNOS (Cell Signaling Technology) and β-actin (Cell Signaling Technology). The protein bands were visualized using 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) with β-actin as a loading control.

Antimicrobial study. The minimum inhibitory concentration (MIC) of SalB was measured using the broth microdilution method. This procedure essentially followed the Clinical and Laboratory Standards Institute guidelines (35). Test bacteria used were two strains of gram-positive bacteria (*Bacillus subtilis* ATCC6633 and *Staphylococcus aureus* ATCC29213) and one strain of gram-negative bacteria (*Escherichia coli* ATCC25922). Cation-adjusted Mueller-Hinton Broth (CAMHB; BD Biosciences) was used as a test medium. For the test, the

Table II. Effects of SalB.

Parameter	Group	Reperfusion, h			
		3	6	24	48
CPK, IU/l	sham	148±20	193±46	208±40	206±43
	s-SalB	134±18	174±41	177±34	134±28
	CS	5,305±1,080[a]	8,368±1,556[a]	12,870±2,281[a]	34,562±3,428[a]
	CS-SalB	3,373±181	3,537±307[b]	11,251±2,309	24,469±2,291[b]
K^+, mEq/l	sham	4.4±0.3	4.3±0.2	4.6±0.2	3.9±0.2
	s-SalB	3.9±0.0	3.6±0.1	3.8±0.2	3.5±0.,1
	CS	5.5±0.2	5.9±0.2	6.4±0.3[a]	7.3±0.7[a]
	CS-SalB	4.8±0.2	5.7±0.2	6.6±0.5	5.6±0.2[b]
pH	sham	7.46±0.04	7.48±0.01	7.50±0.01	7.45±0.01
	s-SalB	7.41±0.02	7.45±0.05	7.44±0.02	7.48±0.03
	CS	7.50±0.02	7.46±0.02	7.24±0.03[a]	7.27±0.07[a]
	CS-SalB	7.46±0.01	7.47±0.02	7.45±0.03[b]	7.47±0.03[b]
BE, mmol/l	sham	5.7±0.3	6.7±0.7	6.7±0.9	4.7±0.3
	s-SalB	1.3±1.8	2.0±2.3	2.3±2.2	6.3±0.7
	CS	1.3±0.9	0.3±1.1	-5.0±1.6[a]	-4.4±2.7[a]
	CS-SalB	1.0±0.8	1.5±1.8	-4.5±1.5	2.0±0.8
MAP, mmHg	sham	131±5	124±5	112±11	116±9
	s-SalB	124±6	110±4	122±11	151±10
	CS	65±6[a]	63±7[a]	58±6[a]	56±14[a]
	CS-SalB	98±3[b]	94±4[b]	89±3[b]	87±3[b]
Hct, %	sham	46.3±0.3	44.7±0.3	43.3±1.2	43.7±0.9
	s-SalB	48.3±1.5	47.0±1.5	48.0±1.2	45.0±2.6
	CS	48.6±1.2	51.9±1.1[a]	51.3±0.9[a]	57.2±7.1[a]
	CS-SalB	45.8±1.7	48.5±1.2	52.3±1.9	46.0±2.1[b]
HR, bpm	sham	400±15	388±17	336±46	329±79
	s-SalB	337±20	309±20	329±5	385±22
	CS	307±18[a]	274±26[a]	344±26	256±38[a]
	CS-SalB	333±5	374±7[b]	443±26[b]	416±30[b]
Temp, ˚C	sham	36.2±0.4	36.4±0.1	36.3±0.3	36.3±0.6
	s-SalB	36.5±0.3	37.5±0.7	37.8±0.5	35.4±0.5
	CS	35.3±0.4	34.6±0.6[a]	35.0±0.8[a]	29.5±1.4[a]
	CS-SalB	36.2±0.1	37.4±0.5[b]	38.2±0.8[b]	35.0±0.5[b]

[a]P<0.05 vs. sham group; [b]P<0.05 vs. CS group (Tukey-Kramer test). Values are presented as the mean ± SEM (n=6 each). CS, crush syndrome; SalB, salvianolic acid B; s-SalB, sham with 20 mg/kg of SalB; CS-SalB, CS with 20 mg/kg of SalB; CPK, creatine phosphokinase; K^+, potassium; BE, base excess; MAP, mean arterial pressure; Hct, hematocrit; HR, heart rate.

solvent used to prepare the agar medium was water. SalB was added to a concentration of 3,000 μg/ml in CAMHB. The mixture was stirred and then diluted with CAMHB to 2,000, 1,000, 500, 300, 100, 50, 30, 10, 1, 0.5, 0.3, and 0.1 μg/ml. The test micro plates were prepared, and an inoculum (5 μl) of the bacterium adjusted to $1.0x10^7$ CFU/ml was spotted onto the test micro plate. After incubating the test plates for 16-20 h at 35˚C, the growth of each strain was observed to determine the MIC.

Statistical analysis. Results are expressed as mean ± standard error of the mean. Survival curves were generated using the Kaplan-Meier method, and survival was compared using the log-rank test. Differences between groups were assessed using the one-way analysis of variance with Tukey's honest significant difference test or Tukey's test. Kidney injury score was assessed using the Dunn's nonparametric comparison for post hoc testing after a Kruskal-Wallis test. DPPH antioxidant assay was assessed using the unpaired Student's t test between two groups. Differences were considered significant at P<0.05 (Statcel 2, 2nd ed. OMS Publishing Inc.).

Results

SalB treatment effects on the survival rate in the CS rat model. The survival rates of rats in the CS group were 92, 84, 46, 15,

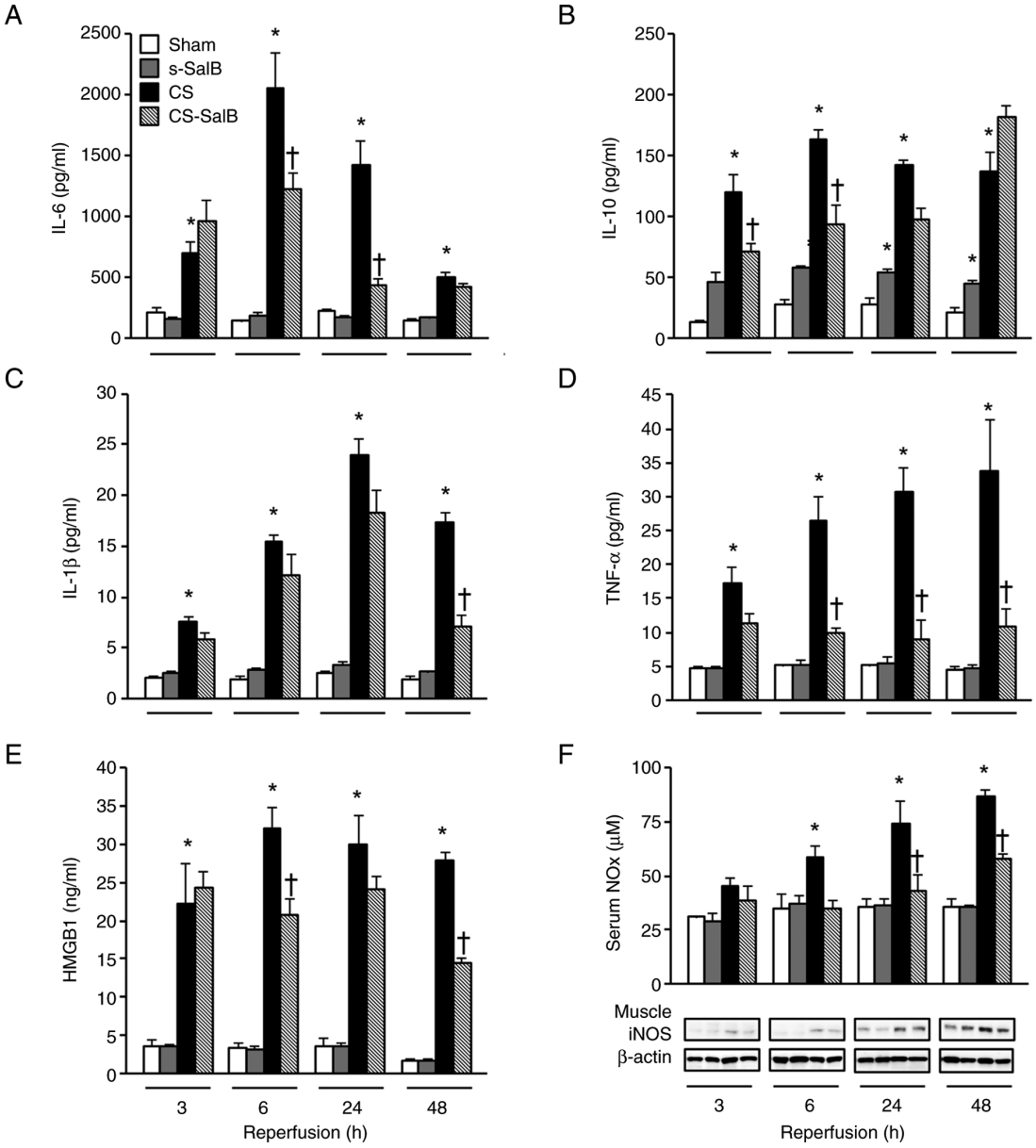

Figure 4. Effect of SalB on inflammatory mediators in the CS rats. (A) Serum IL-6 levels, (B) serum IL-10 levels, (C) serum IL-1β levels, (D) serum TNF-α levels, (E) serum HMGB1 levels, and (F) serum NOx levels and muscle iNOS expression. Values are presented as the mean ± SEM (n=6). *P<0.05 vs. sham group, †P<0.05 vs. CS group (Tukey-Kramer test). CS, crush syndrome; SalB, salvianolic acid B; s-SalB, sham with 20 mg/kg of SalB; CS-SalB, CS with 20 mg/kg of SalB; HMGB1, high mobility group box 1; NOx, nitrogen oxide; iNOS, inducible nitric oxide synthase.

and 15% at 1, 3, 6, 24, and 48 h, respectively, and they were significantly lower than those in the sham group at 6, 24, and 48 h after reperfusion (P<0.05). CS rats died of cardiac failure and hypovolemic shock. The cause of death was related to kidney dysfunction and systemic inflammatory response associated with traumatic rhabdomyolysis caused by crush injury. The survival rates of the CS-SalB group at 6, 24, and 48 h after reperfusion (82, 76, and 71%, respectively) were significantly higher than those of the CS group (P<0.05). No mortality was observed in the sham and s-SalB groups (Fig. 2). These results suggested the effectiveness of SalB in the treatment and prevention of CS symptoms.

SalB treatment effects on kidney disfunction in the CS rat model. The parameters of kidney functions are shown in Fig. 3 and Table I. The SOD activity in the CS group was significantly higher than that in the sham group at 6 and 24 h after reperfusion, and significantly lower than that in the sham group at 48 h after reperfusion (Fig. 3A). The NAG and KIM-1 levels in the CS group were significantly higher than those in the sham group at 6, 24, and 48 h (Fig. 3B and C), and the NGAL level in the CS group was significantly higher than the sham group at 3, 6, and 24 h (Fig. 3D). In the CS-SalB group, the kidney SOD activity was significantly higher than that in the CS group at 48 h after reperfusion, and the NAG, KIM-1, and NGAL levels were significantly lower than those in the CS group. In terms of pathology, the CS group showed moderate pathological dilation in the distal convoluted tubules, and this was improved in the CS-SalB group after 48 h of reperfusion (Fig. 3E). These findings suggest that SalB improves the kidney

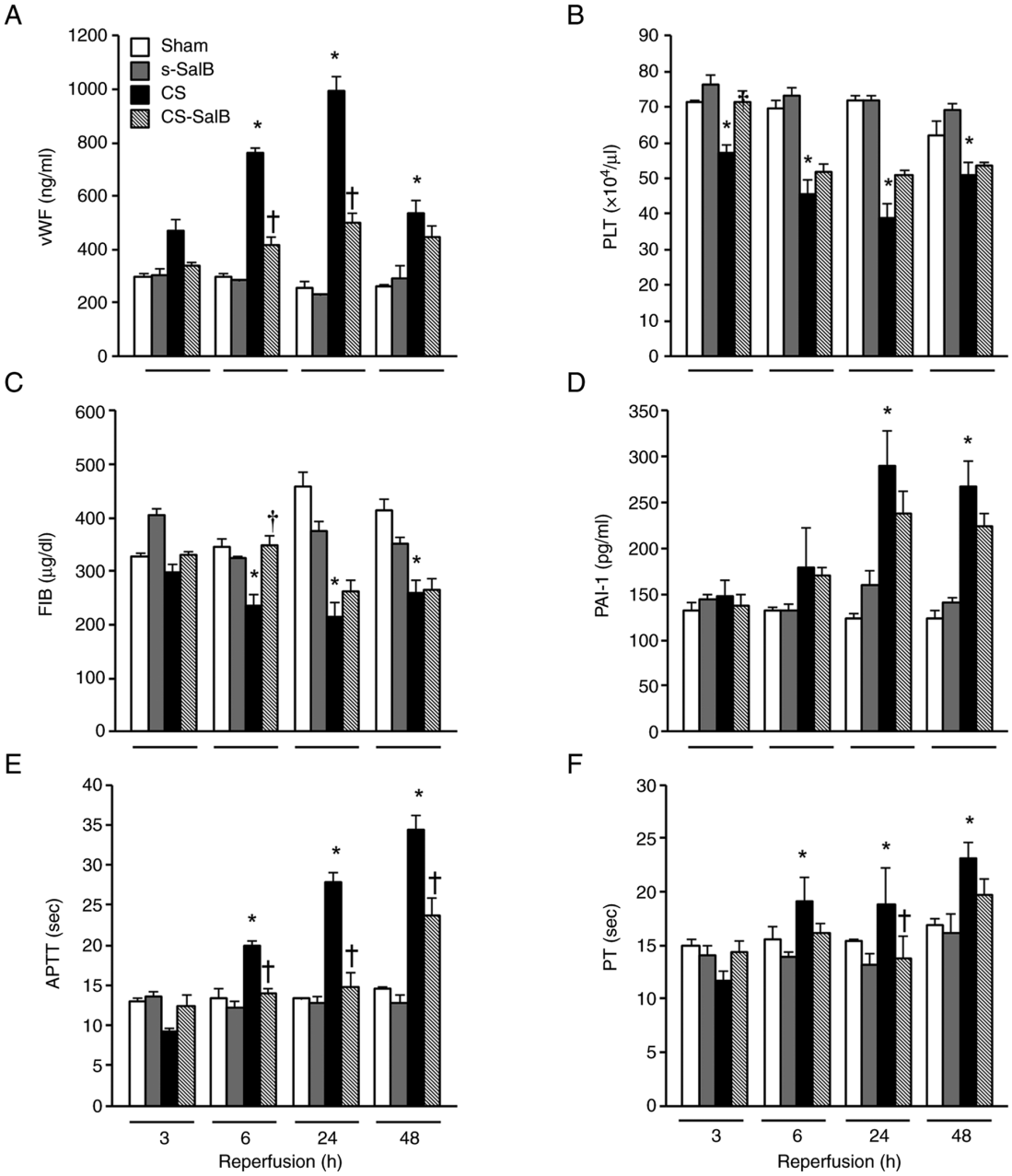

Figure 5. Effect of SalB on the coagulation system in the CS rats. (A) Plasma vWF levels, (B) PLT levels, (C) FIB levels, (D) serum PAI-1 levels, (E) APTT levels and (F) PT levels. Values are presented as the mean ± SEM (n=6). *P<0.05 vs. sham group, †P<0.05 vs. CS group (Tukey-Kramer test). CS, crush syndrome; SalB, salvianolic acid B; s-SalB, sham with 20 mg/kg of SalB; CS-SalB, CS with 20 mg/kg of SalB; PLT, platelet; APTT, activated partial thromboplastin time; PT, prothrombin time; FIB, fibrinogen; vWF, von Willebrand factor; PAI-1, plasminogen activator inhibitor-1.

dysfunction by improving kidney tubule epithelial damage and antioxidative effect in CS.

SalB treatment effects on cardiac failure and shock in the CS rat model. Table II shows the parameters of the acute phase symptoms of CS. The CPK and K^+ levels in the CS group were significantly higher than those in the sham group. In contrast, the MAP in the CS group was significantly lower than that in the sham group. pH, BE, Hct, HR, and Temp in the CS group were significantly lower than those in the sham group (Table II). In contrast, these changes in the CS-SalB group were significantly higher than those in the CS group. In addition, the adverse effects of SalB treatment were not observed in the groups. These finding suggest that SalB improves shock and cardiac failure by decreasing systemic circulation of K^+ following the suppression of muscle cell collapse in CS.

SalB treatment effects on inflammation and coagulation disorder from endothelial cell damage. Endothelial damage with inflammation, and the levels of IL-6, IL-10, IL-1β, TNF-α, HMGB1, and NOx levels are shown in Fig. 4. These parameters in the CS group were significantly higher than those in the sham group at 3-48 h. The IL-6, IL-1β, and HMGB1 levels in the CS-SalB group were significantly lower than those in the CS group at 6-24 h. The TNF-α and NOx levels in the CS-SalB group were significantly lower than those in the CS group and comparable with those in the sham group. Moreover, the IL-10 level in the s-SalB group was significantly higher than that in

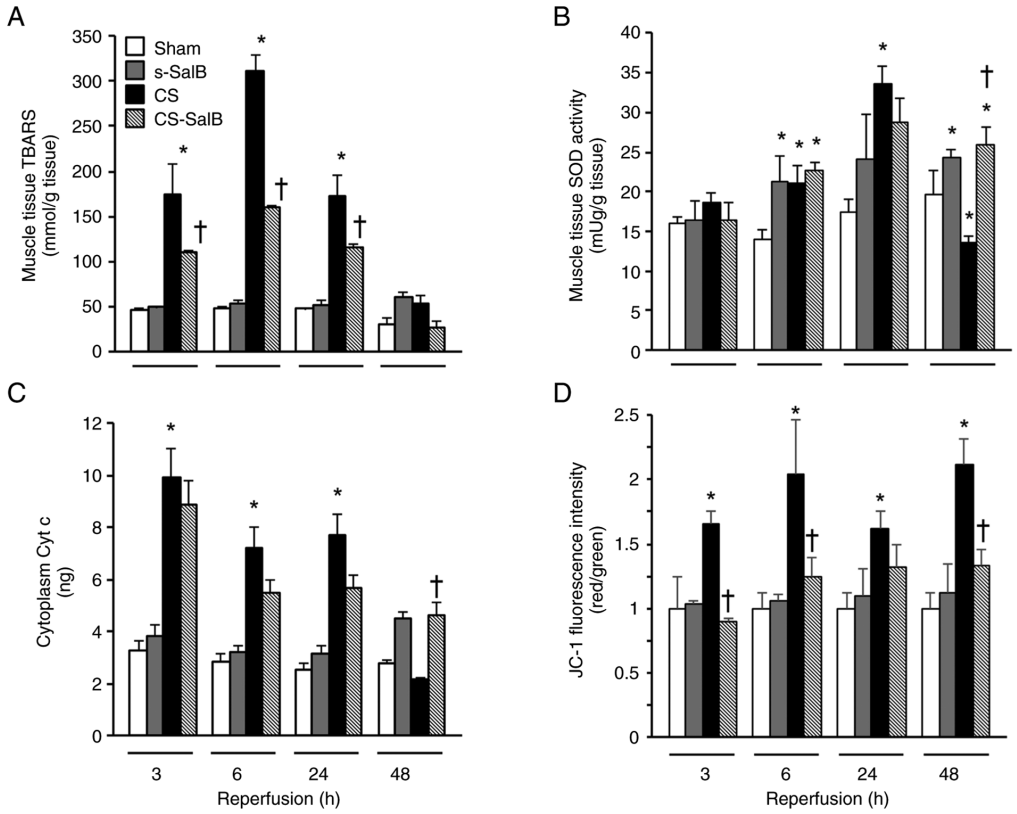

Figure 6. Effect of SalB on antioxidant action and mitochondrial function in the CS rats. (A) Muscle TBARS levels, (B) muscle SOD activity, (C) cytoplasm Cyt c content and (D) muscle JC-1 fluorescence. Values are presented as the mean ± SEM (n=6). *P<0.05 vs. sham group, †P<0.05 vs. CS group (Tukey-Kramer test). CS, crush syndrome; SalB, salvianolic acid B; s-SalB, sham with 20 mg/kg of SalB; CS-SalB, CS with 20 mg/kg of SalB; TBARS, thiobarbituric acid reactive substance; SOD, superoxide dismutase; Cyt c, cytochrome.

the sham group. However, the IL-10 level in the CS-SalB group was not significantly lower than that in the CS group.

Endothelial cell damage was assessed using the vWF, PLT, FIB, PAI-1, APTT, and PT levels (Fig. 5). The vWF level in the CS group was significantly lower than that in the sham group during the experimental period, and temporarily inhibited in the CS-SalB group compared with that in the CS group (Fig. 5A). The PLT and FIB levels in the CS-SalB group were significantly higher than those in the sham group. In particular, the PLT level in the CS-SalB group was significantly higher than that in the CS group at 3-24 h (Fig. 5B and C). The PAI-1 level in the CS group was significantly higher than that in the sham group, and the PAI-1 level in the CS-SalB group showed a decreasing trend, which was not observed in the CS group (Fig. 5D). The APTT and PT levels in the CS group were significantly higher than those in the sham group at 3-48 h, and the former's level in the CS-SalB group was significantly lower than that in the CS group at 3-24 h. In the s-SalB group, these were no differences in these parameters. These finding suggested that SalB improves coagulation disorder by suppressing the increased production of inflammatory cytokines induced by damaged vascular endothelial cells on CS.

Inhibitory effects of SalB on ROS production and mitochondrial dysfunction in the CS rat model. ROS damage was assessed by TBARS production, and muscle SOD activity and mitochondrial damage were assessed using cytoplasm Cyt *c* and JC-1. The CS group showed significantly higher tissue TBARS level than the sham group, and the maximum level of tissue TBARS in the CS-SalB group was significantly lower than that in the CS group. The muscle SOD activity in the CS group was significantly higher than that in the sham group at 6 and 24 h of reperfusion, and significantly lower in the CS group than in the sham group at 48 h of reperfusion. In the CS-SalB group, the SOD activity was significantly higher than that in the CS group at 24 h of reperfusion (Fig. 6B). The cytoplasmic Cyt *c* level in the CS group was significantly higher than that in the sham group, and the cytoplasmic Cyt *c* level in the CS-SalB group showed a tendency to decrease compared with that in the CS group (Fig. 6C). The CS group had significantly higher JC-1 level than the sham group at all experimental periods, and the JC-1 level was inhibited and higher in the CS-SalB group than in the CS group (Fig. 6D). These finding suggested that SalB improves the antioxidation system including the SOD activity, and ROS generation by mitochondrial dysfunction in CS. In addition, the direct radical scavenging ability of the SalB group was significantly higher compared with that in the AA group (Fig. 7).

SalB induced antibacterial action via NETosis process in the CS model. We focused in the NETosis process because it is not only a frequent problem associated with infections such as sepsis in patients with CS, but also related to leukocyte activation in the CS rat model. NETosis was evaluated using NEU, IL-8, blood MPO activity, and tissue MPO activity levels (Fig. 8). These parameters in the CS group were significantly higher than those in the sham group during the experimental period. In contrast,

the NEU and tissue MPO activity levels in the CS-SalB group were significantly lower than those in the CS group, and the IL-8 level in the CS-SalB group was not significantly different from that in the CS group. The antibacterial effect (i.e., MIC) was not observed from 2,000 to 0.1 μg/ml (Table III). These findings suggest that SalB exerts its antibacterial activity via NETosis activation in CS.

Discussion

We demonstrated that a simple therapeutic method of SalB intravenous injection improves severe morbidity and mortality of patients with CS. The 20 mg/kg SalB intravenous injection presented the highest survival rate (Fig. 2), which was related to improve the cause of death of CS rat model of kidney dysfunction and cardiac failure. Moreover, sever systemic inflammation was related to improve endothelial cell damage and oxidation stress. However, 50 mg/kg SalB intravenous injection did not improve the survival rate because of metabolic and respiratory alkalosis (data not shown).

Kidney injury leads to kidney failure, which is a serious complication of CS that results from circulatory shock, renal afferent arteriolar vasoconstriction (urinary concentration), increased urinary myoglobin levels, or metabolic acidosis (urinary acidity) (21-23). All of these induce precipitation in distal convoluted tubules and formation of tubular cast with the subsequent tubular obstruction. Myoglobin accumulation has also been known to trigger oxidative injury (24,25). We demonstrated that an improvement in shock and acidosis by cardioprotective effect (31) for the risk of kidney disfunction (Table I) and the protection for kidney oxidative stress injury was the sustained activation of SOD by SalB (Fig. 3 and Table I), hence indicated a high survival rate. Interestingly, the SOD activity of CS model rats at 48 h was significantly decreased compared with that at 24 h. The CS rats showed persistently high levels of NGAL, KIM-1 and NAG, demonstrated kidney tubule injury. These results were suggested that the persistent injury induced excessive ROS production, and these associated with consumption of SOD (Fig. 3A). Generally, fluid resuscitation (kidney replacement therapy) requires an infusion preparation of 6 l/day or more for each patient with CS (3). SalB also eliminates the need to manage such large amounts of medication. We suggest the use of SalB to simplify the initial treatment of patients with CS who are at an increased risk of a variety of symptoms.

Previously, we reported that survival following CS is increased by the anti-inflammatory effects of treatment agents that prevent systemic inflammatory response syndrome, making systemic management difficult, even after the acute phase (24-27). The cause of the inflammatory response in CS is not only traumatic stress following crush injury but also reactive oxygen injury through mitochondrial dysfunction associated with ischemia-reperfusion injury. HMGB1 is lethally involved in constitutive expression and vascular endothelial cell interactions (36,37) and is a lethal inflammation mediator (38); it is released into the circulation from collapsed muscle cells after crush injury. In this study, SalB significantly improved muscle damage (Table II) and suppressed the serum HMGB1 level (39). On the contrary, the fibrinolytic system via PAI-1 generation was enhanced by HMGB1 (40), because an increase in vWF and a decrease in PLT and FIB promoted

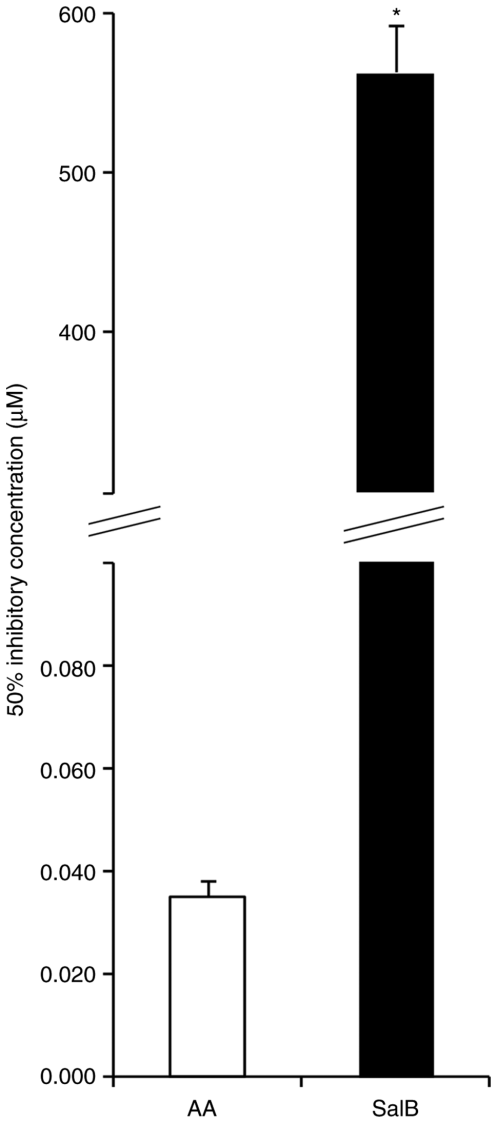

Figure 7. DPPH radical scavenging assay for SalB. Values are presented as the mean ± SEM (n=3). *P<0.05 vs. AA group (Student's t-test). AA, ascorbic acid; SalB, salvianolic acid B; DPPH, 1,1 diphenil-2-picryl-hydrazal.

platelet aggregation and increased vascular endothelial interaction (Figs. 4 and 5). In addition, excessive nitric oxide has also been implicated in the induction of endothelial damage. According to Yang *et al* (41), SalB treatment reduced blood nitric oxide level, and consequently prevented platelet aggregation. In this study, these effected suggested, because serum NOx content and iNOS expression were significantly enhanced toward normal range (Fig. 4F). The inducible factor contributing to inflammation was a mitochondrial function disorder. First, SalB improves mitochondrial membrane potential and mitochondrial outer membrane function (Fig. 6C and D). A direct radical scavenging ability of SalB was only observed at a concentration of 563.4±88.3 μg/ml (Fig. 7), which suggested that these effects of SalB were not observed because a ~16,000-fold higher concentration was needed in order to show a similar effect as ascorbic acid (0.035±0.008 μg/ml). Generally, the primary functions of mitochondria in cell apoptosis include the release of activity factors of caspase, such as Cyt *c*, loss of the mitochondrial transmembrane potential, and

Table III. Antibacterial effects of SalB.

	SalB concentration, μg/ml											
Test bacteria	2000	1000	500	300	100	50	30	10	1	0.5	0.3	0.1
Escherichia coli	-	-	-	-	-	-	-	-	-	-	-	-
Staphylococcus aureus	-	-	-	-	-	-	-	-	-	-	-	-
Bacillus subtilis	-	-	-	-	-	-	-	-	-	-	-	-

-, negative (no antibacterial effect: Bacterial colony growth); SalB, salvianolic acid B.

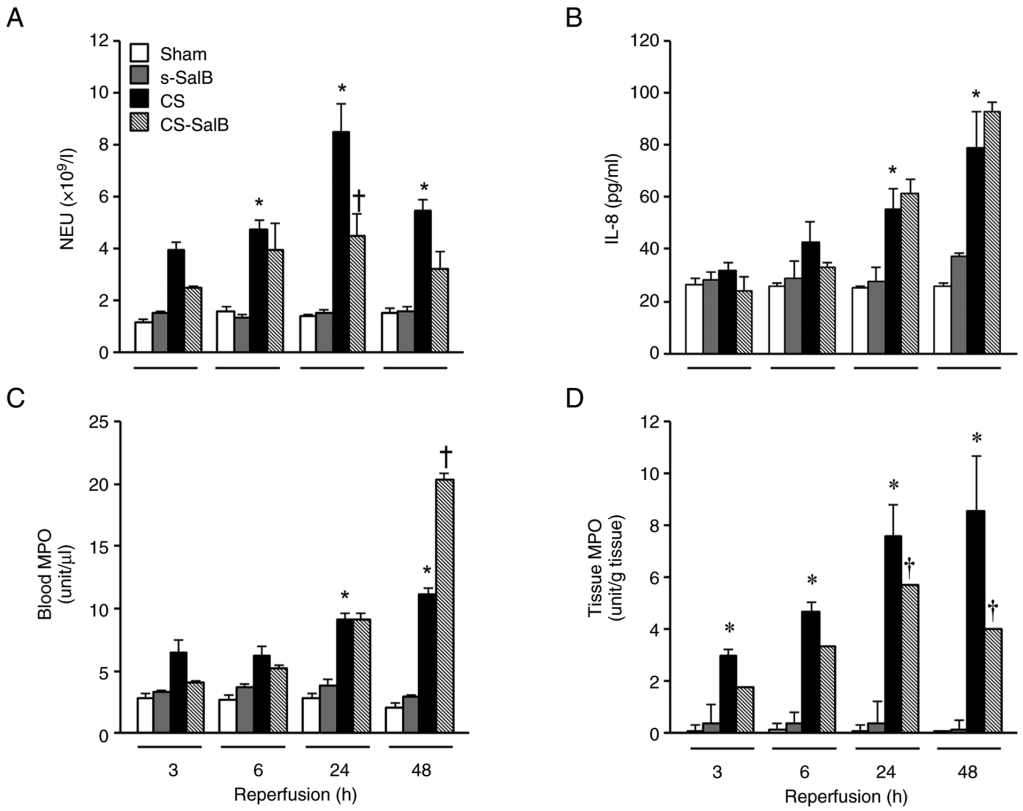

Figure 8. Effect of neutrophil extracellular trap system of on antibacterial potential in the CS rats. (A) NEU levels, (B) serum IL-8 levels, (C) blood MPO activity and (D) muscle MPO activity. Values are presented as the mean ± SEM (n=6). *P<0.05 vs. sham group, †P<0.05 vs. CS group (Tukey-Kramer test). CS, crush syndrome; SalB, salvianolic acid B; s-SalB, sham with 20 mg/kg of SalB; CS-SalB, CS with 20 mg/kg of SalB; NEU, neutrophil; MPO, myeloperoxidase.

dysfunction of oxidative phosphorylation of mitochondria (42). SalB affects mitochondrial function improvement by an antiradical effect (43). In fact, the present study showed that ROS is suppressed by improving mitochondrial membrane potential and reducing cytoplasmic Cyt *c* (Fig. 6). Moreover, the anti-oxidative stress effect was demonstrated by the low TBARS level and high SOD activity induced by SalB treatment. It is suggested that SalB not only suppresses HMGB1 expression and prevents the myocyte collapse by suppressing vascular endothelial cell damage due to anticoagulant action and anti-inflammatory action, but also improves mitochondrial function. According to Huang *et al* (44), SalB reported notably increased SOD ability. SOD activity in CS rats was to exhausted associated with the oxidative damage (45), therefore, SalB treated group were higher than Sham and CS model rat.

Death due to sepsis associated with CS (i.e., infections) begins to occur 3 days after injury and most often within 2 weeks (11). Therefore, the suggested countermeasures against infection include the prevention of nosocomial infection via antibiotic administration, tetanus prevention, maintaining sanitary conditions in affected hospitals (46). According to Huttunen *et al* (22), SalB induces antibacterial activity against *Neisseria meningitidis*. We conducted MIC test using *E. coli*, *S. aureus*, and *B. subtilis* as causative bacteria for septicemia to simulate disaster sites. However, in our study, 0.1-2000 μg/ml SalB showed no antibacterial effect (Table III). Of note, our results showed the potential antibacterial effect of SalB via NEU response. Generally, the MPO level is correlated with the NEU count; however, these levels were not matched. The heterogeneity of neutrophils is characterized by features such as

enhanced NET formation when the state activated by DAMPs and other factors receives new stimuli compared to the steady state. Crush injury causes neutrophils to infiltrate the injury site via vascular endothelial cell interaction by TNF-α, IL-1β and IL-8 (47). Our CS rat model enhanced neutrophil-mediated inflammatory responses associated with the induction of these cytokines (Figs. 4 and 8B). SalB attenuated vascular endothelial interaction of NEU by suppressing IL-1 and TNF-α expression (Fig. 8C and D) (48,49). Nevertheless, SalB administration may have contributed to neutrophil activation and IL-8 production rather than infiltration by mildly inhibiting IL-1β and TNF-α production (50,51). Generally, IL-8, also known as CXCL8, is a proinflammatory chemokine that is produced parenchymal cells and the one produced by monocytes and macrophages. The production of IL-8 is mainly regulated by NF-κB transcription factors. IL-8 is a fundamental chemokine to promote tissue infiltration by polymorphonuclear leukocytes. And IL-8 determines in endothelial cells proangiogenic effects that include the proliferation, survival, and migration of vascular endothelial cells (52). Our rat model has characteristics of neutrophil infiltration and angiogenesis at the site of injury (27), suggested that IL-8 was involved as a factor in the progression of these conditions. Furthermore, the effect of NETosis was not observed in the SalB group (Figs. 4 and 8). Hence, the activated NEU released MPO-containing NETs (i.e., NETosis), which have bactericidal action and pathogen-capture properties via a high level of IL-8 (Fig. 8B) (53-55). Based on these effects, SalB could be a first-choice antimicrobial treatment for CS patients with or without acute kidney failure. A limitation for this study is that the CS model rats were not in a state that simulated infected patients with CS in disaster sites, and thus, further investigations are needed to elucidate the antibacterial efficacy of SalB and the antibacterial function via NET process.

In conclusion, SalB administration to the CS rat model led to a substantial improvement in survival following CS by decreasing kidney and cardiac dysfunction, inflammation, and endothelial dysfunction by improving the mitochondrial function and by antibacterial effects via NETs.

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Availability of data and materials

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

Authors' contributions

IM led the project and designed and performed most of the experiments. TS and YMu assisted with the survival and biochemical marker analyses. YMi, JK, YI and IK conceived the study, participated in its design and coordination, and helped draft the manuscript. IM, TS and YMu confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were approved by the Institutional Animal Care and Use Committee of Josai University (approval no. JU18030; Sakado, Japan).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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