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Sabiporide improves cardiovascular function and attenuates organ injury from severe sepsis

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ABSTRACT

Background: The aim of the present study was to evaluate the efficacy of orally administered sabiporide, a selective Na^+/H^+ exchanger inhibitor on whole body protection from severe sepsis in rats.

Methods: Series 1: Sepsis was induced by cecal ligation and puncture (CLP). Animals received treatment of vehicle or sabiporide (10 mg/kg, p.o.). The experiment was terminated 20 h after CLP. Series 2: At 20 h after CLP, the necrotic cecum was excised and the abdominal cavity was washed. The animals were then returned to their cages. The experiment was terminated 7 d after CLP.

Results: Series 1: Compared with vehicle treatment, administration of sabiporide prevented hemodynamic derangement and improved cardiac function as evidenced by improved arterial pressure, left ventricle systolic pressure, ±dp/dt max, ejection fraction and fractional shorting, attenuated left ventricle end-diastolic pressure elevation, and wall motion abnormality. Furthermore, administration of sabiporide attenuated intestinal mucosal hyperpermeability and reduced accumulation of abdominal ascites. In addition, treatment with sabiporide also reduced plasma levels of tumor necrosis factor-α, interleukin 6, interleukin 10, cardiac troponin, aspartate aminotransferase, alanine aminotransferase, urea, and lactate, and attenuated neutrophil infiltration in the liver and gut. Series 2: Administration of sabiporide improved the 7-day survival rate after CLP in rats (42% in vehicle group *versus* 75% in sabiporide group).

Conclusions: Administration of sabiporide improved cardiovascular performance, lessened the inflammatory response, tissue hypoperfusion and multiorgan injury, and most importantly reduced mortality.

1. Introduction

Sepsis presents a major health care problem and remains one of the leading causes of death within the intensive care unit worldwide. The pathophysiology of sepsis involves a highly complex and integrated response, including metabolic acidosis, calcium overload, the activation of various cell types, inflammatory mediators, and the hemostatic system [1–3]. The development of metabolic acidosis in patients with sepsis and septic shock is often accompanied by impairment in organ function and an increase in morbidity and mortality [4,5]. Recent studies from our laboratory and that of others indicate that the detrimental effects of tissue hypoperfusion and metabolic acidosis have largely been attributed to changes in critical protein functions arising from alterations in extracellular and intracellular pH; and that the activity of

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Na^+/H^+ exchanger (NHE1), a ubiquitous plasma membrane transporter that regulates cytoplasmic pH, is an important determinant of cellular injury [5–7]. NHE1 activation is induced by intracellular acidosis through a proton-dependent regulation pathway resulting in intracellular sodium and calcium overload and tissue injury [8,9]. NHE1 activation is also induced by oxidative stress and endogenous mediators released from hypoxic cells including various autocrine and paracrine factors, such as endothelin 1, angiotensin II, and α_1-adrenergic agonists as well as toxic agents, such as hydrogen peroxide and lysophosphatidylcholine [8,10,11]. Furthermore, there is substantial evidence indicating that NHE1 regulates inflammatory processes, and inhibition of NHE1 attenuates neutrophil activation, chemokine production, and leukocyte-endothelial cell interactions, thus, providing protection from inflammation-related tissue damage [12–14].

Hypoxia, lactic acidosis, and systemic inflammation are hallmarks of severe sepsis. Thus, a variety of intracellular and extracellular factors produced during severe sepsis may contribute to tissue dysfunction through NHE1-dependent processes. Therefore, NHE1 inhibition may provide a novel approach to attenuate tissue injury caused by severe sepsis. Consistent with this concept, Sikes *et al.* [15] examined cardiac function, intracellular Na^+ and Ca^{2+} concentrations, myocardial pH, and high-energy phosphates in perfused hearts harvested from rats with or without sepsis. They showed that induction of sepsis causes a significant increase in serum lactate levels, more than twofold increase in intracellular myocardial Na^+ and Ca^{2+} concentrations, and a drop in left ventricular pressure of ~25%. In addition, they also showed that administration of amiloride to septic animals almost completely blunts the rise in myocardial Na^+ and Ca^{2+} concentrations, substantially attenuates the decrease in left ventricular pressure, and decreases the severity of lactic acidosis [15]. The efficacy of NHE1 inhibition has not been evaluated in a clinically relevant animal model of sepsis *in vivo*. Sabiporide is a potent orally active NHE1 inhibitor [7]. The aim of the present study was to evaluate the protective effects of NHE1 inhibition with sabiporide in a clinically relevant sepsis model of cecal ligation and puncture (CLP) in rats.

2. Methods

2.1. Animals

All animal studies were approved by the Institutional Animal Care and Use Committee and complied with the Animal Welfare Act. Male Sprague–Dawley rats weighing 300–350 g were used in all experiments. The rats were housed under controlled light–dark conditions and fed with standard rat food and water *ad libitum*. All animals were observed daily for general health, and all invasive procedures were performed under aseptic conditions.

2.2. Induction of sepsis

Animals were anesthetized with ketamine (60 mg/kg, intramuscular [i.m.]) plus xylazine (10 mg/kg, i.m.). Sepsis was induced by CLP as previously described by Miksa *et al.* [16]. Briefly, a 2-cm midline abdominal incision was performed. The cecum was then exposed, ligated just distal to the ileocecal valve to avoid intestinal obstruction, punctured twice with an 18-gauge needle, and returned to the abdominal cavity. The incision was then closed in layers. Sham-operated animals underwent the same procedure with the exception that the cecum was neither ligated nor punctured. The animals were resuscitated with 3 mL/100 g body weight normal saline subcutaneously immediately after surgery. The animals were then returned to their cages.

2.3. Experimental protocol

After CLP, animals were randomly assigned to two study groups, and received treatment of vehicle (0.5% Natrosol + 0.01% TWEEN 80, $n = 8$) or sabiporide (selective NHE1 inhibitor, 10 mg/kg, oral administration, $n = 8$) at 1, 10, and 19 h after CLP. The dose was selected based on preclinical pharmacokinetic data. The experiment was terminated 20 h after CLP.

2.4. Echocardiography

Twenty hours after CLP, animals were anesthetized with ketamine (60 mg/kg, i.m.) plus xylazine (10 mg/kg, i.m.). Echocardiography was performed with a Hewlett–Packard echocardiographic system SONOS 2000 with a 7.5/5.5 MHz transducer (Hewlett-Packard Company, Palo Alto, CA). For each animal, a two-dimensional short-axis view was taken at the midpapillary muscle level to obtain left ventricular ejection fraction. Linear dimensions were measured from two-dimensionally guided M-mode tracing, and fractional shortening was obtained. An electrocardiography tracing was recorded simultaneously with the echocardiogram. Wall motion score index was the sum of wall motion scores divided by the number of visualized segments. In this scoring system, higher scores indicate more severe wall motion abnormalities: 1 = normal, 2 = hypokinesis, 3 = akinesis, 4 = dyskinesis, and 5 = aneurysm [17]. All measurements were repeated three times, and the results represent the average of these measurements.

2.5. Hemodynamic assessment and tissue harvest

After echocardiography analysis, the left external jugular vein was cannulated for right atrial pressure measurement. A catheter was inserted into the right common carotid artery for quantifying arterial blood pressure with a Powerlab data acquisition system (ADInstruments Inc, CO). Heart rate was derived from the blood pressure signal. After arterial blood pressure was measured, the catheter was introduced into the left ventricle through the right carotid artery to monitor left ventricular pressure (LVP) and its first derivative (±dp/dp max). At the end of the experiment, blood samples were collected and centrifuged. Plasma samples were stored at −80°C until assayed. Abdominal ascites were also collected and volumes were determined.

Liver and intestinal tissues were collected for immediate assay or snap-frozen until later analysis.

2.6. Determination of intestinal mucosal permeability

Small intestinal mucosal barrier function was assessed by using the *ex vivo* isolated ileum sac as previously described [18]. Briefly, 20 h after CLP or sham operation, isolated ileum sacs were prepared in an ice-cold modified Krebs-Henseleit bicarbonate buffer (KHBB, pH 7.4). Fluorescein isothiocyanate–dextran with a molecular weight of 4000 Da (FD4; Sigma, St. Louis, MO) was used as a permeability probe. The ileal segment, one end closed with silk ligature, was filled with 1.5 mL of KHBB and subsequently suspended in a glass beaker filled with 80 mL of KHBB (containing FD4 [20 μg/mL]) at 37°C and gassed with 95% O_2 and 5% CO_2. The beaker was incubated for 30 min in a shaker bath at a frequency of 80–100 shakes/min. After incubation, the fluid was aspirated from the inside of the ileum sac to determine FD4 concentrations in the serosal side. The length and diameter of the ileum sac were then measured. FD4 concentrations in the serosal side of the ileum as well as the initial and final levels in the glass beaker were determined by fluorescence measurements at an excitation wavelength of 480 nm and an emission wavelength of 520 nm. Intestinal mucosal permeability was expressed as the mucosal-to-serosal clearance of FD4 as previously described [18].

2.7. Biochemical assay

Enzyme immunoassay kits for cardiac troponin I (Life Diagnostics, Inc, West Chester, PA), tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-10 (R & D Systems, Minneapolis, MN) were used to determine the concentrations of these mediators in the plasma. Plasma levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST; Biotron Diagnostics, Hemet, CA) and plasma levels of urea (Bioassay System, Hayward, CA) and lactate (Sigma) were determined by using assay kits according to the manufacturer's instructions. Neutrophil accumulation in the liver and gut was measured by determining myeloperoxidase (MPO) activity according to previously published methods [19,20].

2.8. Survival study

Additional groups of animals were used to evaluate the effect of sabiporide on survival after CLP-induced severe sepsis. Animal preparation was the same as described for the first series of experiments with the following exceptions. Twenty hours after CLP, the necrotic cecum was excised and the abdominal cavity was washed twice with 40 mL of warm, sterilized normal saline solution. The abdominal incision was closed in layers. The cecal excision procedure was performed in CLP animals to mimic clinical situations in which the septic focus is removed whenever possible [18]. The animals were then returned to their cages and allowed food and water. One hour after CLP, animals received daily treatments of vehicle (0.5% Natrosol + 0.01% TWEEN 80) or sabiporide (10 mg/kg, oral administration, twice daily). The experiment was terminated at day 8.

2.9. Statistical analysis

Statistical analysis was undertaken using SPSS Statistics 17.0 (IBM SPSS, Chicago, IL). Analysis of variance was used to assess an overall difference among the groups for each of the variables. Levene test for equality of variance was used to suggest the multiple comparison procedure to be used. If equality of variance among the groups was suggested, multiple comparison procedures were performed (Bonferroni); if inequality of variance was suggested by Levene test, Tamhane multiple comparisons (which do not assume equal variance in each group) were performed [15]. P values <0.05 were considered to indicate statistically significant differences. Survival rates were estimated by the Kaplan-Meier method (GraphPad Prism 5.0, GraphPad Software, Inc., La Jolla, CA).

3. Results

3.1. Hemodynamic and myocardial performance

In the present study, sepsis induced by CLP resulted in a severe impairment of the indices of cardiovascular performance (Table 1, Fig. 1A–D). In vehicle-treated control animals, CLP resulted in decreased mean arterial pressure, left ventricle systolic pressure and ±dp/dt max, and elevated left ventricle end-diastolic pressure. In contrast, sabiporide treatment significantly improved mean arterial pressure, left ventricle systolic pressure, and ±dp/dt max, and attenuated the elevation of left ventricle end-diastolic pressure (Fig. 1A–D).

Echocardiography analysis showed that CLP resulted in impaired left ventricular function as evidenced by reduced ejection fraction and fractional shorting and impaired wall motion in vehicle-treated control animals. The sabiporide treatment group exhibited improvements in all three parameters (Table 1).

3.2. Intestinal mucosal permeability and abdominal ascites accumulation

Intestinal mucosal permeability to the fluorescent macromolecule FD4 was significantly increased 20 h after CLP in vehicle-treated animals as compared with sham controls (Fig. 2A). Similarly, abdominal ascites were not present in sham animals, but extensive accumulation of abdominal ascites was seen in the CLP vehicle-treated group (Fig. 2B). However, treatment with sabiporide significantly attenuated the hyperpermeability

Table 1 – Echocardiographic data of left ventricular function in sham-operated animals (Control) and septic animals treated with saline (Vehicle) or sabiporide 20 h after CLP in rats.

	Control	Vehicle	Sabiporide
Ejection fraction (%)	70.3 ± 4.2	41.7 ± 4.5*	58.4 ± 3.6*,†
Fractional shortening (%)	51.3 ± 3.4	34.6 ± 3.8*	42.8 ± 4.1*,†
Wall motion score index	1.0 ± 0.0	1.8 ± 0.16*	1.0 ± 0.0*,†

All values are mean ± SD, $n = 8$.
* $P < 0.05$ *versus* the sham control group.
† $P < 0.05$ *versus* the vehicle group.

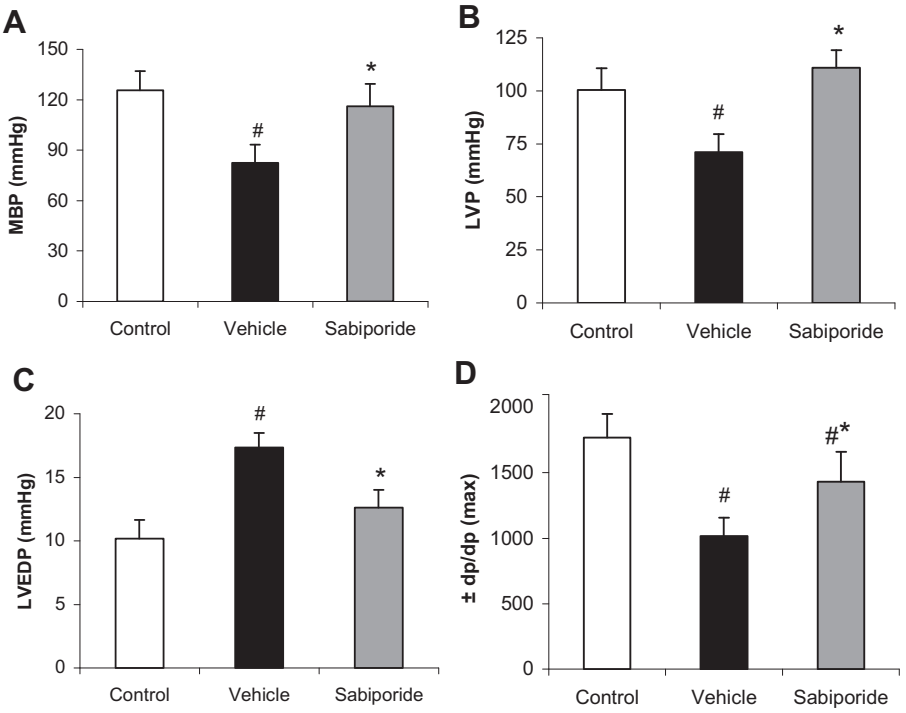

Fig. 1 – Hemodynamic parameters in sham-operated animals (Control) and septic animals treated with saline (Vehicle) or sabiporide 20 h after CLP in rats. All values are mean ± SD, $n = 8$. *$P < 0.05$ *versus* vehicle; #$P < 0.05$ *versus* control. MBP = mean arterial blood pressure; LVP = left ventricular systolic pressure; LVEDP = left ventricle end-diastolic pressure; ±dp/dt max = first derivative of left ventricular pressure.

of intestinal mucosa and reduced accumulation of abdominal ascites (Fig. 2A and B, respectively).

3.3. *Biomarkers for proinflammatory response and organ injury*

Sepsis induced by CLP resulted in a significant inflammatory response. In animals receiving vehicle alone, plasma levels of TNF-α, IL-6, and IL-10 were significantly increased, and there was a significant accumulation of neutrophils in the liver and gut tissues (Fig. 3A and B, Table 2). Treatment with sabiporide significantly reduced plasma levels of TNF-α, IL-6, and IL-10 and attenuated neutrophil infiltration in the liver and gut tissues, as measured by MPO activity (Fig. 3A and B, Table 2).

Plasma levels of cardiac-specific troponin-I were significantly increased 20 h after CLP, indicating myocardial damage. However, release of troponin-I was reduced in animals receiving sabiporide treatment (Table 2). There was also a marked increase in plasma levels of ALT and AST (markers of liver injury), indicating liver injury. Treatment with sabiporide significantly reduced plasma levels of ALT by 36% and decreased AST levels by 27%. We assessed renal dysfunction by measuring the rise in plasma levels of urea (an indicator of impaired excretory function of the kidney or increased catabolism). Plasma levels of urea were significantly elevated after sepsis, and sabiporide treatment reduced urea levels by 34% (Table 2). There was an excessive increase in plasma levels of lactate (a marker for tissue hypoxia) in vehicle-treated rats 20 h after CLP. In contrast, plasma levels of lactate were significantly decreased (by 45%) in rats receiving sabiporide (Table 2).

3.4. *Survival*

CLP and cecal excision led to the death of 58% (7/12) of the vehicle-treated animals, compared with 25% (3/12) for sabiporide-treated animals. No further death occurred after day 4 (Fig. 4). Furthermore, hemodynamic parameters, cardiac performance, and intestinal mucosal permeability were normalized 7 d after CLP in all surviving animals (data not shown). This is in agreement with clinical observations that cardiac function in survivors gradually returns to normal by 10 d after the onset of septic shock [21,22].

4. Discussion

Severe sepsis is among the most common causes of death in intensive care units worldwide. It causes high morbidity, mortality, and social and economic costs. This study was undertaken to demonstrate that pharmacological inhibition of the ubiquitously expressed NHE1 in a clinically relevant rat sepsis model of CLP provides a novel approach for the treatment of severe sepsis. We found that oral administration of sabiporide (a potent NHE1 selective inhibitor) prevented hemodynamic derangement by improving cardiac performance. In addition, sabiporide treatment prevented multiple organ injury by reducing proinflammatory cytokine production and

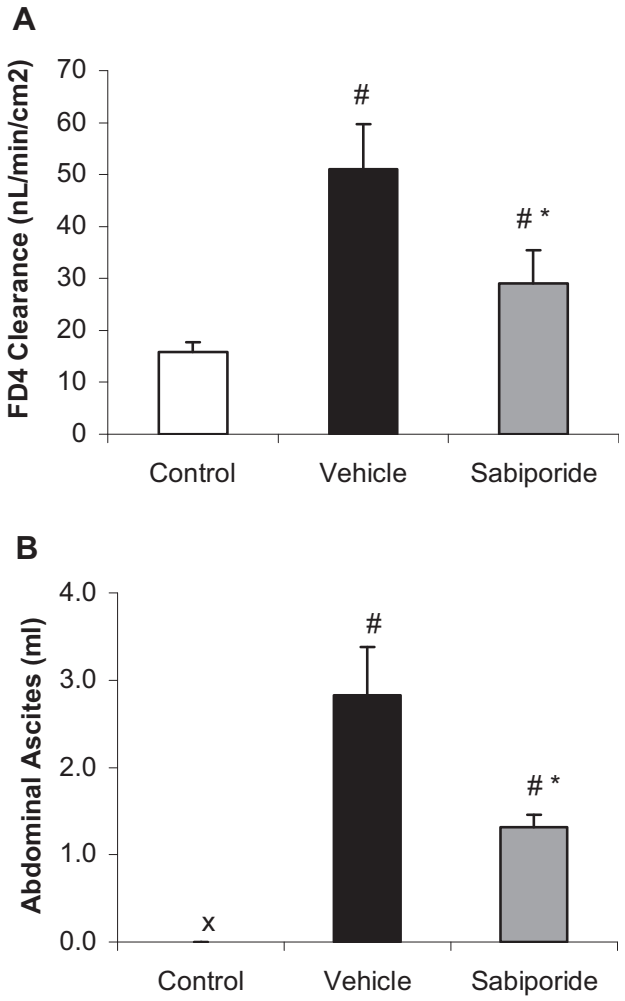

Fig. 2 – Permeability of the intestinal mucosal to the fluorescent macromolecule FD4 and accumulation of abdominal ascites in sham-operated animals (Control) and septic animals treated with saline (Vehicle) or sabiporide 20 h after CLP in rats. All values are mean ± SD, $n = 8$. *$P < 0.05$ *versus* vehicle; #$P < 0.05$ *versus* control.

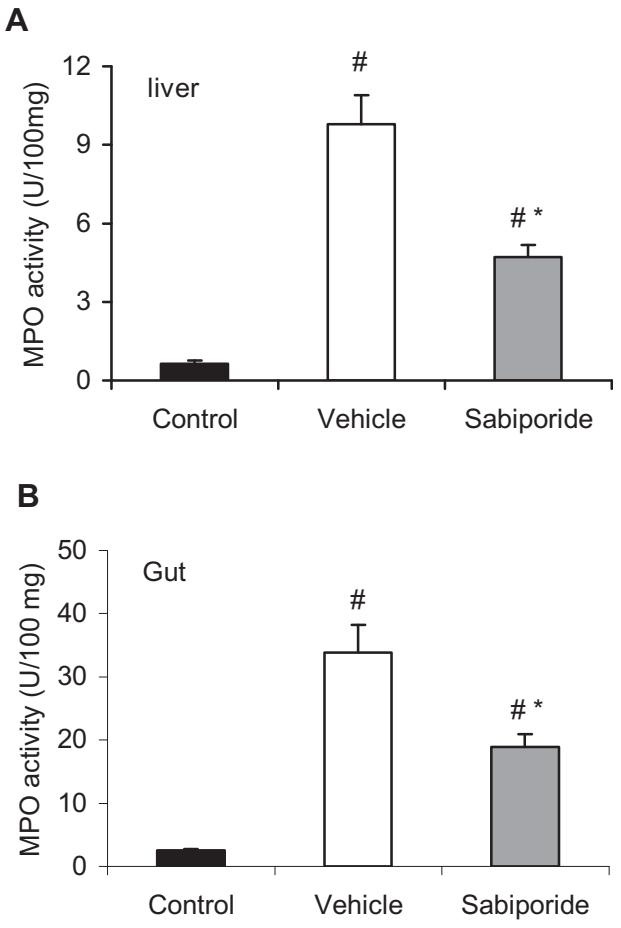

Fig. 3 – MPO activity in liver and gut tissues in sham-operated animals (Control) and septic animals treated with saline (Vehicle) or sabiporide 20 h after CLP in rats. All values are mean ± SD, $n = 8$. *$P < 0.05$ *versus* vehicle; #$P < 0.05$ *versus* control.

neutrophil infiltration, thereby, reducing systemic inflammation, which resulted in improved survival.

4.1. *Sabiporide ameliorates hemodynamic derangement and cardiac dysfunction in sepsis*

Hemodynamic derangement and cardiac dysfunction are frequent complications associated with severe sepsis and important factors contributing to the high mortality associated with multiorgan failure [23]. Whether progressive cardiovascular dysfunction is a principal cause of the rapid deterioration of irreversible septic shock or merely a final step in a decompensating subject remains unclear [22]. Similarly, factors contributing to cardiovascular dysfunction in sepsis have not been completely defined. However, tissue hypoperfusion/hypoxia is the hallmark of severe sepsis. The metabolic consequences of insufficient tissue perfusion are anaerobic glycolysis with increased production of lactate and hydrogen ions, acidosis, impaired mitochondrial energy production, disturbed ionic homeostasis across cell membranes, and reduced functional capacity of tissue cells [24]. Therefore, interventions that ameliorate ischemia–reperfusion injury represent a possible strategy to improve outcomes from severe sepsis.

It is well established that NHE1 activation during ischemia–reperfusion through the pH-regulatory pathway and other pathways mediated by endogenous ischemia metabolites results in increased intracellular Na^+ and Ca^{2+}, leading to myocardial damage [8,10]. NHE1 inhibitors have been shown to protect the myocardium against ischemia–reperfusion damage [9,12,15]. Thus, a major goal of the present study was to determine the effects of sabiporide on attenuating sepsis-induced hemodynamic derangement and cardiac dysfunction in rats. Our results show that severe sepsis had developed by 20 h after CLP, as evidenced by severe tissue hypoperfusion from excessively increased plasma lactate levels. Severe sepsis also resulted in impaired hemodynamic parameters and cardiac performance as measured by vascular and cardiac catheterization and echo analysis. However, administration of sabiporide after CLP prevented hemodynamic derangement

Table 2 – Plasma levels of TNF-α, IL-6, IL-10, troponin I, AST, AST, urea, and lactate in sham-operated animals (Control) and septic animals treated with saline (Vehicle) or sabiporide 20 h after CLP in rats.

	Control	Vehicle	Sabiporide
TNF-α (pg/mL)	5.3 ± 1.4	43.7 ± 5.5*	25.4 ± 3.6*,†
IL-6 (ng/mL)	0.04 ± 0.01	1.93 ± 0.32*	0.96 ± 0.15*,†
IL-10 (pg/mL)	44.5 ± 5.2	279.2 ± 39.9*	195.7 ± 26.7*,†
Troponin I (ng/mL)	1.37 ± 0.18	8.9 ± 1.1*	4.3 ± 0.5*,†
AST (U/L)	97.2 ± 21.5	428.8 ± 51.2*	311.8 ± 57.1*,†
ALT (U/L)	13.5 ± 2.5	58.2 ± 9.4*	37.4 ± 6.8*,†
Urea (mg/dL)	58.9 ± 6.2	225.3 ± 33.6*	149.8 ± 21.2*,†
Lactate (mmol/L)	1.48 ± 0.12	4.87 ± 0.25*	2.35 ± 0.17*,†

All values are mean ± SD, $n = 6–8$.
* $P < 0.05$ *versus* the sham control group.
† $P < 0.05$ *versus* the vehicle group.

and improved cardiac function. The improvement in cardiac function was accompanied by a reduction in the release of cardiac troponin-I, indicating that sepsis-induced myocardial damage can be attenuated by NHE1 inhibition with sabiporide. Furthermore, the improvement in hemodynamic and cardiac performance also resulted in improved tissue perfusion as evidenced by a significant reduction in tissue lactate production in animals receiving sabiporide. Thus, the evidence of less myocardial and liver damage is also consistent with improved tissue perfusion resulting from sabiporide treatment.

4.2. *NHE1 inhibition reduces systemic inflammation and prevents multiple organ injury*

Systemic inflammatory response is a hallmark of sepsis and is mediated by innate immune cells, including neutrophils, monocytes, and macrophages [25]. In severe sepsis, the excessive and prolonged production of proinflammatory cytokines causes capillary leakage, tissue injury, multiple organ failure, and death [25–27]. Studies have shown that elevated proinflammatory cytokine levels directly correlate with severity and mortality in human sepsis [27]. There is substantial evidence indicating that NHE1 regulates inflammatory processes. NHE1 regulates the inflammatory functions of various cell types, including endothelial cells, monocytes, macrophages, and neutrophils [12,28,29]. NHE1 is rapidly activated in response to a variety of inflammatory signals, such as IL-1, TNF-α, interferon-γ, and lipopolysaccharide [30,31]. Increased activity of NHE1 contributes to the production of cytokines in macrophages [29]. NHE1 inhibitors have been shown to inhibit neutrophil accumulation, chemokine production and nuclear factor-kappa κB activation, attenuate leukocyte–endothelial cell interactions, and improve endothelial dysfunction induced by regional or whole body ischemic–reperfusion [28,32,33]. These observations suggest a pathophysiological role of NHE1 activation in inflammation-related tissue damage. In the present study, administration of sabiporide significantly reduced proinflammatory cytokine production and accumulation of tissue neutrophils and abdominal ascites, and attenuated intestinal mucosal hyperpermeability and multiple organ injury as evidenced by reductions in plasma levels of cardiac troponin-I, ALT, AST, and urea; this resulted in improved survival. Our results further support the hypothesis that NHE1 regulates inflammatory processes *in vivo* in sepsis.

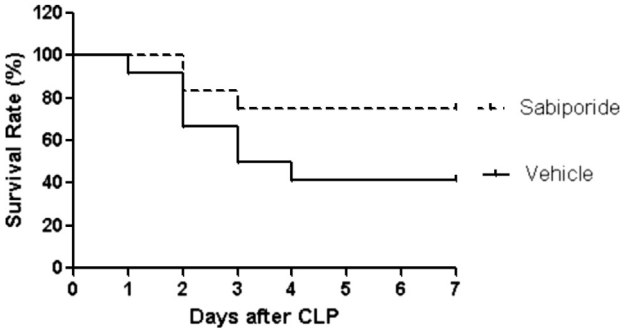

Fig. 4 – Survival rates 7 d after CLP in septic rats treated with saline (Vehicle) *versus* sabiporide; P = 0.10 *versus* vehicle.

It is worth noting that NHE1 is ubiquitously expressed in all mammalian cells, and is the predominant isoform in cardiomyocytes, red blood cells, neutrophils, and neurons [34–36]. Previous studies have shown that NHE1 inhibition reduces tissue damage in brain, heart, lung, and vascular tissues in regional ischemia–reperfusion injury [10,12,36–38]. Thus, our demonstration of the multiorgan protective actions of NHE1 inhibition in a setting of whole body tissue hypoperfusion and ischemia injury in sepsis support the hypothesis that NHE1 is a novel therapeutic target for whole body protection from systemic ischemia injury.

5. Conclusions

In summary, data from the present study demonstrate that oral administration of sabiporide can improve cardiovascular performance, lessen the inflammatory response, prevent or lessen tissue hypoperfusion and multiorgan injury, and reduce mortality. Given the lack of effective therapies for the treatment of severe sepsis, these observations support the use of NHE1 inhibitor as an adjunct therapy to improve cardiovascular and multiorgan function in patients with severe sepsis.

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