Suppressive activity of RGX-365 on HMGB1-mediated septic responses

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RGX-365 is the main fraction of black ginseng conmprising protopanaxatriol (PPT)-type rare ginsenosides (ginsenosides Rg4, Rg6, Rh4, Rh1, and Rg2). No studies on the antiseptic activity of RGX-365 have been reported. High mobility group box 1 (HMGB1) is recognized as a late mediator of sepsis, and the inhibition of HMGB1 release and recovery of vascular barrier integrity have emerged as attractive therapeutic strategies for the management of sepsis. In this study, we examined the effects of RGX-365 on HMGB1-mediated septic responses and survival rate in a mouse sepsis model. RGX-365 was administered to the mice after HMGB1 challenge. The antiseptic activity of RGX-365 was assessed based on the production of HMGB1, measurement of permeability, and septic mouse mortality using a cecal ligation and puncture (CLP)-induced sepsis mouse model and HMGB1-activated human umbilical vein endothelial cells (HUVECs). We found that RGX-365 significantly reduced HMGB1 release from LPS-activated HUVECs and CLP-induced release of HMGB1 in mice. RGX-365 also restored HMGB1-mediated vascular disruption and inhibited hyperpermeability in the mice. In addition, treatment with RGX-365 reduced sepsis-related mortality in vivo. Our results suggest that RGX-365 reduces HMGB1 release and septic mortality in vivo, indicating that it is useful in the treatment of sepsis.

Keywords: RGX-365. Permeability. HMGB1. Sepsis.

INTRODUCTION

Sepsis is an overwhelming systematic inflammatory response to severe infections and continues to be a common cause of morbidity and mortality despite recent advances in antibiotic therapy and intensive care (Russell, 2006). The pathogenesis of sepsis is rather complex, but is partly mediated by endotoxin, which stimulates macrophages/monocytes to sequentially release early (e.g., tumor necrosis factor (TNF)- a and interleukin (IL)-1b) and late (e.g., high mobility group box 1 (HMGB1)) proinflammatory mediators (Abraham et al., 2000; Riedemann et al., 2003). In animal models of endotoxemia or sepsis, circulating HMGB1 increases to

plateau levels between 24 and 36 h, and is distinguishable from TNF and other early cytokines (Bae, 2012; Wang et al., 1999). Further, HMGB1-neutralizing antibodies confer protection against lethal endotoxemia and sepsis even when administered 24 h after the onset of sepsis, suggesting that HMGB1 is a critically important late mediator of lethal sepsis (Bae, 2012; Wang et al., 1999; Yang et al., 2004). Thus, therapeutic agents capable of inhibiting HMGB1 release might have potential in the treatment of lethal systemic inflammatory diseases.

Ginseng (Panax ginseng C.A. Meyer, Araliaceae) is one of the most popular herbs in Oriental medicine. Recently, black ginseng (BG), a ginseng preparation steamed nine times and dried, has received attention from scientists because of its bioactive rare ginsenosides with anti-diabetic, wound healing, immune-stimulatory, and anti-antioxidant properties (Kang et al., 2017; Park et al., 2018; Saba et al., 2018). It is well known that the major protopanaxadiol (PPD)-type rare ginsenosides in BG are Rg3, Rk1, and Rg5, and the major protopanaxatriol (PPT)-type rare ginsenosides in BG are Rg4, Rg6, Rh4, Rh1, and Rg2 (Sun et al., 2009a). In general, during BG production, the ginsenosides are structurally transformed into low-polarity rare ginsenosides via hydrolysis, isomerization, and dehydration at C-20; hydrolysis also occurs at C-3 or C-6. Recently, it was reported that the major PPT-type ginsenosides in ginseng such as Rg1 and Re successfully rescued mice from sepsis-associated death (Li et al., 2017; Su et al., 2015). In addition, in our recent studies, the PPD-type rare ginsenoside Rk1 and Rg5 were found to play an active role in suppression of HMGB1-mediated septic responses (Kim et al., 2019). However, to the best of our knowledge, the protective effect of the PPT-type rare ginsenosides on HMGB1-mediated septic responses has not been reported so far. The octanol/water partition coefficient (log P) of a molecule reflects its ability to pass through the lipid bilayer (Walter, Gutknecht, 1986). Because log P of the low-polarity ginsenoside Rg1 (Molecular weight; 800.49) is 1.12 and log P of the high-polarity ginsenoside Re (Molecular weight; 946.55) is −0.03, ginsenoside Rg1 can enter cells but ginsenoside Re cannot (Su et al., 2015). Therefore, it is of great interest to transform the ginsenoside Re into the low-polarity PPT-type rare ginsenosides Rg4, Rg6, Rh4, Rh1, and Rg2 and investigate their effect on HMGB1-mediated septic responses.

MATERIAL AND METHODS

Reagents

Bacterial lipopolysaccharide (LPS, serotype: 0111:B4, L5293), Evans blue, crystal violet, 2-mercaptoethanol, and antibiotics (penicillin G and streptomycin) were purchased from Sigma (St. Louis, MO). Human recombinant HMGB1 was purchased from Abnova (Taipei City, Taiwan). Fetal bovine serum and Vybrant DiD were purchased from Invitrogen (Carlsbad, CA).

Preparation of RGX-365

RGX-365, the PPT-type rare ginsenoside fraction of black ginseng (ginsenoside Rg4, Rg6, Rh4, Rh1, and Rg2), was prepared from ginsenoside Re using a previously reported method (Lee et al., 2015). Purified ginsenoside Re (1 g) in water (1.5 mL) was steamed at 120 °C for 6 h in an autoclave. The steamed ginsenoside Re fraction was dissolved in water (1 L) and resolved via chromatography on a Diaion HP-20 resin (1 kg) eluting with ethanol in water (0–80 %) to obtain 0.635 g of RGX-365 containing PPT-type rare ginsenosides (Rg2, Rg4, Rg6, Rh1, and Rh4, Figure 1, Table I). The amount of each rare ginsenoside in RGX-365 was analyzed via HPLC. HPLC analysis was carried out on an Agilent Technologies 1260 infinity UV visible spectrometer using an ACE 5-C_{18} column (250 x 4.6 mm) at 40 °C. A binary gradient elution system consisting of water (A) and acetonitrile (B) was used. Separation was achieved using the following gradient program; 0~3 min (20% B), 3~15 min (23 % B), 15~20 min (33 % B), 20~45 min (40 % B), 45~60 min (68 % B), 60~65 min (85 % B), 65~70min (85 % B), 70~73min (20 % B), and 73~75 min (20 % B). The solvent flow rate was held constant at 1 ml/min and the sample injection volume was 10 ml. The HPLC data of RGX-365 are presented in figure 2.
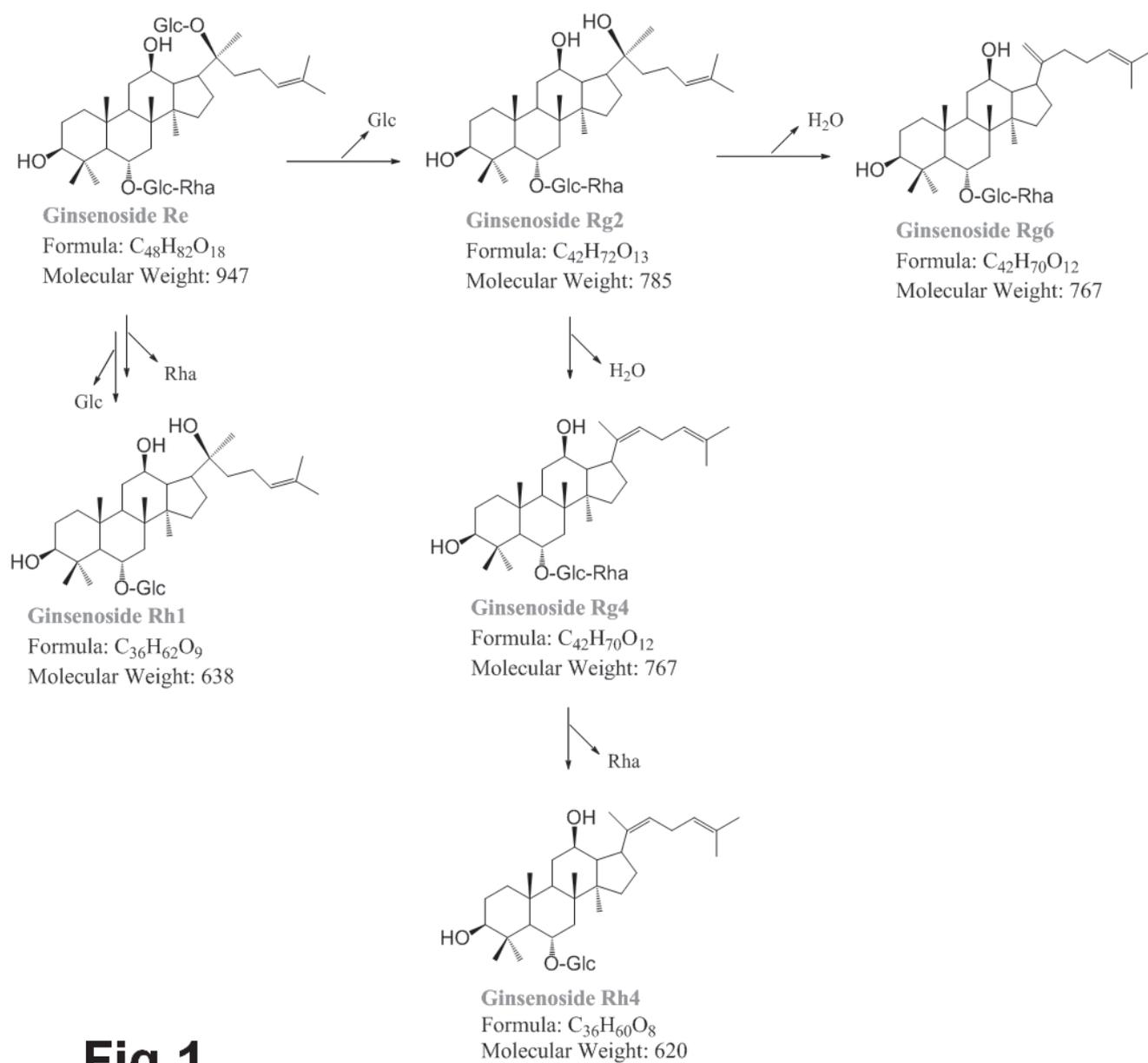
Fig 1

FIGURE 1 - Transformation of ginsenoside Re into the PPT-type rare ginsenoside fraction RGX-365.
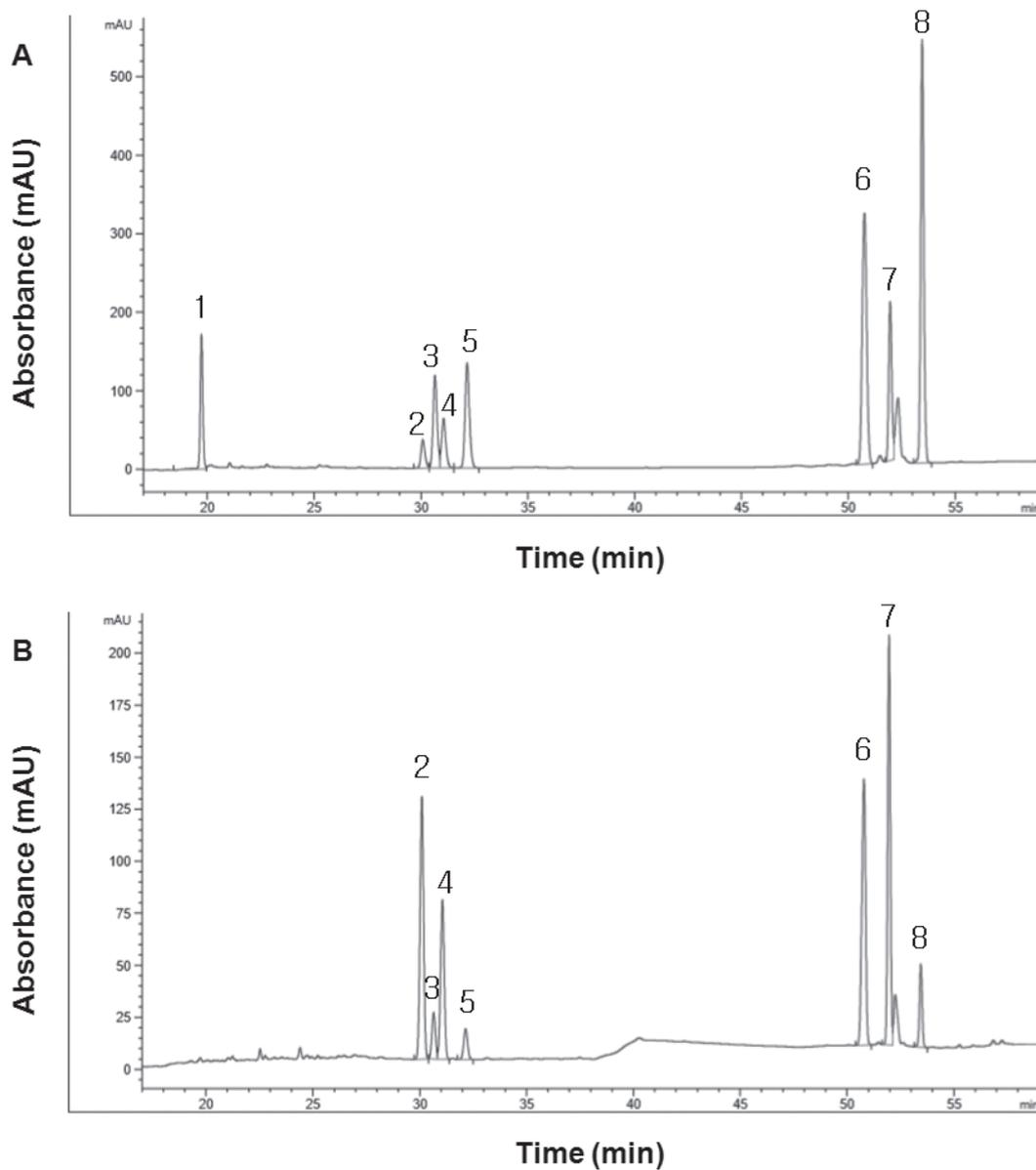
FIGURE 2 - HPLC chromatograms of mixed standards **(A)** and rare ginsenosides in RGX-365 **(B).** Each rare ginsenoside was analyzed using an HPLC system fitted with a C-18 column utilizing a solvent gradient system. Identification of each rare ginsenoside was performed via comparison of retention times with standard ginsenosides. Peaks: 1, Re; 2, 20(*S*)-Rg2; 3, 20(*S*)-Rh1; 4, 20(*R*)-Rg2; 5, 20(*R*)-Rh1; 6, Rg6; 7, Rg4; 8, Rh4.

TABLE I - The amounts of rare ginsenosides in RGX365

Rare ginsenoside	Amounts (mg)
(S)-Rg2	175.7
(S)-Rh1	21.2
(R)-Rg2	88.5
(R)-Rh1	6.9
Rg6	91.3
Rg4	227.7
Rh4	7.8

Cell culture

Primary human umbilical vein endothelial cells (HUVECs) were obtained from Cambrex Bio Science (Charles City, IA) and maintained using a previously described method (Lee *et al.*, 2017b; Lee *et al.*, 2017c). HUVECs were used in cell culture at passages 3–5.

Animals and husbandry

Male C57BL/6 mice (6–7 weeks old, 27 g) purchased from Orient Bio Co. (Sungnam, Republic of Korea) were used after a 12-d acclimatization period. The animals (five per polycarbonate cage) were housed under controlled temperature (20–25°C) and humidity (40%–45% RH), with a 12:12 h light/dark cycle. They received a normal rodent pellet diet and water ad libitum during the acclimatization. All animals were treated in accordance with the 'Guidelines for the Care and Use of Laboratory Animals' issued by Kyungpook National University (IRB No. KNU 2017-102).

Cecal ligation and puncture (CLP)

To induce sepsis, male mice were anesthetized with Zoletil (tiletamine and zolazepam, 1:1 mixture, 30 mg/kg) and Rompum (xylazine, 10 mg/kg). The CLP-induced sepsis model was prepared as previously described (Lee *et al.*, 2017a). In brief, a 2-cm midline incision was made to expose the cecum and adjoining intestine. The cecum was then tightly ligated with a 3.0 silk suture 5.0-mm from the cecal tip and punctured once using a 22-gauge needle to induce high grade sepsis. The cecum was then gently squeezed to extrude a small amount of feces from the perforation site and returned to the peritoneal cavity. The laparotomy site was then sutured with 4.0 silk. In sham control animals, the cecum was exposed but not ligated, manipulated, squeezed or punctured, and was then returned to the abdominal cavity. This protocol was approved by the Animal Care Committee at Kyungpook National University prior to conducting the study (IRB No. KNU 2017-102).

Competitive enzyme-linked immunosorbent assay (ELISA) of HMGB1

A competitive ELISA was performed as previously described to determine the HMGB1 concentrations in cell culture medium or mouse serum (Lee *et al.*, 2017a). HUVEC monolayers were treated with LPS (100 ng/mL) for 16 h, followed by RGX-365 for 6 h. Cell culture media was collected for the determination of HMGB1. To perform the ELISA, 96-well flat plastic microtiter plates (Corning, NY) were coated with HMGB1 protein in 20 mM carbonate-bicarbonate buffer (pH 9.6) containing 0.02% sodium azide overnight at 4°C. The plates were then rinsed three times in PBS with 0.05% Tween 20 (PBST) and kept at 4°C. Lyophilized culture medium was pre-incubated with an anti-HMGB1 antibody (Abnova, diluted 1:1000 in PBST) in 96-well plastic round-bottom microtiter plates for 90 min at 37°C, transferred to the pre-coated plates, and incubated for 30 min at room temperature. The plates were then rinsed three times in PBST, incubated for 90 min at room temperature with peroxidase-conjugated anti-rabbit IgG antibodies (diluted 1:2000 in PBS-T, Amersham Pharmacia Biotech), rinsed three times with PBST, and incubated for 60 min at room temperature in the dark with 200 µL of substrate solution (100 µg/mL o-phenylenediamine and 0.003% H_2O_2). After stopping the reaction with 50 µL of 8 N H_2SO_4, absorbance was measured at 490 nm.

Cell viability assay

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) was used as an indicator

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of cell viability. Cells were grown in 96-well plates at a density of 5×10^3 cells/well. After 24 h, the cells were washed with fresh medium, followed by treatment with RGX-365. After a 48-h incubation period, the cells were washed and 100 µL of MTT (1 mg/mL) was added, followed by incubation for 4 h. Finally, dimethyl sulfoxide (DMSO, 150 µL) was added to solubilize the formazan salt formed. The amount of formazan salt was determined by measuring the OD at 540 nm using a microplate reader (Tecan Austria GmbH, Austria).

In vitro permeability assay

For the spectrophotometric quantification of endothelial cell permeability in response to increasing concentrations of each compound, the flux of Evans blue-bound albumin across functional cell monolayers was measured using a modified two-compartment chamber model, as previously described (Lee *et al.*, 2017a). HUVECs were plated (5×10^4 cells/well) in transwells with a pore size of 3 µm and a diameter of 12 mm for three days. Confluent monolayers of HUVECs were treated with LPS (100 ng/mL) for 4 h or HMGB1 (1 µg/mL) for 16 h, followed by treatment with RGX-365. The transwell inserts were then washed with PBS (pH 7.4), and growth medium containing 0.5 mL of Evans blue (0.67 mg/mL) and 4% BSA was added. Fresh growth medium was then added to the lower chamber, and the medium in the upper chamber was replaced with Evans blue/BSA. Ten minutes later, optical density in the lower chamber was measured at 650 nm.

In vivo permeability assay

For the *in vivo* study, male mice were anesthetized with 2% isoflurane (Forane, JW Pharmaceutical, South Korea) in oxygen delivered via a small rodent gas anesthesia machine (RC2, Vetequip, Pleasanton, CA), first in a breathing chamber and then via facemask. Mice were allowed to breath spontaneously during the procedure. Mice were treated with HMGB1 (2 µg/mouse, i.v.) for 16 h, followed by treatment with RGX-365 (7 or 15 mg/kg, *i.v.*). For the *in vivo* permeability assay, after 6 h, 1% Evans blue dye solution in normal saline was injected intravenously into each mouse. Vascular permeability was expressed as µg of dye in the peritoneal cavity/mouse, and determined using a standard curve as previously described.

ELISA of phosphorylated p38 mitogen-activated protein kinase (MAPK)

The amount of phosphorylated p38 MAPK was quantified following the manufacturer's instructions using a commercially available ELISA kit (Cell Signaling Technology, Danvers, MA). Absorbance values were measured using an ELISA plate reader (Tecan, Austria GmbH, Austria).

Statistical Analysis

All experiments were performed independently at least three times. Values were expressed as means ± standard deviation (SD). The statistical significance of differences between test groups was evaluated using SPSS for Windows, version 16.0 (SPSS, Chicago, IL). Statistical relevance was determined via one-way analysis of variance (ANOVA) and Tukey's post-hoc test. P values less than 0.05 were considered to indicate significance.

RESULTS AND DISCUSSION

Effects of RGX-365 on LPS- and CLP-mediated release of HMGB1

The HMGB1 protein secreted by activated immune cells and damaged cells has been shown to function as a sepsis mediator (Bae, 2012), and LPS is used as research tool for inducing severe vascular inflammation in animal or cell studies (Buras et al., 2005). Our results showed that LPS significantly stimulated HMGB1 secretion, which, in turn, was inhibited by RGX-365 (Figure 3A). To confirm the inhibitory effects of RGX-365 on HMGB1 release in vivo, RGX-365 was intravenously injected into mice 12 h after CLP surgery. RGX-365 was found to significantly reduce CLP-induced HMGB1 secretion (Figure 3B). In addition, to determine the toxicity of RGX-365, a cellular viability assay was performed using

MTT in HUVECs. RGX-365 did not affect the viability of cells treated with concentrations up to 400 µM for 48 h (Figure 3C). Taken together, these results indicate that RGX-365 may be a viable early intervention to prevent the release of HMGB1 and progression to severe sepsis and septic shock.
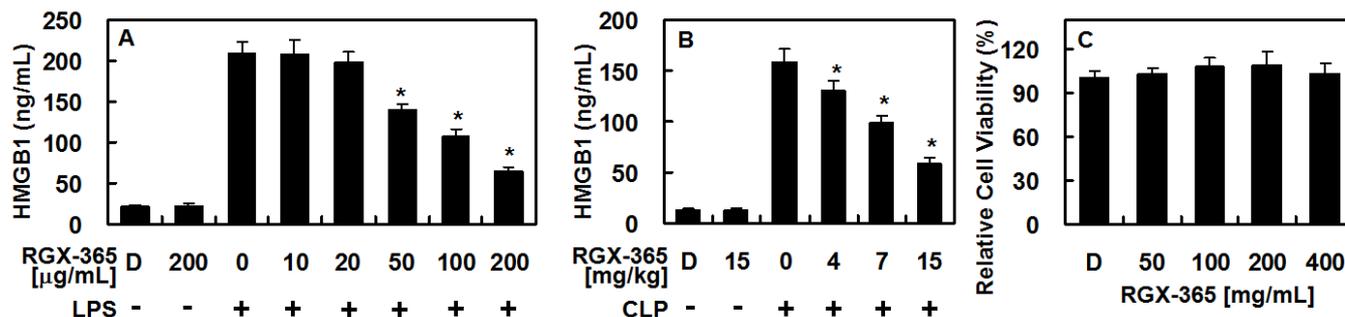
FIGURE 3 - Effects of RGX-365 on HMGB1 release. A, HUVECs were treated with the indicated concentrations of RGX-365 for 6 h, after stimulation with LPS (100 ng/mL, 16 h), and HMGB1 release was measured using ELISA. B, Male C57BL/6 mice that underwent CLP were administered the indicated concentration of RGX-365 (intravenously 12 h after CLP, n = 5). The mice were euthanized 24 h after CLP. Serum HMGB1 levels were measured via ELISA. C, Effect of RGX-365 on cellular viability was measured using MTT assay. The results shown are mean ± SD of three separate experiments in different days with triplicate wells. D represents 0.2% DMSO, the vehicle control. *$p < 0.05$ versus LPS alone (A), CLP alone (B).

Effect of RGX-365 on HMGB1-mediated vascular barrier disruption

Because HMGB1 and LPS are known to destroy the integrity of the vascular barrier (Lee *et al.*, 2014), we analyzed vascular permeability to evaluate the effect of RGX-365 on maintaining barrier consistency of HUVECs. HUVECs were activated with LPS (Figure 4A; 100 ng/mL) or HMGB1 (Figure 4B; 1 µg/mL) and then treated with RGX-365 for 16 h. Our data showed that the LPS- and HMGB1-mediated hyperpermeability was inhibited by RGX-365 (Figs. 4A and 4B). This barrier-protective effect of RGX-365 was confirmed in the mouse model (Figure 4C). Previous reports indicated that the vascular destruction response caused by HMGB1 occurs via the activation of p38 MAPK (Palumbo *et al.*, 2007; Qin *et al.*, 2009; Sun *et al.*, 2009b). Therefore, we determined the effects of RGX-365 on the activation of p38. Our data showed that the activation of p38 was increased by HMGB1, which was reduced by RGX-365 (Figure 4D). The reduction in HMGB1-mediated hyperpermeability and p38 activation indicated a potential role for RGX-365 as an anti-septic agent.
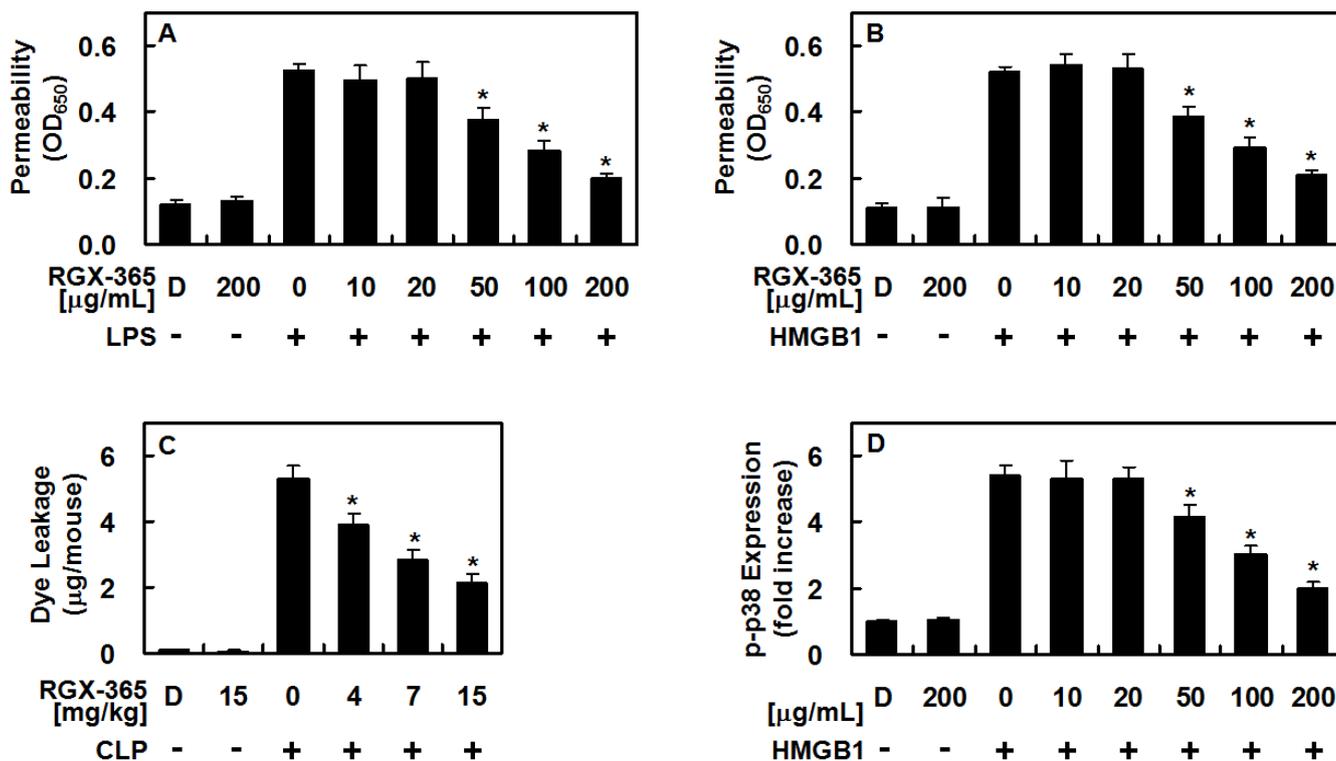
FIGURE 4 - Effects of RGX-365 on HMGB1-mediated permeability *in vitro* and *in vivo*. Effects of treatment with different concentrations of RGX-365 for 6 h on barrier disruption caused by LPS (A, 100 ng/mL, 4 h) or HMGB1 (B, 1 μg/mL, 16 h) were monitored by measuring the flux of Evans blue-bound albumin across HUVECs. C, The effects of RGX-365 on HMGB1-induced (2 μg/mouse, i.v.) vascular permeability in mice were examined by measuring the amount of Evans blue in peritoneal washings (expressed μg/mouse, n = 5). D, HUVECs were activated with HMGB1 (1 μg/mL, 16 h), followed by treatment with different concentrations of RGX-365 for 6 h. The effects of CTXA on HMGB1-mediated expression of phospho-p38 were determined via ELISA. Results are expressed as the mean ± SD of three separate experiments done on different days. *$p < 0.05$ versus LPS (A) or HMGB1 (B, C, D).

Administration of RGX-365 increased survival rate and reduced tissue injury in CLP-induced septic mice

Finally, we tested the protective effects of RGX-365 on CLP-induced septic lethality. RGX-365 was administered to mice after CLP surgery. A single dose of RGX-365 (15 mg/kg, 12 h or 50 h after CLP) did not protect against CLP-induced lethality (data not shown). Therefore, RGX-365 was dosed twice at 12 h and 50 h after CLP. Our data showed that RGX-365 improved survival rate of the septic mice ($p < 0.00001$; Figure 5). These results indicate that although HMGB1 levels were significantly reduced by RGX-365 at 12 h after CLP (Figure 2B), a single treatment with RGX-365 could not inhibit further secretion of HMGB1.

Therefore, it was administered twice to inhibit further secretion of HMGB1 and HMGB1-mediated inflammatory responses. Our findings suggests that RGX-365 is useful in the control of sepsis and septic shock.

The present study aimed to evaluate the barrier-protective effects of RGX-365 against vascular barrier-disruptive responses under septic conditions. Under normal pathophysiological conditions, the vascular endothelium plays pivotal roles in maintaining vascular barrier integrity in response to the vascular endothelial extracellular environment. Therefore, disruption of vascular barrier integrity is the primary and an important process involved in septic responses resulting in vascular hyperpermeability and body-cavity edema in septic patients (Bogatcheva, Verin, 2008). Therefore, recovery

of vascular integrity from the destructive response to inflammatory stimuli, and maintenance of vascular homeostasis is a major strategy for the treatment of sepsis. The results of the present study suggested that the antiseptic effects of RGX-365 occurred through the inhibition of HMGB1 release and HMGB1-mediated hyperpermeability.

Collectively, the results of the present study demonstrate that RGX-365 inhibited both LPS and CLP-mediated release of HMGB1 and HMGB1-mediated barrier disruption by increasing barrier integrity and inhibiting the expression of p38. Further, the barrier-protective effects of RGX-365 were confirmed in a mouse model, in which treatment with RGX-365 reduced CLP-induced mortality. Our findings indicate that RGX-365 may be a potential candidate for use in the treatment of severe vascular inflammatory diseases such as sepsis and septic shock.
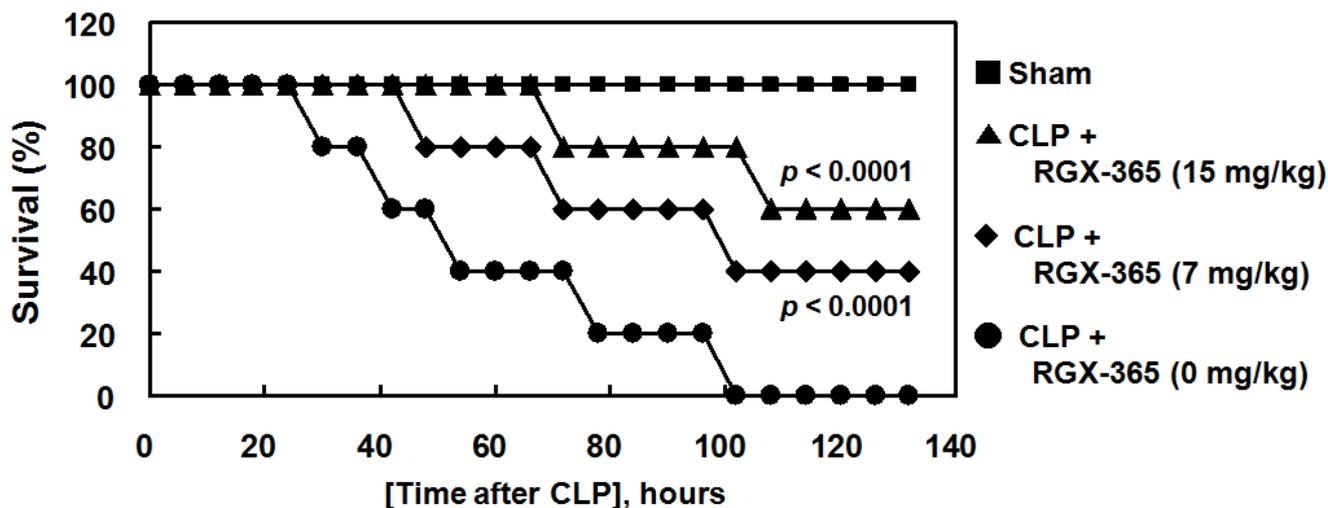
FIGURE 5 - Effects of RGX-365 on lethality after CLP. Male C57BL/6 mice (n = 20) were administered RGX-365 at 0 mg/kg (i.v., ○), 7 mg/kg (i.v.◆), or at 15 mg/kg (i.v. ▲) at 12 h and 50 h after CLP. Animal survival was monitored every 12 h after CLP, for a total of 132 h. Control sham-operated mice (■) were administered sterile saline (n = 20). Kaplan-Meier survival analysis was used to determine the overall survival rates versus CLP-treated mice.

CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

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