EFFECTS OF C1 INHIBITOR ON TISSUE DAMAGE IN A PORCINE MODEL OF CONTROLLED HEMORRHAGE

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ABSTRACT—Activation of the complement system has been associated with tissue injury after hemorrhage and resuscitation in animals. We investigated whether administration of recombinant human C1-esterase inhibitor (rhC1-INH), a regulator of complement and contact activation systems, reduces tissue damage and cytokine release and improves metabolic acidosis in a porcine model of hemorrhagic shock. Male Yorkshire swine were assigned to experimental groups and subjected to controlled, isobaric hemorrhage to a target mean arterial pressure of 35 mmHg. Hypotension was maintained for 20 min followed by a bolus intravenous injection of rhC1-INH or vehicle; animals were then observed for 3 h. Blood chemistry and physiologic parameters were recorded. Lung and small intestine tissue samples were subjected to histopathologic evaluation and immunohistochemistry to determine the extent of injury and deposition of complement proteins. Cytokine levels and quantitative assessment of renal and hepatic function were measured via enzyme-linked immunosorbent assay and chemistry analyzer, respectively. Pharmacokinetics of rhC1-INH revealed dose proportionality for maximum concentration, half-life, and the time span in which the functional C1-INH level was greater than 1 IU/mL. Recombinant human C1-INH significantly reduced renal, intestinal, and lung tissue damage in a dose-dependent manner (100 and 250 IU/kg). In addition, rhC1-INH (250 IU/kg) markedly improved hemorrhage-induced metabolic acidosis and circulating tumor necrosis factor α. The tissue-protective effects of rhC1-INH appear to be related to its ability to reduce tissue complement activation and deposition. Recombinant human C1-INH decreased tissue complement activation and deposition in hemorrhaged animals, improved metabolic acidosis, reduced circulating tumor necrosis factor α, and attenuated tissue damage in this model. The observed beneficial effects of rhC1-INH treatment on tissue injury 20 min into severe hypotension present an attractive model of low-volume resuscitation, particularly in situations with a restrictive medical logistical footprint.

KEYWORDS—Recombinant human C1 inhibitor, hemorrhage, low volume fluid resuscitation, swine, reperfusion injury

INTRODUCTION

Priorities in traumatic hemorrhage patient management are prompt control of hemorrhage and fluid resuscitation traditionally consisting of administration of crystalloids, colloids, Ringer's lactate solution, packed red blood cells, fresh frozen plasma, platelets, and/or whole blood. Unfortunately, both crystalloid and colloid resuscitation fluids have been associated with deleterious effects in animal models and have had questionable results in humans (1). Furthermore, massive transfusions of lactated Ringer's solution have been shown to increase reperfusion injury and leukocyte adhesion (2). The evaluation of resuscitation measures for trauma is typically based on emergency room practices and treatment of battlefield casualties (1). Initially advanced by the military, the treatment strategy of damage control resuscitation promotes hemostatic resuscitation and reduction of coagulopathy by permissive hypotension while avoiding early crystalloid use (2); therefore, blood products are currently the preferred fluid. Transfusing blood products is the standard of care for massive transfusions, but it has its disadvantages, such as allergic reactions and blood-borne diseases (1). Blood products also pose logistical problems such as limited availability, short shelf life, type and cross-match requirements, and need for refrigeration (1). Identification of a pharmacologic agent that promotes homeostasis without conventional resuscitation fluids or as an adjunct to fluid resuscitation reducing the need for copious amounts of fresh blood products would offer an immense therapeutic and logistical advantage.

The first physiologic response to severe blood loss is activation of the neuroendocrine response leading to vasoconstriction in an attempt to shift blood away from the bleeding site toward more vital organs, such as the heart, lungs, and brain. This vasoconstriction also drives blood away from the intestines causing functional intestinal ischemia (3), triggering immune-inflammatory responses rendering the body particularly susceptible to deleterious complications (1). Morbidity and mortality following hemorrhage result from the involvement of multiple inflammatory pathways, including complement.

Complement activation in trauma patients occurs immediately after the traumatic event, and its activity is directly related to the severity of the injury and associated complications. Both local and systemic complement activations are part of a

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common inflammatory cascade in the pathogenesis of tissue damage and organ dysfunction in the early phase after trauma (4). We have implicated complement activation in reperfusion-induced intestinal injury (5) as well as hemorrhage-induced inflammation and intestinal damage in mice (6). Complement inhibition has been shown to be an effective therapeutic strategy in mice for intestinal ischemia/reperfusion (5, 7) as well as in rats for traumatic and hemorrhagic shock (8).

C1 inhibitor (C1-INH), derived from human plasma, is Food and Drug Administration approved for treating patients with hereditary angioedema, but this source has limited availability and higher costs. A recombinant human C1-INH (rhC1-INH) has recently been developed and is approved in Europe for hereditary angioedema treatment and is investigational in the United States. In this study, we demonstrate that infusion of rhC1-INH 20 min after severe hemorrhagic hypotension decreased tissue complement activation and deposition, improved metabolic acidosis, reduced circulating tumor necrosis factor α (TNF-α), and reduced renal, intestinal, and lung injury in swine.

MATERIALS AND METHODS

This study was conducted in compliance with the Animal Welfare Act, the implementing Animal Welfare Regulations, and the principles of the Guide for the Care and Use of Laboratory Animals. After Institutional Animal Care and Use Committee approval, adolescent male swine underwent controlled isobaric arterial hemorrhage with target mean arterial pressure (MAP) of 35 mmHg for 20 min followed by 120 mL bolus of rhC1 INH or vehicle (phosphate buffered saline [PBS]).

Animal preparation and physiologic monitoring

The swine were fasted overnight with free access to water. After induction of anesthesia (20 mg/kg body weight ketamine and 2.2 mg/kg body weight xylazine, intramuscular injection), adolescent male Yorkshire cross bred swine (12 weeks old, 30 38 kg, *Sus scrofa*; ABI, Danboro, Pa) underwent surgical placement of prehirudinized (100 µg of Refludan/mL saline; ZLB Behring GmbH, Marburg, Germany) catheter (Starflex, 9F, 11 cm; Inver Grove Heights, Minn) under sterile conditions into the left femoral artery and vein under iso flurane anesthesia (3% induction, 2% 2.6% maintenance; Minrad, Buffalo, NY).

Immediately after placement and periodically after blood sampling, the catheters were flushed with 2 mL of hirudinized saline to ensure patency. Hemorrhage and withdrawal of the blood samples were performed via the artery, whereas rhC1 INH or vehicle was administered into the vein. A micromanometer (MPC 500; Millar Instruments, Houston, Tex) was inserted into the right femoral artery for hemodynamic monitoring by CARA (Computer Assisted Resuscitation Algo rithm) software (developed by US Army's Walter Reed Institute of Research).

Experimental design

The animals were hemorrhaged using a controlled, isobaric Wiggers model of controlled hemorrhagic shock (Fig. 1). The animals were randomly enrolled in one of four experimental groups: (*a*) H, hemorrhage + vehicle (n = 6); (*b*) H + C1 100, hemorrhage + C1 INH (100 IU/kg body weight, n = 5); (*c*) H + C1 250, hemorrhage + C1 INH (250 IU/kg body weight, n = 5); or (*d*) control, sham operated (cannulated but not hemorrhaged, n = 6). Each dose was infused in a total volume of 120 mL. Shed blood was not returned to the animal.

The animals were spontaneously ventilated. Controlled arterial hemorrhage was automated to ensure reproducibility. In brief, a customized computer protocol (LabView version 8.2; National Instruments, Austin, Tex) monitored MAP. Using a proportional control feedback algorithm, the program controlled the speed and direction of a partial occlusion roller pump (Masterflex Digital Console Drive; Cole Parmer Instruments, Chicago, Ill) that was connected to the femoral arterial catheter. At the start of the hemorrhage phase, the computer started withdrawing blood to decrease MAP to the target pressure over a 15 min period. During the protocol, the volume of withdrawn blood was gravimetrically measured (PB5001 S; Mettler Toledo, Columbus, Ohio), and the data output was stored on computer hard disk with time stamped hemodynamic parameters. Twenty minutes after the blood pressure reached 35 mmHg, a bolus (120 mL) of rhC1 INH or vehicle was administered. The animals were then monitored for 3 h.

As indicated in Figure 1, arterial blood samples were obtained before surgery (60 min), before hemorrhage (15 and 5 min before hemorrhage), during hemorrhage to MAP of 35 mmHg (5 and 15 min), immediately before rhC1 INH/vehicle administration (35 min, posthemorrhage), then at 20 min intervals until the end of the observation period. Blood samples were analyzed for pH, P_{CO_2}, P_{O_2}, bicarbonate (HCO_3), base excess (BE), lactate, glucose, hematocrit (Hct), hemoglobin (Hb), sodium (Na^+), potassium (K^+), and ionized calcium (Ca^{2+}) using i STAT cartridges (Abbott Laboratories, Abbott Park, Ill).

Tissue harvest

The animals were killed with pentobarbital sodium (90 mg/kg i.v.) at the end point following the aforementioned procedures. Tissue samples, including lung and small intestine, were removed and fixed with 10% formalin or 4% para formaldehyde for histological and immunohistochemical analysis, respectively.

Reagents and antibodies

Recombinant human C1 INH was provided by Pharming Technologies BV (Leiden, the Netherlands). CG4+ and CG8+ cartridges were purchased from Abbott
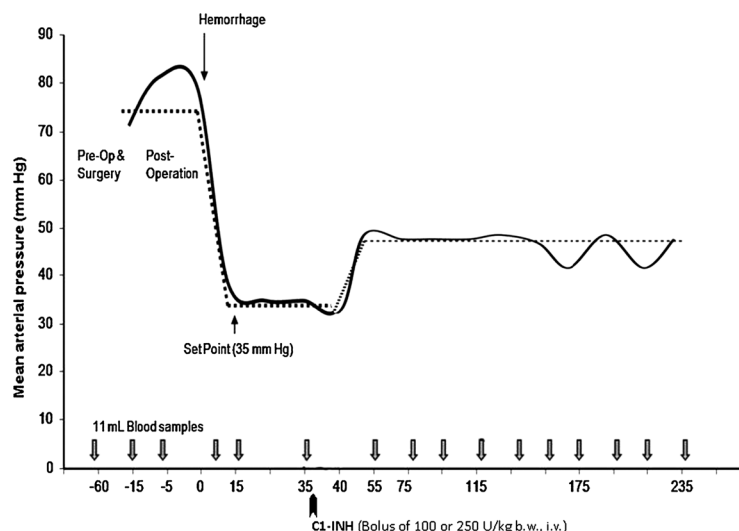

Fig. 1. **Scheme of the experimental design**. Actual (solid line) and target (dotted line) mean arterial blood pressure (MAP) and blood sample collection (down arrows) are shown. Recombinant human C1 INH was given as a bolus of 100 or 250 U/kg body weight in 120 mL of saline 20 min after target MAP was achieved.

(Princeton, NJ). MicroVue C1 INH enzyme immunoassay (EIA) kit was purchased from Quidel Corporation (San Diego, Calif). Chicken anti C3/C3a, mouse anti C5/C5a, mouse anti C5b 9, and mouse anti endothelial cell antibodies were obtained from Abcam Inc (Cambridge, Mass). Goat anti chicken Alexa Fluor 594, goat anti mouse Alexa Fluor 488, goat anti mouse Alexa Fluor 594 immunoglobulin G (H + L) conjugated secondary antibodies, and ProLong Gold antifade reagent were from Invitrogen (Carlsbad, Calif).

Pharmacokinetic analysis in porcine plasma

Blood was collected in EDTA tubes and centrifuged at 3,000 revolutions/min for 10 min at 4°C. Plasma was removed and was stored at 80°C until analysis. Plasma level of functional C1 INH was determined using the EIA kit following the manufacturer's instruction except that rhC1 INH was used as the standard, and a 1:2,000 specimen dilution was used. Maximum concentration (C_{max}) above baseline was derived directly from the concentration time profiles. The time span in which the functional C1 INH concentration exceeded 1 U/mL was determined by linear interpolation. The pharmacokinetic parameters of functional C1 INH were determined by using a 1 compartment pharmacokinetic model. Calculations were performed by using linear model (first order kinetics) estimation.

Histological examination

Formalin fixed tissues were embedded in paraffin, sectioned, and stained with hematoxylin eosin. Histological images were recorded under a light microscope (AX80; Olympus, Center Valley, Pa) with 10× objective by a pathologist blinded to the treatment group. Histological injury scores were graded from 0 to 3 for lung injury and 0 to 6 for tissue injury as previously described (3).

Immunohistochemical staining

Paraformaldehyde fixed lung and small intestine biopsies were snap frozen at 70°C; sections were cut at 5 µm with a cryostat and fixed in cold methanol for 20 min. The fixed sections were permeabilized with 0.2% Triton X 100 in PBS for 10 min and blocked with 2% bovine serum albumin in PBS for 30 min at room temperature. The sections were incubated with the primary antibodies overnight at 4°C, washed, then incubated with the appropriate secondary antibodies labeled with Alexa Fluor 488 and Alexa Fluor 594 for 1h at room temperature. After washing, the sections were mounted with ProLong Gold antifade solution containing 4′, 6′ diamidino 2 phenylindole and visualized under a confocal laser scanning microscope (Radiance 2100; Bio Rad, Hercules, NJ) at 400× magnification. Captured digital images were processed by Image J software (National Institutes of Health, Bethesda, Md).

Immunofluorescent quantification

This procedure was based on a modified method as previously described (7). Briefly, four to six images from each animal were opened using Adobe Photoshop software and adjusted until only the fluorescent deposits and no background tissue were visible. Using Image J software, the image was changed to black and white pixels, with black representing deposits of the target proteins and white representing nonstained areas of the image. Using the image Adjust Threshold command, the image was then changed to red and white, fluorescent deposits being red. The image was analyzed to result in the total red area in pixels squared. Values for total area for all animals in each group were averaged to give the average area of fluorescent deposit.

Quantitative assessment of end tissue damage

Urea nitrogen, creatinine, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were measured from blood samples using Dimension Xpand Plus Integrated Chemistry System (Siemens, Holliston, Mass).

Cytokine assays

Serum TNF α, interleukin 6 (IL 6), and IL 8 levels were assessed by quantitative sandwich enzyme linked immunosorbent assay (R&D Systems, Minneapolis, Minn) according to the manufacturer's instructions.

Statistical analysis

Data were analyzed using GraphPad Prism version 4.0 (GraphPad Software, La Jolla, Calif) and are expressed as mean ± SEM. Statistical analysis of pharmacokinetic parameters was performed using unpaired t test with Welch correction. Other parameters were analyzed using one way analysis of variance (ANOVA) followed by Bonferroni, two way ANOVA using Tukey post hoc test, or unpaired t test (two tailed P value); $P < 0.05$ was considered significant.

RESULTS

To investigate the direct effects of rhC1-INH on hemorrhagic shock, animals were subjected to controlled bleeding to MAP target of 35 mmHg for 20 min followed by bolus i.v. injection (120 mL) of rhC1-INH (100 or 250 IU/kg body weight) or vehicle as outlined in Figure 1. The primary end point was the quantitative assessment of end tissue damage. Secondary end points were physiologic and blood metabolic analysis throughout the course of experiment.

Pharmacokinetics of rhC1-INH

The porcine plasma levels of functional C1-INH were measured by Microvue human C1-INH EIA (Table 1, Figs. 2 and 3). The EIA is specific for human C1-INH; the values from swine at time 0 and swine receiving PBS injection were both zero. Plasma levels of functional C1-INH in group H + C1-INH250 U/kg were significantly higher than those of the 100 U/kg dose group throughout the observation period ($P < 0.001$). The elimination curve (semilog plot) showed a single straight line and a steady decrease in concentration as the C1-INH was eliminated, suggesting one compartment pharmacokinetic model and first-order kinetics of rhC1-INH in this study. The profile of functional C1-INH displayed a dose-dependent increase in C_{max} ($P < 0.001$) and in the time span in which the functional C1-INH level exceeded 1 U/mL ($P < 0.0001$). Concentration was directly proportional to dose. Dose-normalized C_{max} appeared constant ($P = 0.99$), indicating plasma proportionality. The volume distribution was dose-independent; however, the clearance and the half-life were dependent on dose ($P < 0.05$).

Baseline characteristics

There were no significant differences among the hemorrhaged groups for any of the parameters shown in Tables 2 and 3 during the prehemorrhagic phase among sham, control, and rhC1-INH–treated groups.

TABLE 1. **Summary of pharmacokinetic parameters of functional C1-INH in each dose group**

Dose, U/kg	n	Baseline, U/mL	C_{max}, U/mL	Dose normal C_{max}, U/mL per U per kg	Time above 1 U/mL, min	Clearance, mL/min	Volume distribution, L	Half-life, min
100	5	0.05 ± 0.03	1.62 ± 0.11	0.02 ± 0.00	49.07 ± 5.75	33.29 ± 3.41	2.16 ± 0.12	49.30 ± 3.8
250	5	0.01 ± 0.01	4.05 ± 0.07*	0.02 ± 0.00	161.74 ± 7.05*	21.47 ± 2.47*	2.16 ± 0.07	72.01 ± 6.45*

Data are expressed as mean ± SEM and compared using unpaired t test with Welch correction.
*$P < 0.05$, H + C1-INH250 versus H + C1-INH100.
Time above 1 U/mL indicates time span in which the functional C1-INH concentration exceeded 1 U/mL.
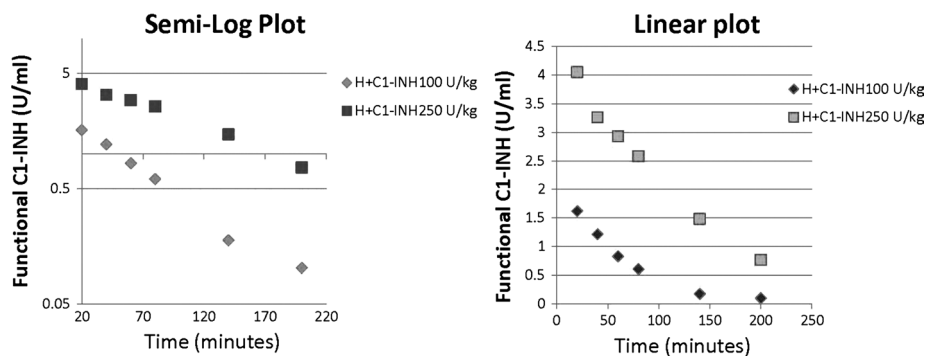
FIG. 2. **The elimination curve of rhC1-INH**. The elimination curve is a fairly straight line (semilog) and steadily decreases in concentration as rhC1 INH is eliminated, suggesting one compartment pharmacokinetic model and first order kinetics in this porcine model.

Effect of rhC1-INH on metabolic and hemodynamic parameters

As shown in Table 2, MAP and pulse pressure (PP) decreased, whereas shock index (SI; defined as the ratio of heart rate to systolic blood pressure) increased in the hemorrhaged animals compared with the sham group ($P < 0.05$) at the end of hemorrhage phase. Although treatment with rhC1-INH did not significantly affect these hemodynamic parameters, there was a trend toward an improvement of MAP, SI, and PP in the H + C1-250 group. As seen in Table 3, the administration of a higher dose, but not a lower dose, of rhC1-INH significantly increased final plasma level of BE and HCO_3^-. Final lactate in the H + C1-250 group normalized to sham level when compared with the H group. Final blood potassium level was significantly higher in the hemorrhaged, H + C1-100, and H + C1-250 animals compared with the sham group. There was also a significant change in posthemorrhage K^+ in the hemorrhaged animals treated with rhC1-INH (100 IU/kg).

Effect of rhC1-INH on hemorrhage-induced intestinal damage

Intestinal tissue from hemorrhaged animals displayed obvious damage with loss of mucosal villi and severely injured crypts in most areas accompanied by massive inflammatory cell infiltration, necrosis, and moderate edema in the mucosa and submucosa layers. In contrast, intestinal tissue damage remained superficial, and villi were well preserved in hemorrhaged animals treated with 250 IU/kg rhC1-INH (Fig. 4A).

Effect of rhC1-INH on hemorrhage-induced lung injury

Hematoxylin-eosin–stained histological images and injury score (Fig. 4B) showed that hemorrhaged pig lung injury was characterized by destruction of the alveolar architecture with severe alveolar hemorrhage, septal edema, and moderate inflammation when compared with the sham group. Treatment with 100 IU/kg rhC1-INH did not result in a beneficial effect on tissue injury; however, lung damage was significantly improved at a dose of 250 IU/kg.

Effect of rhC1-INH on complement activation and deposition in the small intestine tissue

Intravenous injection of rhC1-INH at a dose of 250 IU/kg at the end of posthemorrhage phase led to an attenuation of hemorrhage-induced C3 and C5 deposition (Fig. 5, A and B).

C5b-9 formation was also inhibited by rhC1-INH in a similar dose-dependent manner (Fig. 5C).

Effect of rhC1-INH on complement activation and deposition in lung tissue

Lung deposition of C3 (Fig. 6A) and C5 (Fig. 6B) in hemorrhaged animals treated with 250 IU/kg rhC1-INH was reduced when compared with hemorrhaged animals. Figure 6C shows an increased deposition of C5b-9 in lung tissue of the H group compared with sham, whereas the hemorrhaged animals that received rhC1-INH exhibited a reduction of C5b-9 formation. It also shows C5b-9 accumulated and colocalized at the vascular endothelium.

Effect of rhC1-INH on renal and hepatic function

Renal function was determined by urea nitrogen and creatinine detected in blood samples (Fig. 7, A and B). In the hemorrhaged group, levels of urea nitrogen (Fig. 7A) and creatinine (Fig. 7B) were significantly increased when compared with the sham group, whereas treatment with the higher dose of C1-INH significantly decreased these levels (Fig. 7, A and B). Liver function was ascertained by ALT and AST in blood samples. There were no statistically significant changes in either enzyme among the groups (Fig. 7, C and D).

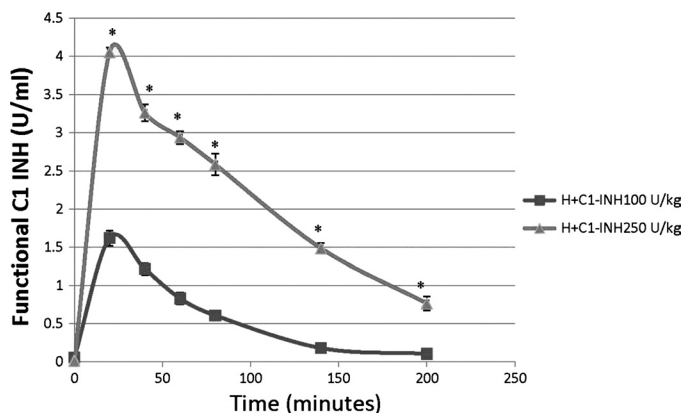FIG. 3. **Time profiles of mean functional C1-INH in the distinct dosage groups**. Porcine plasma levels of functional rhC1 INH were determined by a modified Microvue C1 INH EIA kit. Group data are expressed as mean ± SEM and compared using one way ANOVA followed by Bonferroni multiple comparison test with $P < 0.05$ considered significant. $*P < 0.001$, H + C1 INH250 U/kg (n = ~4 5) versus H + C1 INH100 U/kg (n = ~4 5).

Table 2. **Prehemorrhage and posthemorrhage hemodynamic data of hemorrhaged animals**

	Group				*P*
	Sham	H	H + C1-100	H + C1-250	
n	5	5 6	4 5	4 5	
Body weight, kg	35.48 ± 1.58	34.53 ± 1.71	34.42 ± 0.69	34.34 ± 1.37	NS
Total shed blood, mL		735.82 ± 242.79	778.40 ± 246.46	559.92 ± 73.84	NS
Prehemorrhage MAP, mmHg	68.01 ± 2.76	70.79 ± 3.19	76.76 ± 5.58	72.43 ± 8.72	NS
Posthemorrhage MAP, mmHg	69.87 ± 3.58	35.45 ± 0.55*	36.27 ± 0.28*	36.04 ± 0.35*	S
Final MAP, mmHg	53.69 ± 1.91	41.29 ± 7.18	42.20 ± 3.37	47.35 ± 2.18	NS
Prehemorrhage SI	1.23 ± 0.04	1.03 ± 0.07	0.93 ± 0.06	1.11 ± 0.09	NS
Posthemorrhage SI	1.14 ± 0.09	2.54 ± 0.40*	2.44 ± 0.38*	2 ± 0.20	S
Final SI	1.46 ± 0.10	3.26 ± 0.41*	3.30 ± 0.51*	2.33 ± 0.29	S
Prehemorrhage PP, mmHg	43.30 ± 5.95	43.04 ± 1.59	42.28 ± 1.44	39.25 ± 2.46	NS
Posthemorrhage PP, mmHg	42.27 ± 4.39	21.54 ± 1.50*	18.42 ± 2.29*	21.89 ± 2.04*	S
Final PP, mmHg	37.18 ± 3.65	23.13 ± 4.49*	23.94 ± 1.88*	27.57 ± 1.05	S

Data are expressed as mean ± SEM. Group data are compared using one-way ANOVA with Bonferroni post hoc test.
*$P < 0.05$ versus sham.
NS indicates not significant; H, hemorrhage.

Effect of rhC1-INH on cytokine levels

As shown in Figure 8, hemorrhage resulted in significant increases in circulating TNF-α levels. Treatment with the higher dose of C1-INH resulted in significantly lower TNF-α levels compared with the hemorrhaged group. There was no change in IL-6 or IL-8 among the groups (data not shown).

DISCUSSION

Hemorrhagic shock is second only to central nervous system injury as cause of death in trauma patients (9). Treatments are aimed at restoring intravascular volume and maintaining vital organ perfusion (1). Complement activation has been implicated during hemorrhage in rats (8) and swine (3, 10) and relates to morbidity and mortality of trauma patients because of severe inflammatory tissue destruction (4). Peckham et al (8) showed that complement inhibition decreased resuscitation fluid requirements of hemorrhaged rats. We demonstrated that decay-accelerating factor attenuated C-reactive protein–enhanced tissue damage in murine mesenteric ischemia/reperfusion (5) and protected neurons from hypoxia-mediated injury *in vitro* (11). We also found that decay-accelerating factor mitigates hemorrhage-associated intestinal and lung tissue damage and hyperkalemia in a controlled hemorrhage swine model (3).

The aforementioned logically leads to the conclusion that complement inhibition would be beneficial during typical high-volume damage control resuscitation. However, the goal of this study was to evaluate a novel low-volume infusion damage control resuscitation treatment. It is well known that first responders attending to multiple traumas on the battlefield or in remote locations may not have sufficient fluids for adequate resuscitation. They would therefore benefit from a small-volume resuscitation product that could be given after initial hemorrhage control to sustain the wounded and prevent tissue injury arising from immune-inflammatory responses for an extended period until the wounded can be evacuated to a medical treatment facility. Small-volume (<4 mL/kg) resuscitation products given immediately after hemorrhage without additional fluids are advocated for by the Defense Advanced Research Projects Agency Surviving Blood Loss program (12). The goal is to provide small-volume drugs or adjuncts that afford tissue cytoprotection and/or immune modulation and result in at least 3 h of survival. Previous studies from our laboratories have also focused on this effort (12, 13). The current study was also performed with low-volume infusion of a complement inhibitor with the same Defense Advanced Research Projects Agency goals.

C1 inhibitor is a member of the serpin family of protease inhibitors and inactivates a variety of proteases including complement system proteases (C1r, C1s, MASP2), contact system proteases (factor XII, plasma kallikrein), an intrinsic coagulation protease (factor XI), and fibrinolytic proteases (plasmin, tissue plasminogen activator). C1 inhibitor is primarily synthesized in the liver; normal human plasma levels are about 240 µg/mL, which corresponds to the functional unit 1 IU/mL (14). C1 inhibitor derived from human blood is Food and Drug Administration approved for treating hereditary angioedema. We used rhC1-INH in this study, which revealed dose proportionality for C_{max}, half-life, and the time span in which the functional C1-INH level was greater than 1 IU/mL. After a 120-mL bolus injection of rhC1-INH, functional plasma C1-INH levels remained greater than 1 IU/mL for 162 min at the 250 IU/kg dose compared with 49 min at the 100 IU/kg dose. Elimination of rhC1-INH was shown to be similar to that of human-derived C1-INH in our study. This dose-dependent clearance is most likely due to a saturable mechanism of elimination. The half-life of rhC1-INH was 72 min at a dosage of 250 IU/kg compared with 49 min at the 100 IU/kg dose. These findings indicated that rhC1-INH has a slightly different pharmacokinetic profile than plasma-derived C1-INH products. The half-life of rhC1-INH is less than that of human

TABLE 3. **Prehemorrhage and posthemorrhage metabolic data of hemorrhaged animals**

	Group				
	Sham	H	H + C1-100	H + C1-250	*P*
n	5	5 6	4 5	4 5	
Prehemorrhage Hb, g/dL	8.16 ± 0.46	8.27 ± 0.26	7.68 ± 0.39	7.96 ± 0.47	NS
Posthemorrhage Hb, g/dL	8.22 ± 0.57	7.47 ± 0.29	7.28 ± 0.28	7.08 ± 0.38	NS
Final Hb, g/dL	8.24 ± 0.45	8.36 ± 0.34	8.40 ± 0.64	8.50 ± 0.66	NS
Prehemorrhage Hct, %	24 ± 1.38	24.33 ± 0.76	22.60 ± 1.17	23.40 ± 1.40	NS
Posthemorrhage Hct, %	24.20 ± 1.66	22 ± 0.86	21.40 ± 0.81	20.75 ± 1.11	NS
Final Hct, %	24.20 ± 1.32	24.60 ± 0.98	24.75 ± 1.89	25 ± 1.92	NS
Prehemorrhage Na$^+$, mM	135 ± 1.18	136.20 ± 0.34	136 ± 1.52	137 ± 0.52	NS
Posthemorrhage Na$^+$, mM	135 ± 0.97	135 ± 0.58	134.33 ± 0.94	135 ± 0.50	NS
Final Na$^+$, mM	132.90 ± 1.10	133.25 ± 0.56	133.67 ± 1.26	134 ± 0.37	NS
Prehemorrhage K$^+$, mM	3.82 ± 0.12	4.40 ± 0.21	3.96 ± 0.15	4.08 ± 0.11	NS
Posthemorrhage K$^+$, mM	4.02 ± 0.16	4.60 ± 0.14	4.78 ± 0.11*	4.70 ± 0.17	S
Final K$^+$, mM	4.80 ± 0.25	6.14 ± 0.40*	5.95 ± 0.29*	5.88 ± 0.15*	S
Prehemorrhage iCa^{2+}, mM	1.33 ± 0.02	1.36 ± 0.05	1.38 ± 0.48	1.39 ± 0.02	NS
Posthemorrhage iCa^{2+}, mM	1.32 ± 0.03	1.37 ± 0.02	1.35 ± 0.03	1.35 ± 0.03	NS
Final iCa^{2+}, mM	1.30 ± 0.03	1.33 ± 0.03	1.29 ± 0.02	1.32 ± 0.03	NS
Prehemorrhage pH	7.46 ± 0.03	7.40 ± 0.02	7.40 ± 0.03	7.44 ± 0.04	NS
Posthemorrhage pH	7.48 ± 0.03	7.41 ± 0.01	7.39 ± 0.03	7.44 ± 0.04	NS
Final pH	7.50 ± 0.02	7.42 ± 0.02	7.42 ± 0.03	7.46 ± 0.03	NS
Prehemorrhage Po_2, mmHg	76.20 ± 4.21	80.08 ± 5.79	78.60 ± 1.35	76.10 ± 4.64	NS
Posthemorrhage Po_2, mmHg	80.20 ± 5.66	89.67 ± 13.79	74.70 ± 2.03	80.88 ± 3.44	NS
Final Po_2, mmHg	79.20 ± 3.48	82.40 ± 4.70	67.63 ± 6.71	76.10 ± 2.66	NS
Prehemorrhage So_2, %	95 ± 1.27	94.58 ± 1.27	95 ± 0.47	94.70 ± 1.01	NS
Posthemorrhage So_2, %	96 ± 0.88	94.92 ± 1.04	94.20 ± 0.12	96.25 ± 0.25	NS
Final So_2, %	96.30 ± 0.62	95.90 ± 0.46	92.50 ± 1.62*	95.50 ± 0.47	S
Prehemorrhage Pco_2, mmHg	45.87 ± 3.41	52.30 ± 3.42	52.33 ± 3.51	49.80 ± 4.03	NS
Posthemorrhage Pco_2, mmHg	44.70 ± 3.88	48 ± 0.87	50.07 ± 2.54	45.85 ± 4.12	NS
Final Pco_2, mmHg	42.66 ± 2.93	46.54 ± 1.82	47.13 ± 1.38	48.40 ± 3.89	NS
Prehemorrhage BE, mEq/L	5.96 ± 0.45	4.39 ± 0.94	5.07 ± 1.45	6.52 ± 0.98	NS
Posthemorrhage BE, mEq/L	6.30 ± 0.82	3.44 ± 1.06	2.91 ± 1.31	4.51 ± 1.01	NS
Final BE, mEq/L	7.23 ± 0.59	3.17 ± 1.79	3.25 ± 2.26	7.36 ± 0.80†	S
Prehemorrhage HCO$_3$, mM	32.48 ± 0.55	32.14 ± 1.21	32.53 ± 1.31	33.54 ± 0.72	NS
Posthemorrhage HCO$_3$, mM	32.52 ± 0.94	30.43 ± 0.91	30.16 ± 1.15	30.84 ± 0.58	NS
Final HCO$_3$, mM	33.06 ± 0.95	30.29 ± 1.58	30.51 ± 1.89	34.28 ± 0.55†	S
Prehemorrhage glucose, mg/dL	103 ± 8.72	92.33 ± 15.7	86.80 ± 10.21	88.80 ± 8.55	NS
Posthemorrhage glucose, mg/dL	114.80 ± 8.65	137 ± 11.39	121.40 ± 13.16	128.25 ± 7.04	NS
Final glucose, mg/dL	124.60 ± 8.16	125 ± 21.14	122 ± 29.24	126.80 ± 13.05	NS
Prehemorrhage lactate, mM	1.01 ± 0.16	1.55 ± 0.52	0.88 ± 0.10	1.12 ± 0.25	NS
Posthemorrhage lactate, mM	1.32 ± 0.26	3.03 ± 1.05	2.25 ± 0.29	2.34 ± 0.23	NS
Final lactate, mM	1.42 ± 0.40	3.08 ± 0.82	2.87 ± 1.04	1.50 ± 0.19†	S

Data are expressed as mean ± SEM. Group data were compared using one-way ANOVA with Bonferroni post hoc test.
*$P < 0.05$ versus sham.
$^†P < 0.05$ versus H.
NS indicates not significant; H, hemorrhage.

plasma-derived C1-INH, which is 24 h (15). This difference was expected and can be attributed to the presence of oligo-mannose, hybrid-type N-linked glycans, and a low degree of glycosylation in rhC1-INH (16) compared with plasma-derived C1-INH. Uptake of rhC1-INH by galactose and mannose receptors expressed on hepatocytes most likely represents the

A

B
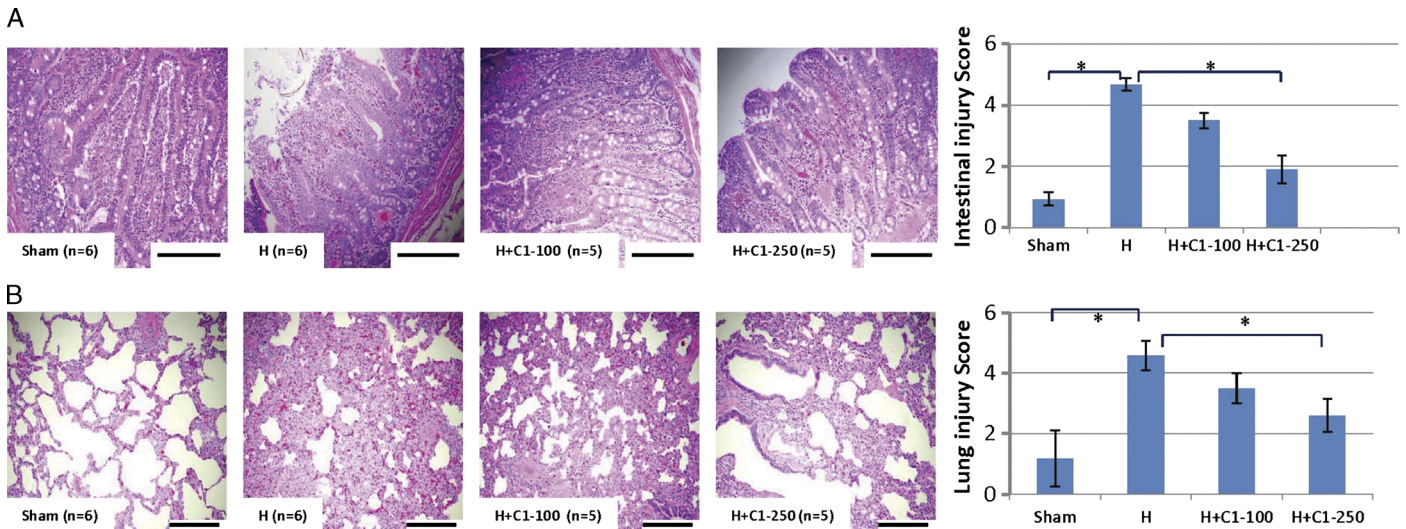
Fig. 4. **Recombinant human C1-INH treatment attenuates hemorrhage-mediated injury of small intestine and lung**. A, Representative histopathologic changes of the small intestines are shown (left). Original magnification ×100. Scale bars = 500 μm. Intestinal injury scores were evaluated by a standard as described in the Materials and Methods and are shown at the right of the images. B, Representative histological microphotographs (hematoxylin eosin staining) of the lungs (left). Original magnification ×200. Scale bars = 200 μm. Lung injury scores were calculated using the scale stated in the Materials and Methods for each animal (right). Group data are expressed as mean ± SEM and compared using one way ANOVA followed by Tukey multiple comparison test with $P < 0.05$ considered significant. *$P < 0.05$.

major clearance pathway of rhC1-INH. It should be noted that even if the increased clearance pathway occurred via hepatocytes, liver function did not decline in our model.

Human and porcine C1-INHs are highly homologous (17). Recombinant human C1-INH displayed different pharmacokinetics in humans compared with our porcine model. The half-life in our model was about three times shorter than that in humans (18). The faster clearance of rhC1-INH in this model is probably due to (*a*) faster hemodynamics after hemorrhagic shock; (*b*) higher blood levels of kallikrein, factor XIIa, and complement C1s subcomponent increasing rhC1-INH complex formation, which could boost C1-INH clearance via serpin

A

B

C
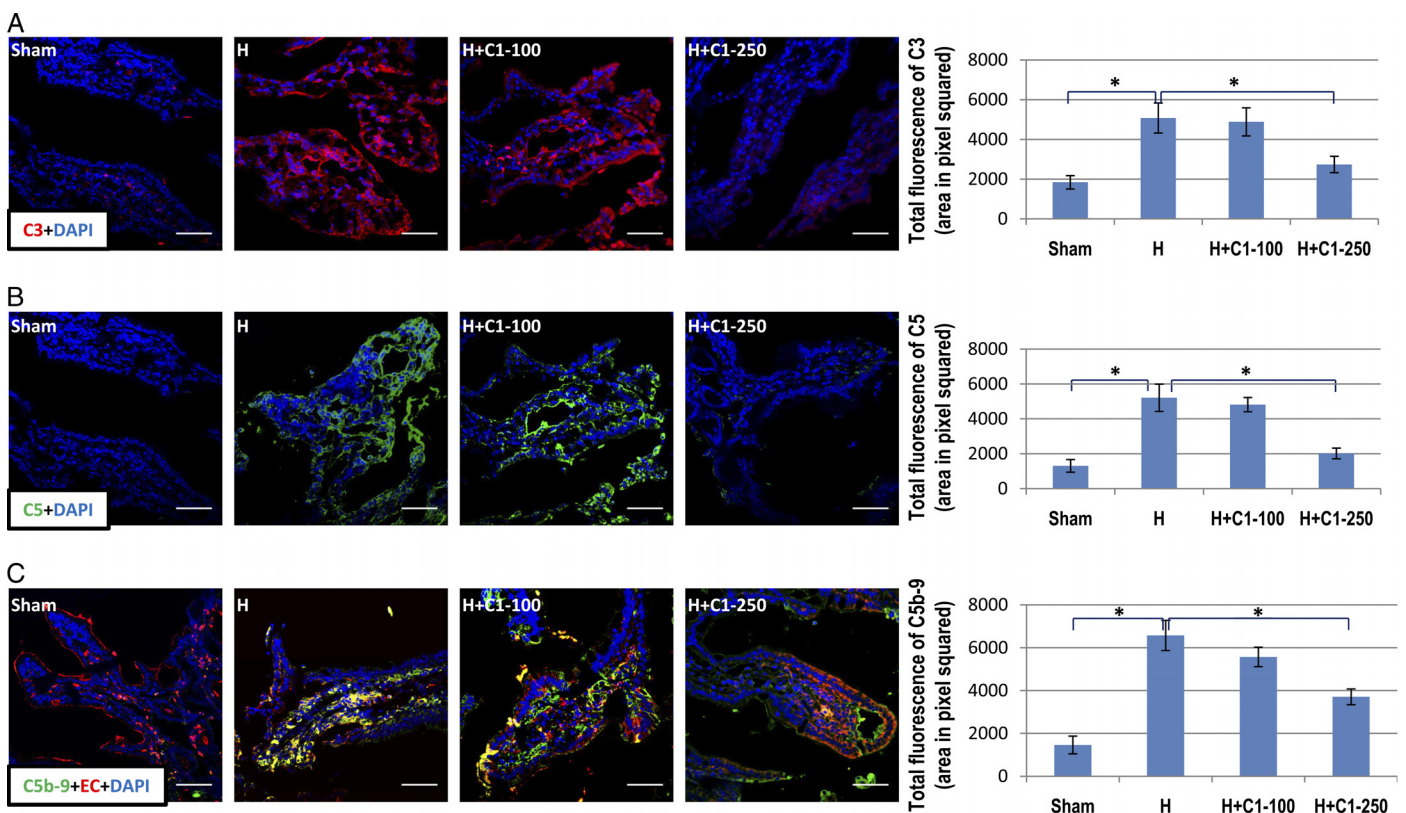
Fig. 5. **Recombinant human C1-INH treatment decreases complement C3, C5, and C5b-9 deposition in small intestinal tissue from hemorrhaged animals**. Representative immunohistochemical images of C3 (A, left), C5 (B, left), and C5b 9 (C, left) deposition in small intestine tissues. The total fluorescence quantification of deposition was measured and shown to the right of the microphotographs. Original magnification ×400. Scale bars = 50 μm. Group data are expressed as mean ± SEM and compared using one way ANOVA followed by Tukey multiple comparison test with $P < 0.05$ considered significant. *$P < 0.05$.
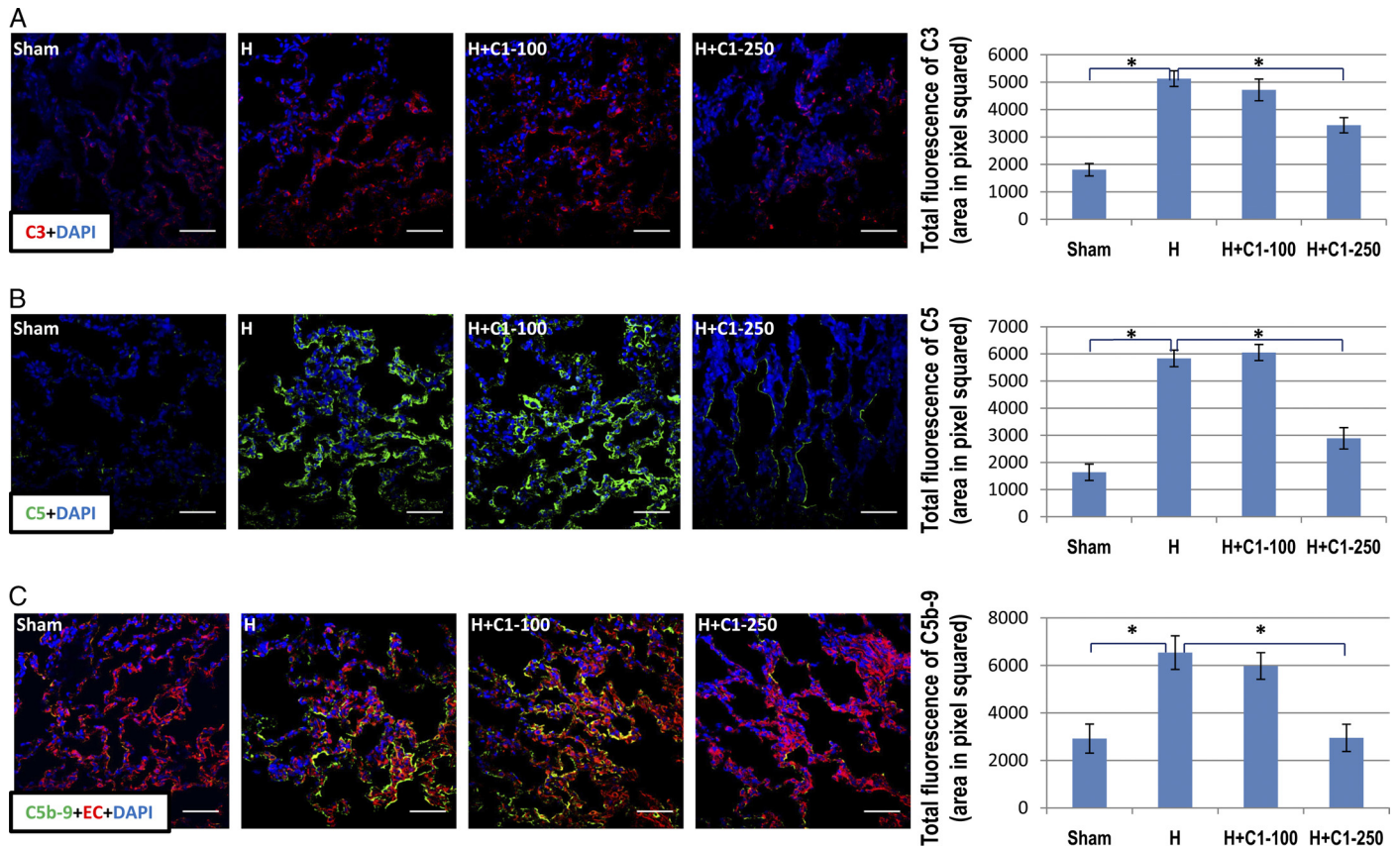
FIG. 6. **C1 inhibitor treatment reduces complement deposition in lung tissue from hemorrhaged animals**. Representative immunohistochemical images of C3 deposition (A, left), C5 deposition (B, left), and C5b 9 deposition (C, left) in the lung tissues. The total fluorescence quantification of deposition was measured and shown to the right of the microphotographs. Original magnification ×400. Scale bars = 50 μm. Group data are expressed as mean ± SEM and compared using one way ANOVA followed by Tukey multiple comparison test with $P < 0.05$ considered significant. *$P < 0.05$.
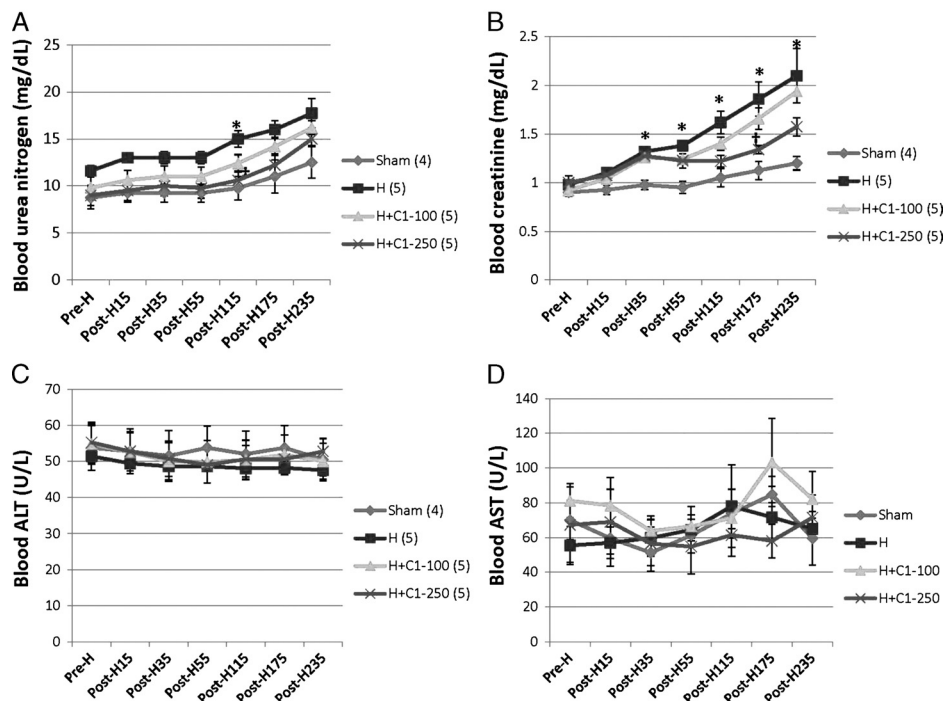

FIG. 7. **C1 inhibitor treatment lowers blood levels of urea nitrogen and creatinine of hemorrhaged animals**. Blood urea nitrogen (A), creatinine (B), ALT (C), and AST (D) were determined by Siemens Dimension Xpand Plus Chemistry Analyzer. Group data are expressed as mean ± SEM and compared using one way ANOVA followed by Bonferroni multiple comparison test with $P < 0.05$ considered significant. *$P < 0.05$ versus sham; [†]H + C1 250 versus H, $P < 0.05$.
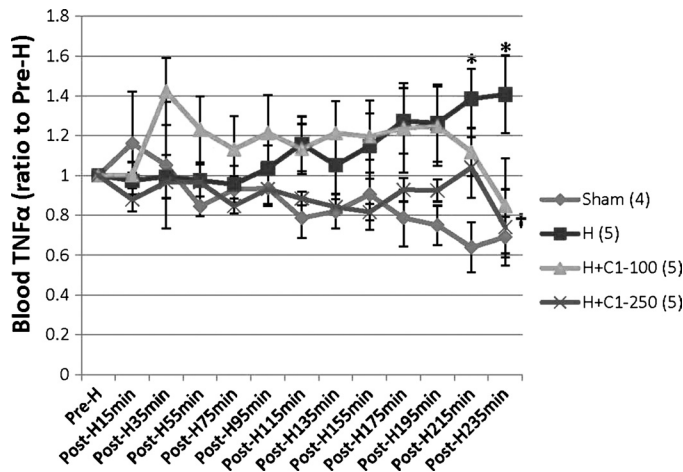
Fig. 8. **C1 inhibitor treatment decreases blood TNF-α levels in hemorrhaged animals**. Serum TNF α levels were detected by enzyme linked immunosorbent assay according to manufacturer's instruction. Group data are expressed as mean ± SEM and compared using unpaired t test (two tailed) with $P < 0.05$ considered significant. *$P < 0.05$ versus sham; †H + C1 250 versus H, $P < 0.05$.

complex–specific receptors present on hepatocytes (19); and/or (c) species-specific effects resulting in accelerated clearance in nonhuman species. These assumptions are speculative and need further investigation.

Beneficial effects of C1-INH in experimental settings have been reported in myocardial ischemia/reperfusion (20), thermal trauma (21), acute traumatic spinal cord injury (22), traumatic brain injury (23), and hemorrhagic shock–induced leukocyte adhesion and rolling (24). The present study was designed to investigate the effects of rhC1-INH on tissue injury as well as on hemodynamic and metabolic parameters in a porcine controlled hemorrhage "prehospital" phase model. It demonstrated that administration of a 250IU/kg dose of rhC1-INH (a) attenuated hemorrhage-associated lung and intestinal injury and reversed biomarkers of hemorrhage-induced renal dysfunction, (b) corrected metabolic acidosis, (c) suppressed deposition and activation of complement at the tissue level, and (d) lowered production of circulating TNF-α.

A direct correlation has been shown between early complement activation (<30 min after trauma) and injury severity in patients (4). Furthermore, concentrations of C3a and C3a/C3 ratio in the blood plasma have been shown to relate directly to the clinical severity of trauma in patients (25).

C1 inhibitor is the only known inhibitor of both the classic and lectin complement pathway by inactivation of C1r/C1s and mannan-binding protein-associated serine protease 2. In addition, C1-INH also suppresses alternative complement pathway activation (20). In this study, we show that a single dose of rhC1-INH after hemorrhagic hypotension resulted in a dose-dependent reduction of complement activation in gut and lung tissues. Decreased deposition of C3 and C5 may be associated with the alleviated inflammatory response, and limited deposition of C5b-9 may be related to decreased tissue damage.

Late shock is characterized by release of bradykinin and impaired cell metabolism (26). Complement and contact system activation appear to contribute to metabolic acidosis induced by hypoxia/ischemia-induced acidosis (27) and hemorrhagic

shock (28). In this study, administration of rhC1-INH resulted in significant improvement of hemorrhage-induced metabolic acidosis in a dose-dependent manner. Our findings are consistent with other reports that C1-INH improved tissue circulation and efficiently prevented metabolic acidosis in rat ischemia/reperfusion (29). The normal BE for immature swine is 7 mEq/L (30). In our model, the BE fell 4 mEq/L posthemorrhage but was reversed after a 250 U/kg dose of rhC1-INH. The improved metabolic acidosis and reduced tissue damage observed in this study may be due to secondary effects of C1-INH, not complement inhibition. However, this assumption is speculative and needs further investigation.

Renal hypoperfusion, which frequently occurs in hemorrhage, causes cellular injury and organ dysfunction. Common clinical assays to evaluate renal function include monitoring plasma levels of ALT and AST enzymes. Our results demonstrate that treatment with the higher dose of C1-INH significantly lowered the hemorrhage-induced increases in urea nitrogen and creatinine. This is consistent with other reports that C1-INH treatment markedly decreased plasma creatinine concentration in patients with severe septic shock (31).

Complement activation has been associated with the cytokine storm (32). The cytokine storm is a potentially fatal reaction that results from an exaggerated systemic immune response. The primary contributors to the cytokine storm are TNF-α and IL-6. C1 inhibitor has been reported to reduce the release of circulating TNF-α in human endotoxemia (33). In the current study, administration of 250 IU/kg C1-INH reduced circulating TNF-α levels after hemorrhage. Although the effectiveness of C1-INH on inflammatory response has largely been assumed to result from inhibition of complement activation, other C1-INH activities such as interaction with circulating cells may play a role.

Hemorrhagic shock initially results in a reduction of intravascular volume, which triggers a decrease in capillary hydrostatic pressure. To compensate this loss, Starling forces cause an influx of fluid from the interstitial space to the intravascular space. This influx may contribute to the immediate Hct drop after acute hemorrhagic shock in humans. However, in the current study, Hct remained unchanged after hemorrhage. Unlike the human spleen, the porcine spleen acts as a reservoir for red blood cells at a volume of 4.5 ± 0.89 mL/kg with a turnover of 9.76 ± 1.93 min (34). Furthermore, the porcine spleen will autotransfuse in response to massive blood loss. These physiologic anomalies combined with the small-volume resuscitation used would be the most probable explanation for the Hct observation in this study.

In addition to complement inhibition, C1-INH has other functions such as inhibition of contact system proteases (factor XII and plasma kallikrein), intrinsic coagulation protease factor XI, and fibrinolytic proteases (plasmin, tissue plasminogen activator) (20). A recent study demonstrated that the anti-inflammatory effects of C1-INH in porcine and human whole blood are largely independent of protease inhibition activity, which is consistent with the findings that C1-INH inhibits binding of factor B to C3b and that C1-INH interacts with circulating leukocytes, endothelial cells, bacteria, and endotoxins (20).

In conclusion, rhC1-INH given as a small-volume bolus injection to hemorrhaged swine attenuated tissue damage, lowered

tissue complement activation and deposition, corrected metabolic acidosis, and reversed circulating TNF-α release. The observed beneficial effects of rhC1-INH on tissue injury provide rationale for clinical testing, either alone or combined with resuscitation fluids, in a prehospital setting.

ACKNOWLEDGMENTS

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