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Decay-accelerating factor limits hemorrhage-instigated tissue injury and improves resuscitation clinical parameters

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ABSTRACT

Background: Complement is invariably activated during trauma and contributes to tissue injury. Recombinant human decay-accelerating factor (DAF), a complement regulatory protein that inhibits both classical and alternative pathways, improves survival and reduces tissue damage in animal models of tissue injury. The extent to which DAF may facilitate resuscitation in hemorrhaged large animals is not known.

Methods: Male Yorkshire swine assigned to one of six groups were subjected to controlled, isobaric hemorrhage over 15 min to a target mean arterial pressure (MAP) of 35 mm Hg. Hypotension was maintained for 20 min followed by a bolus intravenous injection of DAF or vehicle followed by Hextend resuscitation. Animals were observed for 3 h after hypotensive Hextend resuscitation. Survival, blood chemistry, and physiological parameters were recorded. Additionally, tissue from lung, small intestine, liver, and kidney were subjected to histopathologic evaluation and tissue deposition of complement proteins was determined by immunohistochemistry, dot-blot, and Western blot analyses.

Results: Administration of DAF (25 µg/kg) to animals subjected to hemorrhage prior to Hextend infusion significantly improved survival (73% versus 27%); protected gut, lung, liver, and kidney tissue from damage; and resulted in reduced resuscitation fluid requirements when compared with animals subjected to hemorrhage and resuscitation with Hextend alone. Animals treated with a higher dose of DAF (50 µg/kg) followed by Hextend fluid resuscitation did not experience the same benefit, suggesting a narrow therapeutic range for use of DAF as adjunct to Hextend fluid.

Conclusion: DAF improved survival and reduced early Hextend fluid resuscitation requirements in swine subjected to hemorrhagic shock. These benefits are attributed to decreased complement deposition and limited organ damage.

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1. Introduction

Hemorrhage is responsible for 50% of combat field deaths [1]. In an analysis of in-hospital deaths at a modern combat support hospital, Martin *et al.* found that deaths with high preventability scores (mean > 54) were primarily related to delays in hemorrhage control during the transportation (47%) or resuscitation phase (43%) [2]. A significant number of trauma victims that survive the initial hemorrhage subsequently die of some form of systemic inflammatory response due to complications of ischemia and/or reperfusion injury, ultimately leading to multiple organ dysfunction syndrome or acute respiratory distress syndrome [3,4]. This associated mortality secondary to hemorrhagic shock occurs despite seemingly acceptable medical therapy and surgical intervention [5]. Maintenance of tissue oxygenation with adequate fluid resuscitation is believed to be one of the critical elements in the management of hemorrhagic patients. However, the most effective resuscitation strategies remain controversial. Pope *et al.* demonstrated that conventional resuscitation strategies, such as intravenous administration of crystalloids and colloids, can exacerbate cell injury caused by hemorrhagic shock [6]. Therefore, strategies aimed at reducing or eliminating the need for resuscitation fluid infusion have been identified as a major requirement for both military and civilian emergency medicine [6].

Late morbidity and mortality following hemorrhage is an outcome of a vicious cycle involving the activation of multiple inflammatory pathways, including the complement and coagulation systems. We and others have reported that complement activation is critical in the pathogenesis of ischemia/reperfusion-instigated intestinal injury and hemorrhage-induced intestinal damage and inflammation in mice [7–9]. Both local and systemic complement activation are part of a common inflammatory cascade in the pathogenesis of tissue damage and organ dysfunction in the early phase after trauma [10,11]. Complement system activation in patients has been observed immediately after trauma and its activity correlates to the severity of the injury and complications [12].

Complement inhibition has been shown to be a promising therapeutic strategy in intestinal ischemia/reperfusion in mice and hemorrhagic shock in rats and swine [9,13,14]. Decay-accelerating factor (CD55/DAF) is a complement membrane protein that decays C3/5 convertases in both the classical and alternative pathways and prevents the assembly of the C3/5 convertases, thus blocking the formation of the generation of C3a and C5a and the formation of the membrane attack complex [15]. We have previously demonstrated that DAF treatment attenuates tissue damage and lowers tissue complement activation and deposition in hemorrhaged swine [16]. In this study, we evaluated the ability of DAF to reduce or eliminate Hextend fluid resuscitation requirements in swine subjected to controlled severe hemorrhage.

2. Materials and methods

After Institutional Animal Care and Use Committee approval, adolescent male swine underwent controlled isobaric arterial

hemorrhage with target mean arterial pressure of 35 mm Hg for 20 min followed by a bolus of DAF with or without Hextend infusion (Hospira, Lake Forest, IL), a plasma expander. All animals were cared for according to the Animal Welfare Act, the Animal Welfare Regulations, and the principles of the Guide for the Care and Use of Laboratory Animals.

2.1. Animal preparation and physiological monitoring

The swine were fasted overnight with free access to water. After induction of anesthesia (ketamine and xylazine), adolescent male Yorkshire cross-bred swine (12 wk old, 30–38 kg, *Sus scrofa*; ABI, Danboro, PA) underwent surgical placement of pre-hirudinized (100 µg Refludan/mL saline; ZLB Behring GmbH, Marburg, Germany) catheter (Starflex, 9Fr, 11 cm; Inver Grove Heights, MN) under sterile conditions into the left femoral artery and vein under isoflurane 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 hirudinized saline to ensure patency. Hemorrhage and withdrawal of the blood samples were performed via the artery. DAF and resuscitation fluid (Hextend) were administered into the vein. A micromanometer (MPC-500; Millar Instruments, Houston, TX) was inserted into the right femoral artery for hemodynamic monitoring by Computer Assisted Resuscitation Algorithm software (Walter Reed Army Institute of Research, MD).

2.2. Experimental design

The animals were hemorrhaged using a controlled, isobaric Wiggers model of controlled hemorrhagic shock (Fig. 1). The animals were enrolled in one of six experimental groups: (1) Control, sham operated (not hemorrhaged, $n = 5$); (2) H, hemorrhage + vehicle (60 mL saline) ($n = 6$); (3) H + Hex, hemorrhage + Hextend ($n = 11$); (4) H + D5 + Hex, hemorrhage + DAF (5 µg/kg body weight [b.w.]) + Hextend ($n = 7$); (5) H + D25 + Hex, hemorrhage + DAF (25 µg/kg b.w.) + Hextend ($n = 11$); (6) H + D50 + Hex, hemorrhage + DAF (50 µg/kg b.w.) + Hextend ($n = 6$). Each dose was infused in a total volume of 60 mL. Shed blood was not returned to the animal. The power analysis of the study required six swine per group.

The animals were spontaneously ventilated to represent a relevant prehospital setting where resuscitation fluids would be given. Controlled arterial hemorrhage was automated to insure reproducibility. In brief, a customized computer protocol (Labview v. 8.2; National Instruments, Austin, TX) monitored the mean arterial pressure (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, IL) connected to the femoral arterial catheter. At the start of the hemorrhage phase, the pump began withdrawing blood to decrease the MAP to the target pressure over a 15-min period. The same pump was used in the resuscitation phase. During the protocol, the volume of withdrawn blood or infused fluid was gravimetrically measured (PB5001-S; Mettler-Toledo, Columbus, OH) and the data output was stored on computer hard disk with time-stamped

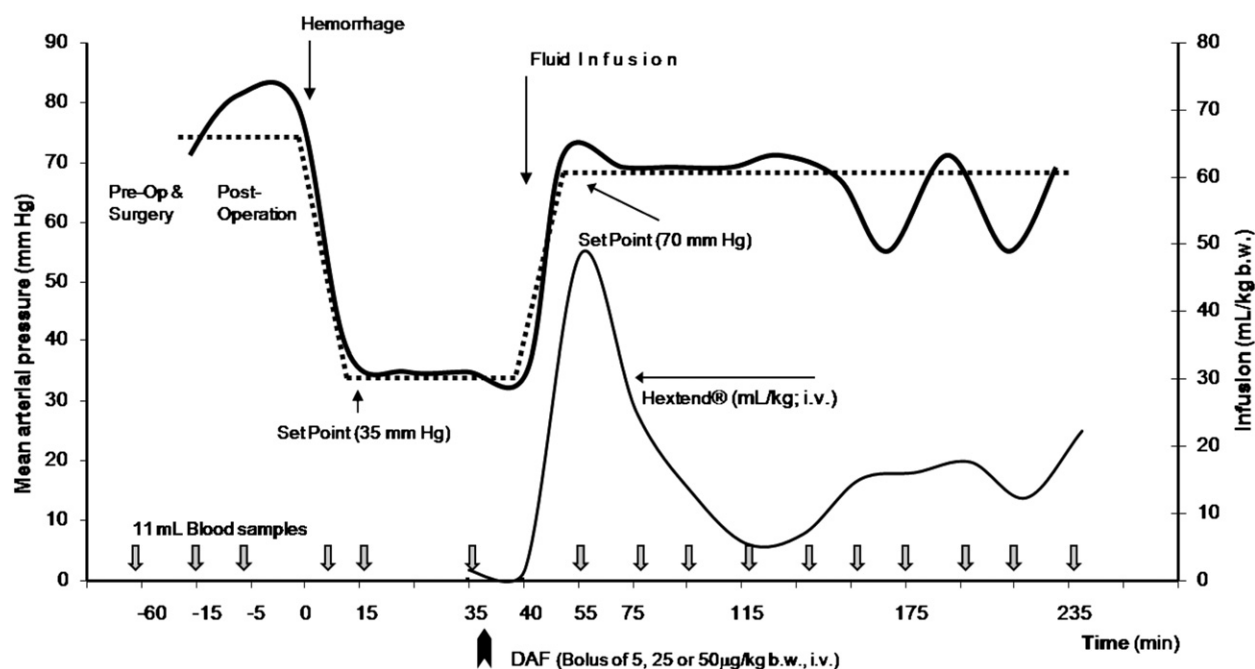


Fig. 1 – Scheme of the experimental design. Actual, target mean arterial blood pressure and the volume of infused Hextend are shown. Recombinant human DAF was given as a bolus in 60 mL of saline immediately before Hextend infusion.

hemodynamic parameters. The resuscitation phase started with a bolus of saline (60 mL, vehicle) or recombinant human DAF (rhDAF) in saline (60 mL) followed by Hextend infusion to the target MAP of 70 mm Hg over a 15-min period and then maintained at a minimum MAP of 70 mm Hg for 180 min. The resuscitation target pressure (70 mm Hg) corresponds with the physiological observation that blood flow through the heart and brain is maintained at normal levels above 70 mm Hg [17]. This hypotensive resuscitation strategy provides a consistent survival benefit independent of hemorrhage severity [18].

As seen in Figure 1, arterial blood samples were obtained prior to surgery (–60 min), prior to hemorrhage (–15 and –5 min prehemorrhage), hemorrhage to MAP of 35 mm Hg (5 and 15 min), immediately before resuscitation (35 min post-hemorrhage), resuscitation to MAP of 70 mm Hg (55 min post-hemorrhage, 20 min postresuscitation), and at 20-min intervals following resuscitation. Blood samples were analyzed for the following metabolic and hemodynamic parameters using i-STAT cartridges (Abbott Laboratories, Abbott Park, IL): pH, pO_2 , pCO_2 , base excess (BE), HCO_3^- , lactate, hematocrit (Hct), hemoglobin (Hb), and sodium (Na^+).

2.3. DAF dosage

Human DAF has cross-species reactivity [19]. The doses of DAF used in this study are based on our previous experience with this complement regulatory protein in rat hemorrhagic shock (unpublished) and a mouse model of ischemia/reperfusion [20]. The calculated concentration of DAF is based on the amount that induces a response halfway between the baseline and maximum responses (ED_{50}) using the lactate concentrations and fluid requirements observed in hemorrhaged animals resuscitated with Hextend alone and those who received DAF as well. Based on the fluid requirement, the ED_{50}

for DAF was 11.53 μ g and the lactate level–based ED_{50} was 9.61 μ g. The 95% confidence interval of the ED_{50} for DAF, based on the lactate level, completely overlapped with the 95% confidence interval of ED_{50} estimated by using the fluid requirement changes (not shown).

2.4. Tissue harvest

The animals were euthanized with isoflurane at the endpoint following the aforementioned procedures. Tissue samples, including lung, small intestine, liver, and kidney, were removed, frozen on dry ice, and either stored at -80°C for determining protein expression or fixed with 10% formalin or 4% paraformaldehyde for histologic and immunohistochemical analysis.

2.5. Tissue protein extraction

Frozen tissue samples were thawed, washed with ice-cold phosphate-buffered saline (PBS), suspended in RIPA buffer containing protease inhibitors (2 μ g/mL aprotinin, 10 μ M leupeptin, 1 mM phenylmethylsulfonyl fluoride), and minced on ice. The samples were sonicated on ice (Ultrasonics Co, Danbury, CT) at setting 5, for 4×10 s at continuous output with 10-s pauses between the cycles. The samples were then centrifuged at 13,000 rpm for 10 min at 4°C . The supernatants were frozen and stored at -80°C until assayed. Aliquots were used to determine protein concentration.

2.6. Reagents and antibodies

Recombinant human DAF/CD55 and mouse anti-human DAF were obtained from R&D Systems (Minneapolis, MN).

Hetastarch, 6% in lactated electrolyte injection (Hextend), was obtained from Hospira, Inc (Berkeley, CA). CG4+ and CG8+ cartridges were purchased from Abbott (Princeton, NJ). Chicken anti-C3/C3a, mouse anti-C5/C5a, mouse anti-C5b-9, and mouse anti-endothelial cell antibodies were obtained from Abcam Inc (Cambridge, MA). Streptavidin Alexa Fluor 594, goat anti-chicken Alexa Fluor 594, goat anti-mouse Alexa Fluor 488, and goat anti-mouse Alexa Fluor 594 IgG (H + L) conjugated secondary antibodies and ProLong Gold antifade reagent were from Invitrogen (Carlsbad, CA).

2.7. Western blotting

Tissue extracts were separated by electrophoresis on sodium dodecyl sulfate–polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with Tween-20 for 1 h, then incubated with primary antibodies for 1 h followed by incubation with appropriate horseradish peroxidase–conjugated secondary antibodies for 1 h. Protein bands were visualized by the ECL method (Amersham Biosciences, Piscataway, NJ) and captured with a Fujifilm LAS-3000 System configured for chemiluminescence (Fujifilm Life Science, Edison, NJ). The density of each band was measured using QuantityOne software (BioRad, Hercules, CA).

2.8. Dot-blotting

For efficient transfer of proteins onto nitrocellulose membrane, Dot Blot 96 System (Labrepco Inc, Horsham, PA) was used. Briefly, 200 μ L of sample containing 1 μ g protein was loaded into each well using multichannel pipettors; the sample was then vacuumed with a vacuum pump. After the membrane dried, it was blocked with 5% nonfat dry milk in Tris-buffered saline–Tween-20 for 1 h at room temperature. It was then incubated with primary antibodies for 1 h followed by incubation with appropriate horseradish peroxidase–conjugated secondary antibodies for 1 h at room temperature. Specific protein dots were visualized by the ECL method (Amersham Biosciences) and captured with a Fujifilm LAS-3000 System configured for chemiluminescence (Fujifilm Life Science). The density of each dot was measured using QuantityOne Software (BioRad).

2.9. Histologic examination

Ten percent formalin-fixed tissues were embedded in paraffin, sectioned, and stained with hematoxylin-eosin (H&E). Histologic images were recorded under a light microscope (Olympus AX80; Olympus, Center Valley, PA) by a pathologist masked to the treatment group. Histologic injury scores were graded according to the following:

- For lung injury, scoring was performed as previously described [21]. Four parameters (alveolar fibrin edema, alveolar hemorrhage, septal thickening, and intra-alveolar inflammatory cells) were scored on each H&E-stained slide for: (1) severity (0: absent; 1, 2, and 3 for more severe changes); and (2) extent of injury (0: absent; 1: <25%; 2: 25%–50%; 3: >50%). Total injury score for each slide was

calculated as the sum of the extent plus the severity of injury.

- Mucosal damage of small intestine for each slide was graded on a six-tiered scale [9]. A score of 0 was assigned to a normal villus; villi with tip distortion were scored as 1; villi lacking goblet cells and containing Guggenheim spaces were scored as 2; villi with patch disruption of the epithelial cells were scored as 3; villi with exposed but intact lamina propria and epithelial cell sloughing were assigned a score of 4; villi in which the lamina propria was exuding were scored as 5; villi displaying hemorrhage or denuded were scored as 6. Neutrophil infiltration of small intestine and lung in the H&E-stained slides was assessed at 1000 $\times$ magnification with 10 randomly selected fields per sample.
- Four parameters were considered for hepatic injury severity score, as follows: vascular congestion, hepatocyte death, degeneration, and inflammation were assayed for severity (score 0 for no change, score 1, 2, 3 for more severe changes) and for extent of injury (0: absent; 1: <25%; 2: 25%–50%; 3: >50%).
- Renal damage was scored based on the following criteria: (1) severity (0 = normal; 1 = slight alteration [loss of brush border, mild hydropic degeneration]; 2 = mild [intensive hydropic degeneration, mild vacuolization]; 3 = moderate [shrunken nuclei, intensive vacuolization]; 4 = severe [necrotic/apoptotic cells, denudation/rupture of basement membrane]; 5 = necrosis [total necrosis of the tubule]); and (2) extent of injury (0: absent; 1: <25%; 2: 25%–50%; 3: >50%).

2.10. Immunohistochemical staining

Paraformaldehyde-fixed lung and small intestine biopsies were snap-frozen at -70°C , and sections were cut at 5 μ m thickness 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, then blocked with 2% bovine serum albumin in PBS for 30 min at room temperature. The sections were incubated with the primary antibodies (anti-C5b-9, C3a, and C5a) overnight at 4°C , washed, and then incubated with the appropriate secondary antibodies labeled with Alexa Fluor 488 and 594 for 1 h 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) at $\times 400$ magnification. Negative controls were conducted by substituting the primary antibodies with corresponding immunoglobulin isotypes. Captured digital images were processed by Image J software (NIH, Bethesda, MD).

2.11. Immunofluorescent quantification

This procedure was based on a modified method as previously described [20]. 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.

2.12. Statistical analysis

Data are expressed as mean $\pm$ SEM. The log-rank test or 1- or 2-way analysis of variance (ANOVA) followed by Bonferroni and Tukey *post hoc* tests were performed using GraphPad Prism (4.0; GraphPad Software, San Diego, CA). *P* value < 0.05 was considered as significant.

3. Results

3.1. DAF prolongs Hextend fluid–achieved resuscitation of swine subjected to hemorrhage

Animals were subjected to controlled bleeding to a MAP target fixed at 35 mm Hg for 20 min followed by bolus intravenous injection of saline or DAF (5, 25, 50 $\mu\text{g/kg}$), as outlined in Figure 1. This procedure was not lethal during the 200-min posthemorrhage observation period. The administration of Hextend resuscitation fluid alone resulted in a 27% survival rate (three out of 11 animals survived the observation period) (Fig. 2). The average time to death for Hextend fluid–resuscitated animals was 161 ± 17 min. Interestingly, the combination of DAF (25 $\mu\text{g/kg}$) along with Hextend fluid increased the survival rate to 73% (Fig. 2) with an average time of death of 203 ± 20 min. Administration of a higher dose of DAF (50 $\mu\text{g/kg}$) did not accomplish the same clinical benefit.

3.2. Recombinant human DAF deposits in porcine tissues

Deposition of rhDAF in intestine and lung was determined by immunohistologic staining. As shown in Figure 3, DAF deposition in the small intestine and lung of DAF-treated animals was observed in a dose-dependent manner. No DAF deposition was evident in the sham- and saline-treated animals.

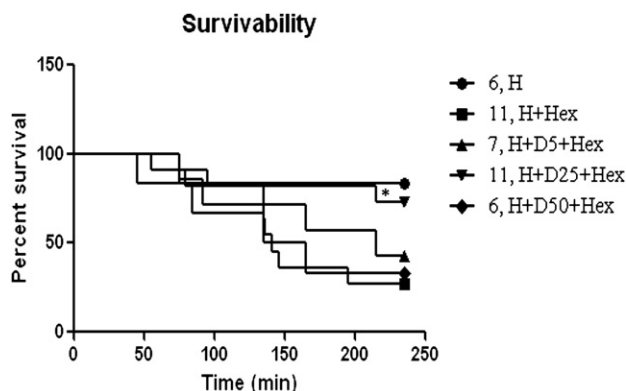


Fig. 2 – DAF with Hextend resuscitation improves survival of hemorrhaged animals. Kaplan-Meier survival curves.
**P* < 0.05 versus Hex (log-rank test).

3.3. DAF reduces required Hextend resuscitation fluid volumes

To determine whether DAF treatment reduces the resuscitation fluid volume requirements, we calculated the ratio of cumulative fluid (Hextend) infusion (mL/kg b.w.) to total blood loss for each group at 20, 80, 140, and 200 min from the onset of resuscitation (Fig. 4A). Both lower doses of DAF (5 and 25 $\mu\text{g/kg}$) reduced significantly the volume requirement for resuscitation fluids at 80 min (H + D5 + Hex and H + D25 + Hex versus H + Hex, *P* < 0.05). Although not significant, a reduction of cumulative fluid volume in the H + D25 + Hex group was also observed at 140 and 200 min. The highest dose of DAF (50 $\mu\text{g/kg}$) did not reduce fluid volume requirements when compared with the H + Hex group.

In this model, animals that underwent hemorrhage followed by Hextend resuscitation developed dilutional anemia compared with animals subjected to hemorrhage without resuscitation, demonstrated by the decreased Hct and Hb 80 min from the beginning of resuscitation (Fig. 4B and C) (H + Hex versus H, *P* < 0.05). Treatment with moderate (25 $\mu\text{g/kg}$ DAF) but not with lower or higher doses of DAF corrected the anemia (Fig. 4B and C), correlating with the reduced volume of fluid infusion observed with this dose of DAF (Fig. 4A).

3.4. DAF does not alter baseline metabolic parameters

No differences were observed in weight, total shed blood, MAP, shock index (SI), pulse pressure (PP), arterial pH, pO_2 , sO_2 , pCO_2 , BE, HCO_3^- , lactate, Hb, Hct, or K^+ during the baseline phase of prehemorrhage among all experimental groups (Sham, hemorrhage, and DAF-treated groups) (Tables 1 and 2, Fig. 4B and C). In the posthemorrhage phase, all experimental groups (excluding Sham) had similar values of SI, PP, pH, pO_2 , sO_2 , pCO_2 , Hb, Hct, and K^+ . However, groups differed with respect to BE, HCO_3^- , and lactate in the posthemorrhage phase. For example, resuscitation with Hextend improved BE as well as HCO_3^- levels in comparison with hemorrhage without resuscitation. Interestingly, administration of DAF increased lactate levels during the posthemorrhage phase compared with H and H + Hex.

3.5. DAF improves hemodynamic parameters during resuscitation

As shown in Tables 1 and 2, many statistically significant hemodynamic and metabolic changes occurred. The Hextend-resuscitated group had a statistically higher MAP compared with the H group (H + Hex: 66.79 ± 3.38 versus H: 44.51 ± 1.34 , *P* < 0.05) at 80 min postresuscitation. Hextend infusion significantly improved SI (H + Hex: 1.25 ± 0.05 versus H: 2.9 ± 0.51 , *P* < 0.05) and PP (H + Hex: 43.20 ± 4.63 versus H: 19.94 ± 1.72 , *P* < 0.05). Hextend combined with DAF significantly elevated MAP of the hemorrhaged animals compared with the nontreated Hemorrhage group and further increased MAP in comparison to the group treated with Hextend only (H + D5 + Hex: 71.09 ± 1.0 , H + D25 + Hex: 69.61 ± 0.33 , H + D50 + Hex: 69.36 ± 0.68 versus H: 44.51 ± 1.34 , H + Hex: 66.79 ± 3.83 , *P* < 0.05). Administration of 5 $\mu\text{g/kg}$ DAF and

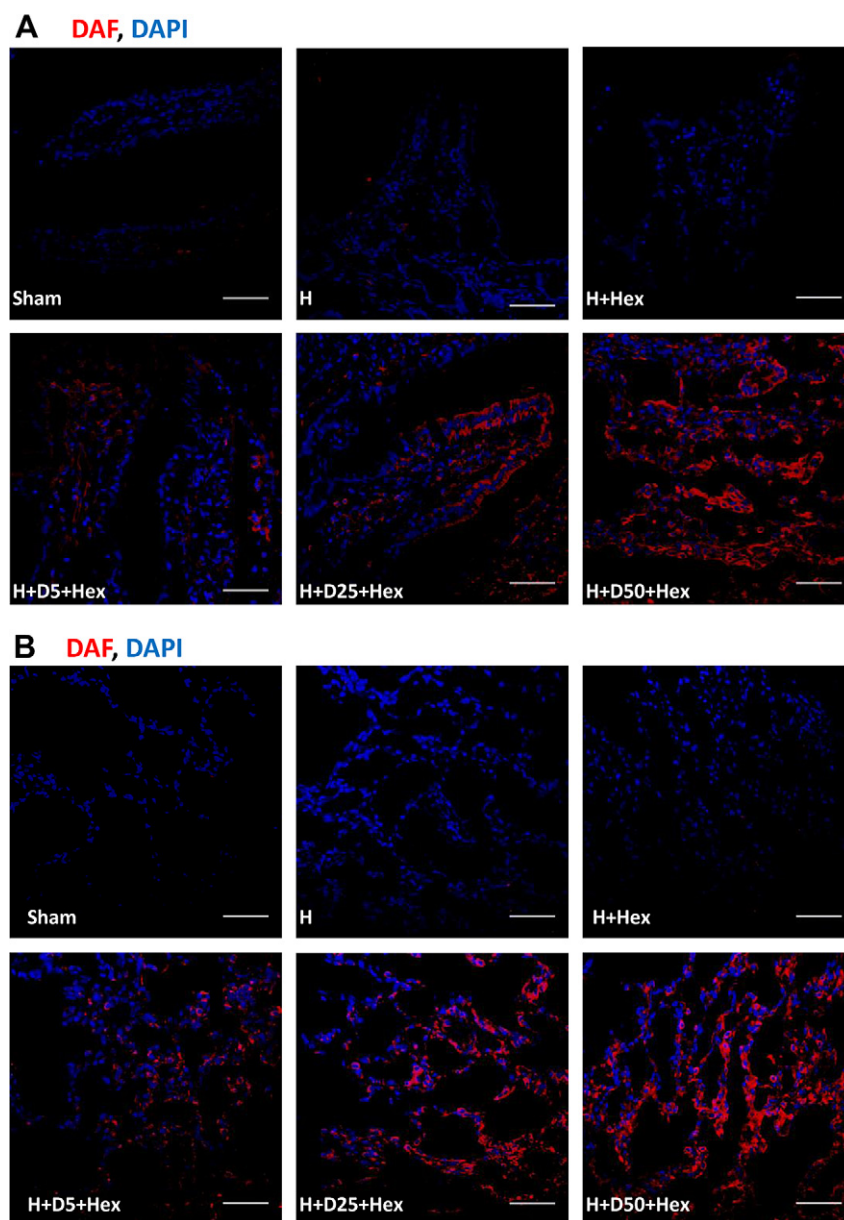


Fig. 3 – DAF is deposited in small intestinal and lung tissues of DAF-treated animals with hemorrhage and Hextend resuscitation. Administration of rhDAF displayed tissue deposition in a dose-dependent manner in small intestine (A) and lung (B). Original magnification $\times 400$. Scale bars in the pictures, 50 μm .

Hextend significantly decreased blood K^+ level when compared with the H group (H + D5 + Hex: 3.88 ± 0.12 versus H: 4.68 ± 0.25 , $P < 0.05$). There were no clear differences in other metabolic parameters between the animals treated with vehicle, Hextend, or DAF + Hextend.

3.6. DAF limits hemorrhage/resuscitation-induced intestinal damage

Figure 5 is representative of the tissue damage observed in intestinal tissue from hemorrhage and Hextend-resuscitated animals. The intestinal tissue from the animals subjected to hemorrhage and Hextend resuscitation exhibited

remarkable mucosal villi loss in nearly all regions accompanied by massive inflammatory cell infiltration, necrosis, and moderate hemorrhage in the mucosa and submucosa layers. Furthermore, the crypts were severely damaged when compared with the Sham animals (injury scores: 4.86 ± 0.34 for H + Hex versus 1.0 ± 0.41 for Sham, $P < 0.05$). Interestingly, Hextend resuscitation did not improve intestinal damage (H: 4.9 ± 0.38 versus H + Hex: 4.86 ± 0.34 , $P > 0.05$). The hemorrhaged animals treated with DAF (5 $\mu\text{g}/\text{kg}$ or 25 $\mu\text{g}/\text{kg}$) immediately before Hextend resuscitation displayed mitigated intestinal injury (injury scores: H + D5 + Hex: 3.14 ± 0.26 versus H: 4.9 ± 0.38 , $P < 0.05$; H + D25 + Hex: 1.88 ± 0.3 versus H + Hex: 4.86 ± 0.34 , $P < 0.05$). However, the highest dose of

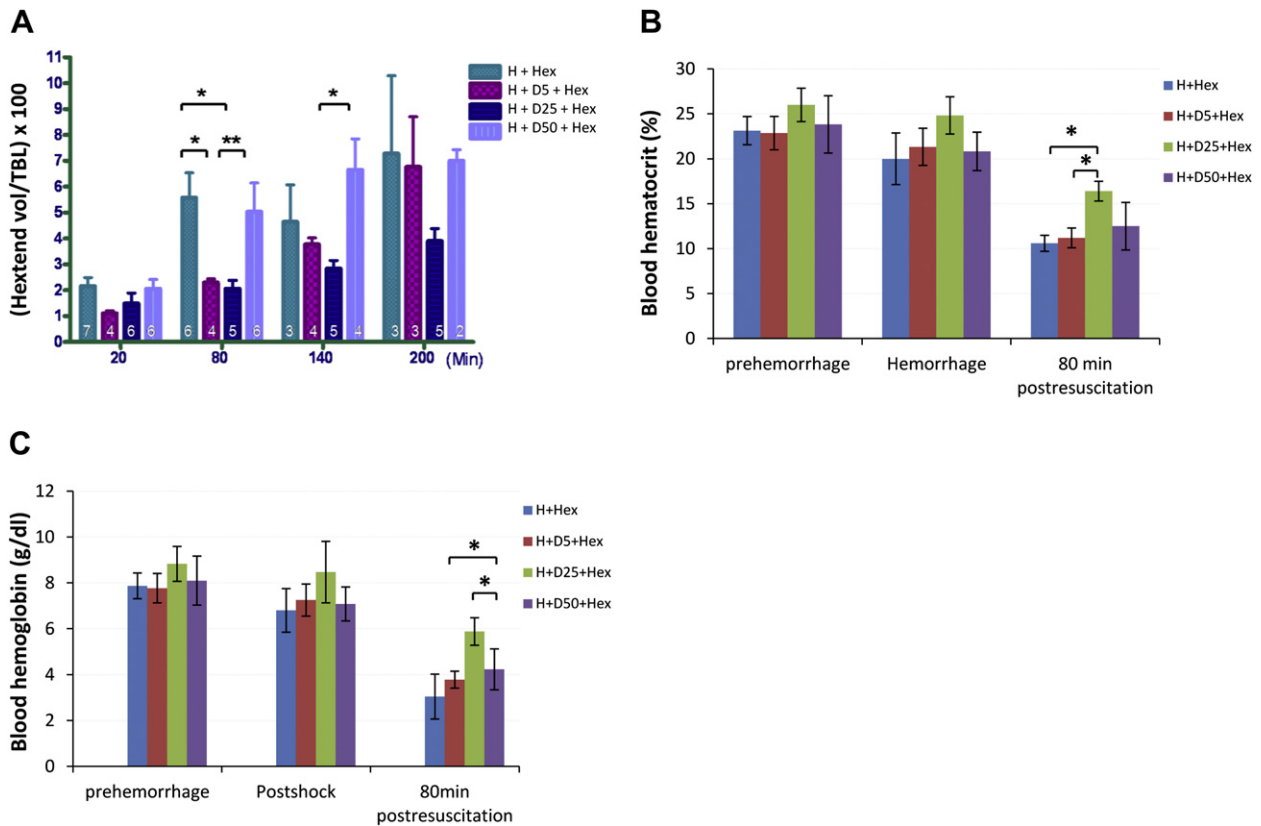


Fig. 4 – DAF treatment reduces fluid requirement and corrects diluted anemia in porcine hemorrhagic shock managed with hypotensive resuscitation. (A) The ratio of cumulative fluid infusion to total blood loss (TBL) was calculated for each group at 20, 80, 140, and 200 min after the onset of resuscitation started with DAF bolus. Mean $\pm$ SEM are given. The number of animals is given on the bars. **(B)** Porcine blood hematocrit. **(C)** Porcine blood hemoglobin. Mean $\pm$ SEM are given. The number of animals is given on the bars. * $P < 0.05$; ** $P < 0.01$ (2-way ANOVA; Bonferroni post hoc tests).

Table 1 – Pre- and posthemorrhage hemodynamic changes.

	Group						P
	Sham	H	H + Hex	H + D5 + Hex	H + D25 + Hex	H + D50 + Hex	
n	5	6	11	7	11	6	
Body weight (kg)	34.85 $\pm$ 1.12	33.55 $\pm$ 0.56	33.05 $\pm$ 0.74	34.66 $\pm$ 0.49	36.13 $\pm$ 1.33	34.52 $\pm$ 0.76	ns
Total shed blood (mL)	n/a	858.87 $\pm$ 104.85	787.92 $\pm$ 49.56	1001.27 $\pm$ 63.06	773.89 $\pm$ 104.37	784.95 $\pm$ 42.85	ns
Prehemorrhage	69.68 $\pm$ 2.52	84.02 $\pm$ 5.72	79.19 $\pm$ 3.98	79.88 $\pm$ 3.85	82.58 $\pm$ 4.30	81.79 $\pm$ 7.90	ns
MAP (mm Hg)							
Posthemorrhage	76.66 $\pm$ 3.14*	36.26 $\pm$ 0.35	35.31 $\pm$ 0.42	35.77 $\pm$ 0.85	37.00 $\pm$ 1.21	35.03 $\pm$ 1.03	s
MAP (mm Hg)							
Final MAP (mm Hg)	57.97 $\pm$ 1.94*	44.51 $\pm$ 1.34	66.79 $\pm$ 3.83 [†]	71.09 $\pm$ 1.00 [†]	69.61 $\pm$ 0.33 [†]	69.36 $\pm$ 0.68 [†]	s
Prehemorrhage SI	1.10 $\pm$ 0.05	0.9 $\pm$ 0.05	0.96 $\pm$ 0.04	0.94 $\pm$ 0.05	0.91 $\pm$ 0.05	0.91 $\pm$ 0.05	ns
Posthemorrhage SI	1.02 $\pm$ 0.06*	2.34 $\pm$ 0.27	2.40 $\pm$ 0.19	2.80 $\pm$ 0.33	1.99 $\pm$ 0.13	2.36 $\pm$ 0.29	s
Final SI	1.23 $\pm$ 0.07 [†]	2.90 $\pm$ 0.51	1.25 $\pm$ 0.05 [†]	1.29 $\pm$ 0.11 [†]	1.39 $\pm$ 0.17 [†]	1.24 $\pm$ 0.05 [†]	s
Prehemorrhage	45.07 $\pm$ 2.75	40.03 $\pm$ 2.13	42.71 $\pm$ 1.90	40.98 $\pm$ 3.49	43.20 $\pm$ 1.77	41.82 $\pm$ 1.53	ns
PP (mm Hg)							
Posthemorrhage	41.5 $\pm$ 4.04*	16.01 $\pm$ 1.46	21.44 $\pm$ 2.27	15.77 $\pm$ 2.20	22.90 $\pm$ 1.59	19.32 $\pm$ 1.69	s
PP (mm Hg)							
Final PP (mm Hg)	41.5 $\pm$ 2 [†]	19.94 $\pm$ 1.72	43.20 $\pm$ 4.63 [†]	44.41 $\pm$ 4.83 [†]	44.35 $\pm$ 2.86 [†]	48.18 $\pm$ 2.74 [†]	s

n = number of animals; ns = not significant; s = significant ($P < 0.05$); H = hemorrhage; D = DAF.

Data are expressed as mean $\pm$ SEM.

Group data were compared using 1-way ANOVA (MedCalc; MedCalc Software bvba, Mariakerke, Belgium).

* $P < 0.05$ versus all hemorrhaged swine.

[†] $P < 0.05$ versus H.

Table 2 – Pre- and posthemorrhage metabolic changes.

	Group						P
	Sham	H	H + Hex	H + D5 + Hex	H + D25 + Hex	H + D50 + Hex	
n	5	6	11	7	11	6	
Prehemorrhage pH	7.45 ± 0.01	7.43 ± 0.02	7.45 ± 0.02	7.42 ± 0.02	7.43 ± 0.01	7.42 ± 0.01	ns
Posthemorrhage pH	7.46 ± 0.02	7.39 ± 0.02	7.42 ± 0.01	7.42 ± 0.03	7.42 ± 0.02	7.41 ± 0.03	ns
Final pH	7.50 ± 0.02	7.46 ± 0.02	7.43 ± 0.02	7.49 ± 0.01	7.46 ± 0.02	7.46 ± 0.02	ns
Prehemorrhage pO ₂ (mm Hg)	70.15 ± 3.56	82.75 ± 3.36	86.46 ± 3.27	106.71 ± 23.57	80.96 ± 5.59	82 ± 4.50	ns
Posthemorrhage pO ₂ (mm Hg)	73.70 ± 3.97	79.67 ± 5.01	76.75 ± 3.21	69.50 ± 3.86	75.68 ± 3.21	74.17 ± 5.05	ns
Final pO ₂ (mm Hg)	69.85 ± 3.12	93.20 ± 5.72	80.33 ± 7.19	76.90 ± 4.37	88.22 ± 5.35	91.13 ± 5.06	ns
Prehemorrhage sO ₂ (%)	93.30 ± 1.16	95.92 ± 0.71	96.41 ± 0.45	96.14 ± 0.99	92.05 ± 4.19	95.67 ± 0.75	ns
Posthemorrhage sO ₂ (%)	94.30 ± 1.02	94.83 ± 0.91	94.60 ± 0.69	93.17 ± 1.19	94.14 ± 1.20	93.92 ± 1.25	ns
Final sO ₂ (%)	94.50 ± 0.89	97.40 ± 0.51	93.22 ± 2.93	95.90 ± 0.68	96.72 ± 0.76	97.00 ± 0.71	ns
Prehemorrhage pCO ₂ (mm Hg)	47.28 ± 1.82	46.95 ± 2.76	49.34 ± 1.94	49.18 ± 1.33	48.48 ± 1.15	47.37 ± 2.07	ns
Posthemorrhage pCO ₂ (mm Hg)	46.55 ± 1.98	46.65 ± 1.74	49.73 ± 1.91	45.48 ± 3.07	44.63 ± 1.45	45.78 ± 4.13	ns
Final pCO ₂ (mm Hg)	42.64 ± 2.13	41.74 ± 2.56	45.90 ± 1.56	43.30 ± 0.97	42.90 ± 1.96	41.80 ± 1.93	ns
Prehemorrhage base excess (mEq/L)	6.04 ± 0.35	3.79 ± 0.98	6.34 ± 0.40	4.76 ± 1.22	5.23 ± 0.40	3.45 ± 0.42	ns
Posthemorrhage base excess (mEq/L)	6.14 ± 0.30	0.90 ± 1.56*	5.57 ± 0.36	2.36 ± 0.87*	3.86 ± 0.85*	2.12 ± 0.77*	s
Final base excess (mEq/L)	6.65 ± 0.32	2.93 ± 0.70*†	6.08 ± 0.42	8.08 ± 0.30	5.66 ± 0.58	4.92 ± 0.57	s
Prehemorrhage bicarbonate (mM)	32.76 ± 0.39	30.73 ± 0.82	33.45 ± 0.62	31.86 ± 1.16	31.86 ± 0.64	30.56 ± 0.62	ns
Posthemorrhage bicarbonate (mM)	32.83 ± 0.51	28.09 ± 1.39*	31.75 ± 0.67	28.98 ± 0.52*‡	28.88 ± 1.06*‡	28.78 ± 0.96*	s
Final bicarbonate (mM)	33.03 ± 0.50	29.20 ± 0.75*	31.01 ± 0.69	32.67 ± 0.26	30.29 ± 1.18	29.81 ± 0.59	s
Prehemorrhage lactate (mM)	0.95 ± 0.11	1.14 ± 0.13	0.83 ± 0.08	0.89 ± 0.08	1.16 ± 0.16	1.37 ± 0.20	ns
Posthemorrhage lactate (mM)	1.18 ± 0.14	2.18 ± 0.64	1.99 ± 0.28	4.08 ± 0.74*	3.11 ± 0.60*	3.11 ± 0.23*	s
Final lactate (mM)	1.34 ± 0.15	2.20 ± 0.47	2.28 ± 0.51	2.63 ± 0.55	2.41 ± 0.78	3.44 ± 1.60	ns
Prehemorrhage K ⁺ (mM)	3.83 ± 0.08	4.02 ± 0.13	3.96 ± 0.10	3.74 ± 0.06	3.96 ± 0.04	4.14 ± 0.07	ns
Posthemorrhage K ⁺ (mM)	4.04 ± 0.09	4.95 ± 0.35*	4.53 ± 0.10*	4.63 ± 0.23*	4.90 ± 0.14*	4.74 ± 0.12*	s
Final K ⁺ (mM)	4.21 ± 0.08	4.68 ± 0.25§	4.19 ± 0.09*	3.88 ± 0.12	4.41 ± 0.05	4.32 ± 0.20	s

n = number of animals; ns = not significant; s = significant ($P < 0.05$); H = hemorrhage; D = DAF.

Data are expressed as mean ± SEM.

Groups were compared using 1-way ANOVA (MedCalc; MedCalc Software bvba, Mariakerke, Belgium).

* $P < 0.05$ versus Sham.

† $P < 0.05$ versus Sham, H + D5 + Hex.

‡ $P < 0.05$ versus H + Hex.

§ $P < 0.05$ versus Sham, treated animals.

DAF (50 µg/kg) followed by Hextend fluid resuscitation did not show significant protection against intestinal damage when compared with the Hextend alone. As expected, the injury observed in the H and H + Hex groups had high neutrophil infiltration, which was significantly attenuated in animals treated with lower doses of DAF (5 µg/kg or 25 µg/kg) but not with the highest dose (50 µg/kg) (Fig. 5C).

3.7. DAF complement activation and deposition in intestinal tissues

To understand whether DAF exerts its major molecular function on complement inhibition, we determined deposition of C3 and C5 as well as formation of C5b-9 in the intestinal tissue by immunohistofluorescent staining and dot-blot (Fig. 6). We found that deposition of C3 and C5 as well as

formation of C5b-9 were significantly higher in the small intestine from all hemorrhaged animals when compared with the Control (sham) group (Fig. 6A–D). The majority of C5b-9 in the small intestine tissue was co-localized with the vascular endothelium (Fig. 6C).

Hextend resuscitation further enhanced hemorrhage-induced C3 and C5 deposition in the intestinal tissue (Fig. 6A–C). We also observed an increased C5b-9 deposition in small intestine from the H + Hex group when compared with the H group (Fig. 6C). The hemorrhaged animals treated with the combination of DAF (5 or 25 µg/kg) and Hextend had significantly reduced intestinal deposition of C3, C5, and C5b-9 compared with the H + Hex group (Fig. 6A–D). The highest dose of DAF (50 µg/kg) combined with Hextend did not decrease deposition of C3 and C5 (Fig. 6A and B) but reduced the amount of C5b-9 (Fig. 6C and D).

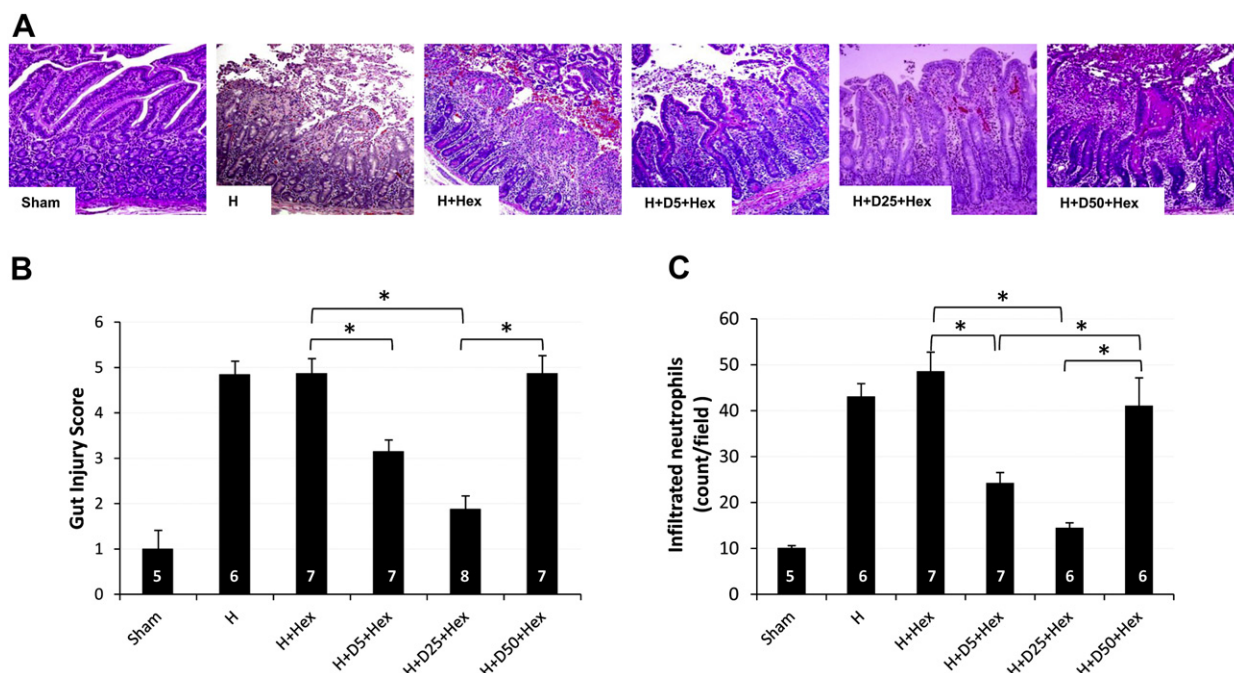


Fig. 5 – DAF treatment alleviates hemorrhage/resuscitation-mediated porcine intestinal injury. (A) Representative histologic changes of porcine small intestines are shown. Original magnification $\times 100$. **(B)** Intestinal injury scores. **(C)** Neutrophil infiltration was counted at $1000\times$ magnification in 10 randomly selected fields and is expressed as the number of neutrophils per field. Group data were expressed as mean $\pm$ SEM and compared using 1-way ANOVA followed by Tukey multiple comparison test. $*P < 0.05$.

3.8. DAF limits hemorrhage and Hextend resuscitation-induced lung injury

Animals subjected to hemorrhage exhibited marked lung injury characterized by destruction of the alveolar architecture with mild alveolar hemorrhage and severe inflammation, as seen in H&E-stained histologic images of the lung and overall tissue injury score (Fig. 7A and B). There was no clear difference in lung injury score between the H and H + Hex groups. In contrast, administration of DAF (5 $\mu\text{g/kg}$ and 25 $\mu\text{g/kg}$) prior to resuscitation with Hextend fluid clearly attenuated lung damage induced by hemorrhage (H + D5 + Hex: 3.29 ± 0.42 and H + D25 + Hex: 2.38 ± 0.42 versus H: 4.83 ± 0.31 , $P < 0.05$) or hemorrhage with Hextend resuscitation (H + D5 + Hex: 3.29 ± 0.42 and H + D25 + Hex: 2.38 ± 0.42 versus H + Hex: 5.0 ± 0.38 , $P < 0.05$). Treatment with a higher dose of DAF (50 $\mu\text{g/kg}$) in the Hextend-resuscitated animals did not show improvement of tissue damage when compared with the H + Hex group. As shown in Figure 7C, The H and H + Hex groups demonstrated marked neutrophil infiltration in lung tissue, which was significantly attenuated by treatment with lower doses of DAF (5 $\mu\text{g/kg}$ and 25 $\mu\text{g/kg}$) but not with the highest dose of DAF (50 $\mu\text{g/kg}$).

3.9. DAF limits complement activation and deposition in the lung tissue

Hextend infusion did not significantly alter hemorrhage-induced deposition of C3 (Fig. 8A) and C5 (Fig. 8C) or the

formation of C5b-9 (Fig. 8D), though a trend towards higher C3a generation in lung tissue from the H + Hex when compared with the H group was observed (Fig. 8B). Lower doses of DAF (5 or 25 $\mu\text{g/kg}$), but not 50 $\mu\text{g/kg}$, followed by Hextend resuscitation noticeably decreased the deposition of C3 and C5 as well as the generation of C5b-9 and C3a compared with H + Hex (Fig. 8). The observed distribution pattern of C5b-9 in the lung tissue was associated with the vascular endothelium.

3.10. DAF suppresses hepatic and renal injury

As shown in Figure 9A and B, animals subjected to hemorrhage showed marked hepatocyte hydropic alteration and vascular congestion. Administration of Hextend resuscitation significantly exacerbated this tissue damage, characterized by cell vacuolization, necrosis/apoptosis, and inflammatory cell infiltration (H + Hex: 6.26 ± 0.42 versus H: 4.17 ± 0.31 , $P < 0.05$). Interestingly, the administration of DAF at the lower doses (5 and 25 $\mu\text{g/kg}$), but not the higher dose (50 $\mu\text{g/kg}$), before resuscitation significantly attenuated the cell-injurious effects of Hextend and resulted in well-preserved hepatic structures.

As shown in Figure 9C and D, hemorrhaged animals demonstrated marked renal tubular injury with loss of brush border, hydropic degeneration, and vascular congestion when compared with the sham group. Hextend resuscitation further aggravated tubular damages, with additional damage observed as cellular vacuolization, necrosis/apoptosis,

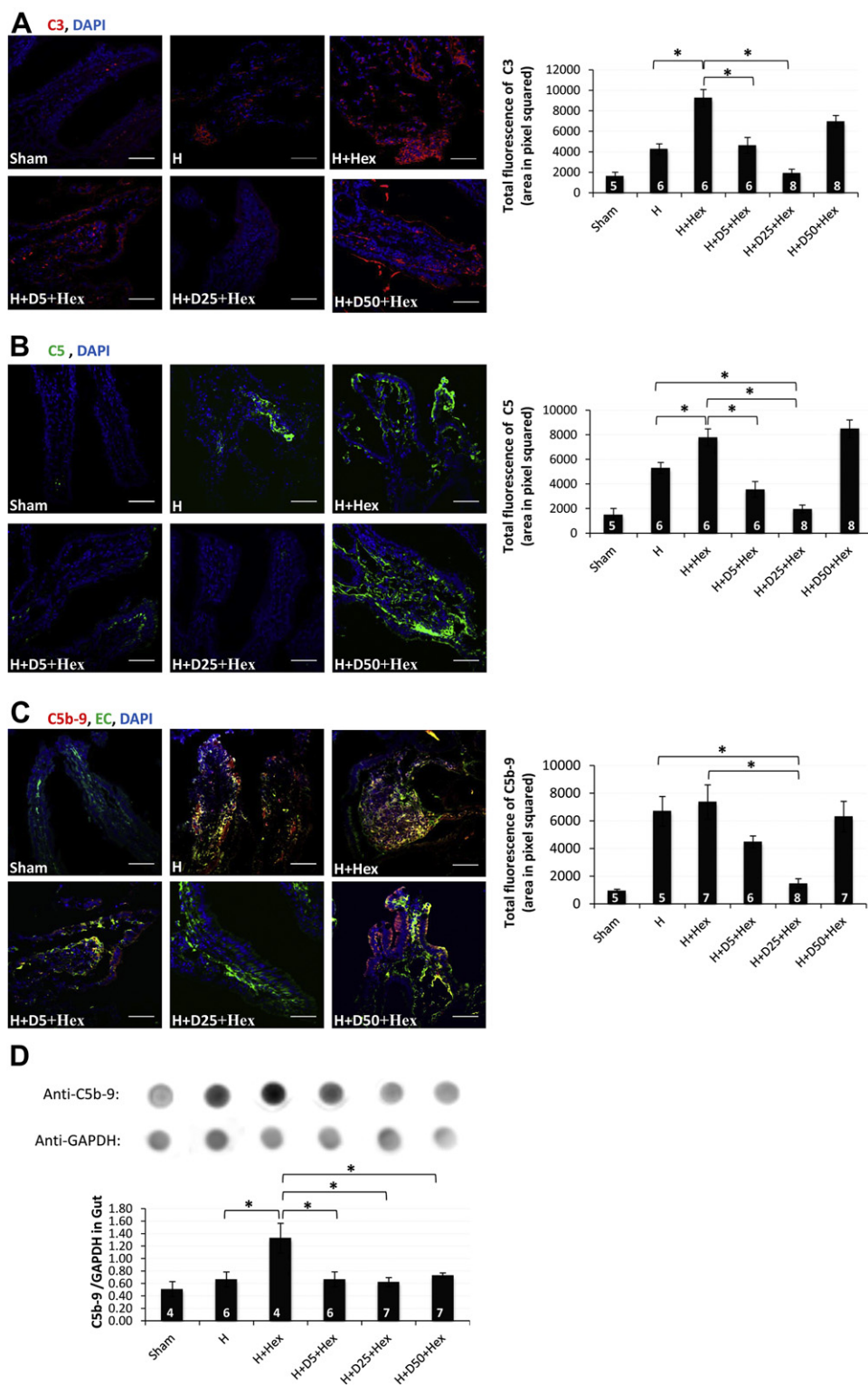


Fig. 6 – DAF treatment limits complement deposition and activation in intestinal tissue from hemorrhaged animals with Hextend resuscitation. Representative immunohistochemistry for C3 deposition (A, top), C5 deposition (B, top), and C5b-9 formation/distribution (C, top) in the small intestine. The total fluorescence quantification of the aforementioned complement deposition is shown at the bottom of the photographs. Original magnification $\times 400$. Scale bars in the pictures, $50\ \mu\text{m}$. C5b-9 level in the intestinal tissue was detected by dot-blot (D). Group data were expressed as mean $\pm$ SEM and compared using 1-way ANOVA followed by Tukey multiple comparison test. $*P < 0.05$.

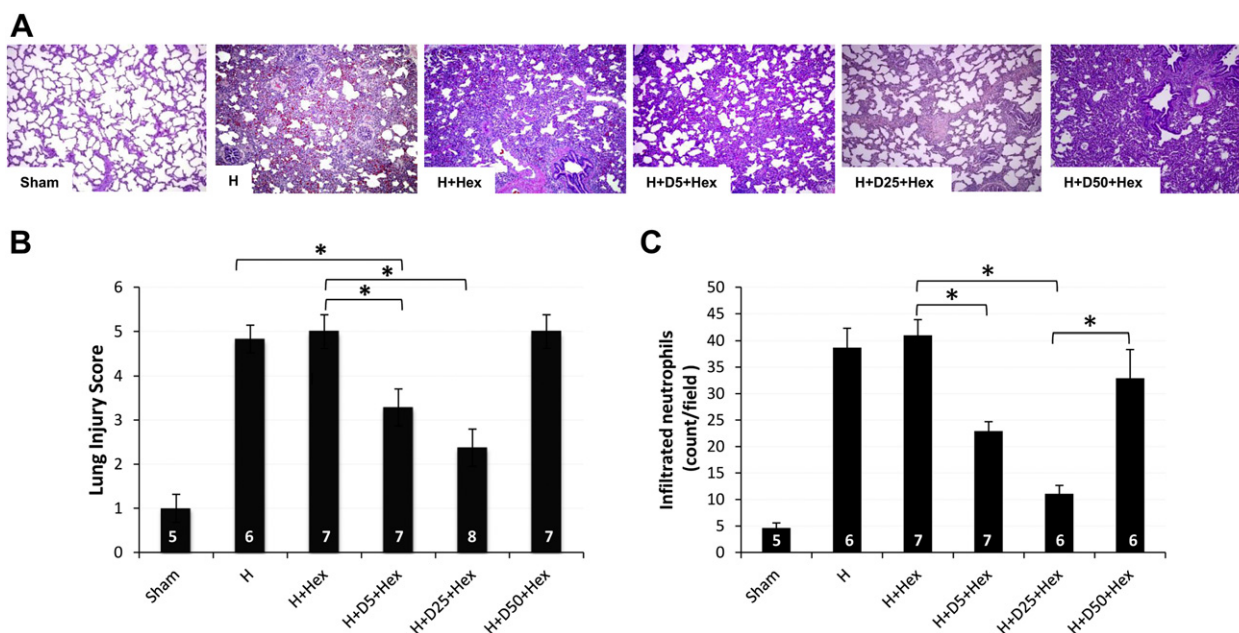


Fig. 7 – DAF treatment mitigates lung injury induced by hemorrhage combined with Hextend in a swine hemorrhagic model. (A) Representative histologic sections with H&E stain from the porcine lungs are shown. Original magnification $\times 100$. **(B)** Lung injury scores. **(C)** Neutrophil infiltration was counted at $\times 1000$ magnification in 10 randomly selected fields and is expressed as the number of neutrophils per field. Group data were expressed as mean $\pm$ SEM and compared using 1-way ANOVA followed by Tukey multiple comparison test. * $P < 0.05$.

interstitial edema, and mononuclear cell infiltration (H + Hex: 3.29 ± 0.29 versus H: 1.83 ± 0.29 , $P < 0.05$). However, lower doses of DAF (5 and 25 $\mu\text{g/kg}$) significantly limited the aforementioned alterations (H + D5 + Hex: 2 ± 0.31 and H + D25 + Hex: 1.25 ± 0.16 versus H + Hex: 3.29 ± 0.29 , $P < 0.05$).

4. Discussion

The present study was designed to investigate the therapeutic potential of complement inhibition in a resuscitated porcine model of controlled hemorrhage. Complement activation has been an important contributor to the pathophysiology of hemorrhagic shock. Complement activation significantly contributes to the mechanisms of systemic and local post-injury complications, such as ischemia/reperfusion injury, sepsis, and multiple organ failure [7,22]. As an ongoing effort to learn more about the role of complement activation in hemorrhagic shock and the possibility to manipulate its activation, we specifically investigated the effect of DAF, an inhibitor of the classic and alternative complement pathways, on animal survival, hemodynamic and metabolic parameters, and tissue injury in a controlled swine hemorrhagic shock model. Unfortunately, because of the lethality of Hextend infusion, this animal model has limited our ability to discriminate all of the beneficial effects of complement inhibition. However, despite the lethality of Hextend infusion, the administration of DAF after injury provided several survival benefits.

Lower and moderate doses of DAF were able to improve cell tolerance to shock, affording vital organ protection and

increased survival during the early phase of traumatic hemorrhage. Specifically, our findings indicate: (1) bolus injection of a moderate dose of DAF (25 $\mu\text{g/kg}$) significantly increases survival in the hemorrhaged animal resuscitated with Hextend; (2) administration of low and moderate doses of DAF (5 and 25 $\mu\text{g/kg}$, respectively) reduces fluid volume requirement and corrects dilutional anemia in hemorrhaged animals resuscitated with Hextend; (3) low and moderate doses of DAF (5 and 25 $\mu\text{g/kg}$) in Hextend-resuscitated animals remarkably limit tissue injury; and (4) deposition and activation of complement at the tissue level were suppressed by low and moderate doses of DAF.

Hypotensive fluid resuscitation is one of the critical treatment strategies for hemorrhagic shock in restoring intravascular volume and maintaining vital organ perfusion [23]. Hypotensive fluid resuscitation has been an attractive hemorrhage control strategy as it can: (1) minimize dilutional anemia and coagulopathy; and (2) minimize logistical challenges in prehospital care, specifically for the military. As recently as 2011, Tactical Combat Casualty Care guidelines identified Hextend as the prehospital fluid of choice. Although prehospital fluids have been widely used, their benefit in improving survival in trauma patients has yet to be fully established or characterized. Most of the emphasis of the benefits of fluid replacement therapy has been based on positive outcomes seen in animal models of controlled hemorrhage [24]. The data presented here suggest that hypotensive fluid resuscitation with Hextend provides no benefit in hemorrhage control. The porcine model of controlled hemorrhage used in this study was not lethal (five out of six swine, 83%). However, resuscitation with Hextend

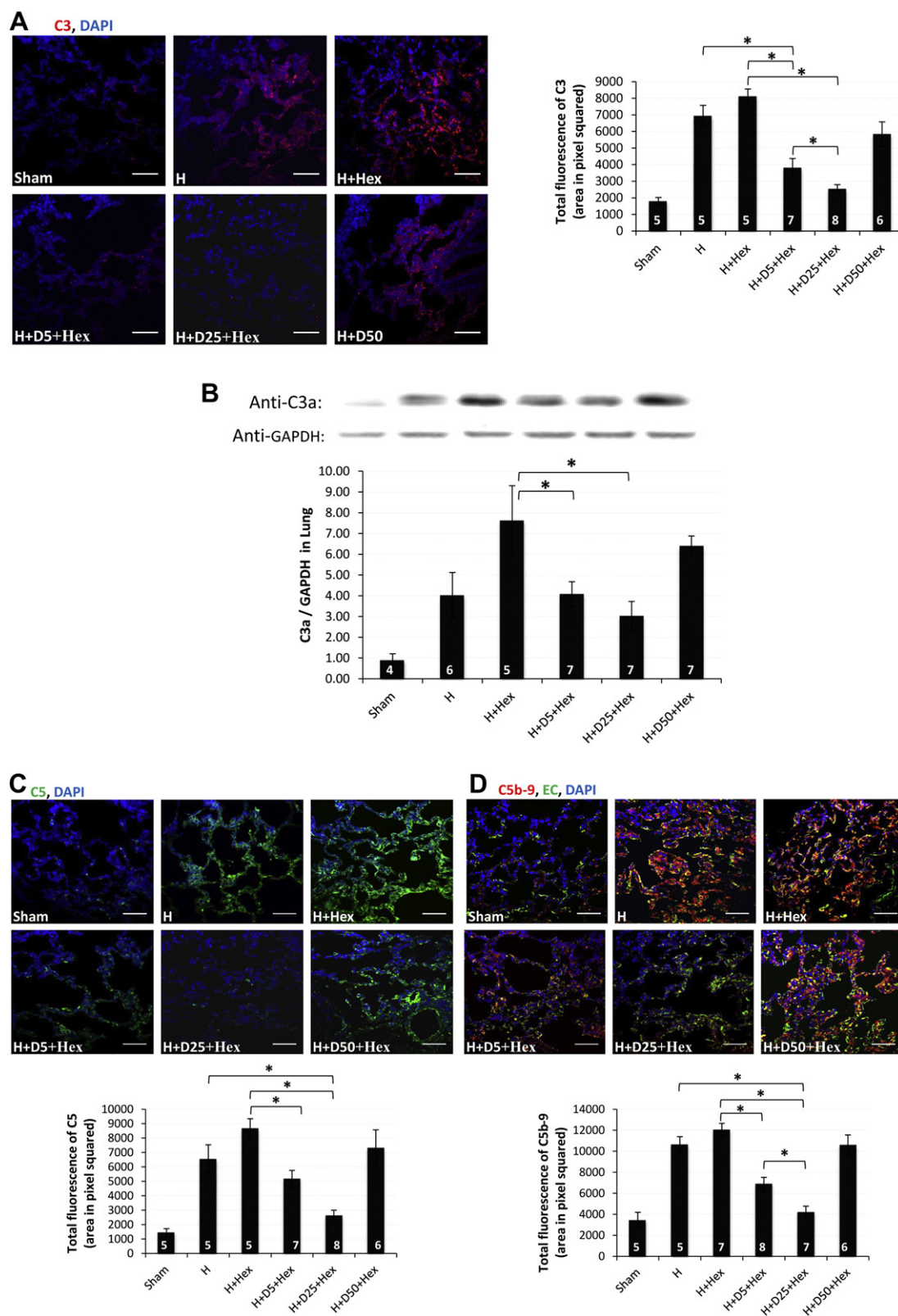


Fig. 8 – DAF treatment attenuates complement deposition and activation in lungs from Hextend-resuscitated animals. Representative immunohistochemistry for C3 deposition (A), C5 deposition (C), and C5b-9 formation/distribution (D) in the lung tissues. The total fluorescent quantification of the aforementioned complement deposition is displayed at the bottom of the photographs. Original magnification $\times 400$. Scale bars in the pictures, 50 μm . Local complement C3a generation was measured by Western blot (B). Group data were expressed as mean $\pm$ SEM and compared using 1-way ANOVA followed by Tukey multiple comparison test. * $P < 0.05$.

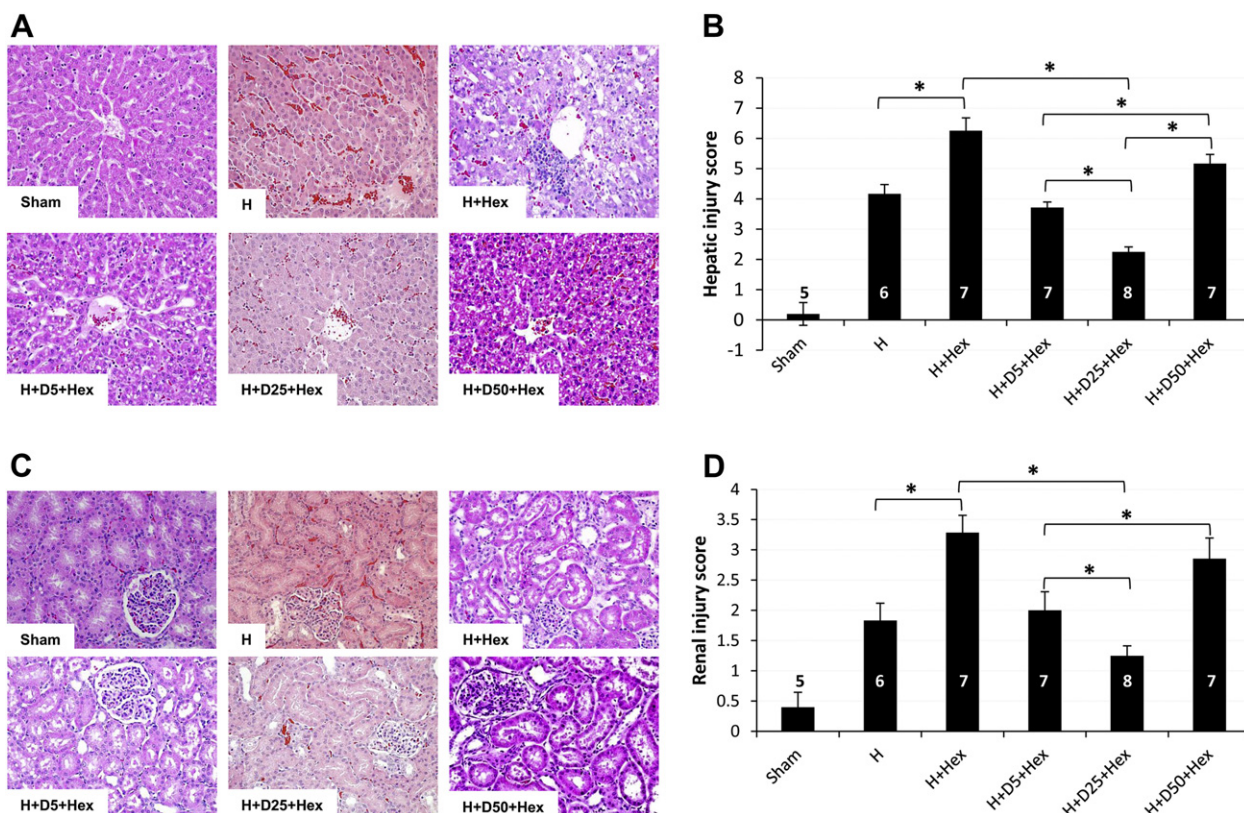


Fig. 9 – DAF treatment reduces hepatic and renal injury after hemorrhage followed by Hextend resuscitation in pigs. (A) Representative liver histologic images. (B) Hepatic injury scores. (C) Representative kidney histologic images. (D) Renal injury scores. Original magnification $\times 400$. Data were expressed as mean $\pm$ SEM. Group data were compared using 1-way ANOVA with Bonferroni *post hoc* test. $^{\dagger}P < 0.05$, H + Hex versus H.

was lethal (three out of 11 swine, 27%), suggesting that spontaneous compensation of the volume lost after hemorrhage might be more beneficial than resuscitation with Hextend (Fig. 2). Resuscitation with Hextend did not reduce hemorrhage-induced tissue damage but, interestingly, contributed to further renal and hepatic injury, supporting limited use of Hextend hypotensive resuscitation.

Many of the beneficial effects of DAF observed in this model may be the result of its ability to lower the amount of Hextend administered. The exact mechanism in which DAF maintains homeostasis and effectively lowers the requirement for fluid resuscitation is currently unknown. Nevertheless, it can be speculated that the inhibition of complement activation by DAF limits local C3a/C5a and membrane attack complex (MAC) production as well as MAC accumulation on the endothelium, which could be playing a major role in maintaining vascular integrity and effectively limiting the loss of fluids from the intravascular space. Alternatively, the lower fluid requirements could be a result of the interplay between the complement system and coagulation system. Both cascades contain a series of serine proteases with evidence of shared activators and inhibitors. Examples include factor (F) XIIa, activating complement factor (C)1q, and C1 esterase inhibitor. C1 esterase inhibitor acts not only as an inhibitor for two established complement pathways but also for the endogenous coagulation activation pathway. In addition, the powerful anaphylatoxins (C5a and C3a)

generated by early hyperactivation of complement contribute to the disturbance of the coagulation system. In particular, thrombin has been found to induce expression of DAF, which in turn helps maintain vascular integrity [25]. Therefore administration of DAF could potentially contribute to maintenance of vascular integrity through preservation of the coagulation system.

Resuscitation with Hextend failed to reduce hemorrhage-induced damage in the small intestine and lung tissue. However, administration of DAF (5 and 25 $\mu\text{g}/\text{kg}$) prior to Hextend infusion decreased tissue damage in hemorrhaged animals when compared with those resuscitated only with Hextend. In our study, complement was significantly increased in hemorrhaged animals, a result that is in alignment with clinical studies showing that complement is activated immediately after injury and that the severity of the trauma is directly proportional to the level of complement activation [10]. The lower dose range of DAF decreased the generation of C3a and C5 as well as the formation of C5b-9 in the small intestine and the lung in Hextend-resuscitated animals.

DAF inhibits complement activation, limiting local C3a/C5a and MAC production, thereby blocking the downstream signaling of the C5a/C5a receptor (C5aR) and MAC formation [15], resulting in the prevention of local and remote organ injury. Taken together, these findings raise the possibility that the beneficial effects of DAF reported in the present study relate to its immune-inflammatory modulatory actions.

DAF (5 and 25 $\mu\text{g/kg}$) decreased initial fluid requirements, but the higher dose of DAF (50 $\mu\text{g/kg}$) failed to produce similar benefits. Although we cannot explain with certainty the underlying mechanisms of these findings, one possibility is that the high dose of DAF combined with Hextend induced hydroxyethyl starch deposition in the interstitial and intracellular spaces of liver, kidney, and spleen (data not shown) via reduction of plasma opsonins (C3b, iC3b, C4b) and/or increased binding of DAF to C3b. The hydroxyethyl starch deposition may worsen organ dysfunction by triggering resident macrophage activation, which would in turn lead to cytokine release and tissue inflammation [26]. This could also cause severe portal hypertension, affecting blood redistribution/mobilization from relatively less vital organs to more vital organs in hemorrhaged animals. Another possibility is that the high dose of DAF may increase its ability to interact with CD97 and this interaction would contribute to leukocyte activation, adhesion, migration, and cytokine release [27]. The unexpected result could also be due to DAF activating leukocytes by interaction with lipid rafts [28], tyrosine kinases [29], and T cell receptor [30] of leukocytes via its membrane anchor domain (C-terminal). Another possibility could be that the high dose of DAF may have caused translocation of microorganisms in the Hextend-infused animals since bacteria/viruses are able to bind to SCR domains of DAF [31]. However, these assumptions are speculative and need further investigation as higher dosages of DAF (50 $\mu\text{g/kg}$) in an unresuscitated hemorrhage swine model produced more beneficial effects in comparison to low and moderate dosages (5 and 25 $\mu\text{g/kg}$) [16].

The concept of controlling complement activation in order to control fluid requirements and correct dilutional anemia is quite interesting, given that complement is a central regulator of the immune response. Despite its generic beneficial functions, including pathogen elimination and immediate response to danger signals, complement activation appears to be exerting detrimental effects after trauma, mounting innocent bystander attacks on host tissue. Conventional fluid resuscitation is designed to reestablish tissue perfusion, but it fails to prevent inflammatory responses and coagulopathy. Conversely, fluid resuscitation can further increase inflammatory responses and worsen coagulopathy, which could be more dangerous than the original injury. Complement inhibition presents a novel pharmacologic modality that can help maintain homeostasis not only by decreasing the excessively generated systemic inflammatory response after major trauma but by preventing further damage by decreasing fluid resuscitation requirements, effectively reducing mortality and morbidity of severely injured casualties.

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