Bovine polymerized hemoglobin (hemoglobin-based oxygen carrier-201) resuscitation in three swine models of hemorrhagic shock with militarily relevant delayed evacuation—Effects on histopathology and organ function*

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Objective: **To test our hypothesis that hemoglobin-based oxygen carrier (HBOC)-201 resuscitation in hemorrhagic shock (HS) will not lead to increased organ injury and dysfunction.**

Design: **Three swine HS models simulating military-relevant delayed evacuation: a) moderate controlled HS, b) severe controlled HS, and c) severe uncontrolled HS.**

Setting: **Military research laboratory.**

Subjects: **Swine.**

Interventions: **Swine were anesthetized/intubated and instrumented. To induce HS, in two controlled hemorrhage experiments, 40% (moderate controlled HS) or 55% (severe controlled HS) of blood volume was withdrawn; in an uncontrolled HS experiment, the liver was crushed/lacerated. During a 4-hr "prehospital phase," pigs were resuscitated with HBOC-201 (HBOC) or Hextend (HEX) or were nonresuscitated (NON). Upon "hospital arrival," liver injury was repaired (severe uncontrolled HS), blood or saline was infused, hemodynamics were monitored, and blood was collected. Upon animal death and/or 72 hrs, necropsy was followed by histopathologic evaluation of organ injury (hematoxylin and eosin, electron microscopy) and immunohistochemistry of oxidative potential (3-nitrotyrosine). Significance ($p < .05$) was assessed by Kruskal-Wallis, analysis of variance/Bonferroni, and mixed procedure tests.**

Measurements and Main Results: **Survival was significantly higher with HBOC than HEX only with severe uncontrolled HS ($p = .002$). Myocardial necrosis/fibroplasia, fluid requirements, cardiac output, and cardiac enzymes were generally similar or lower in HBOC than HEX pigs, but creatine kinase-MB (but not creatine kinase-MB/creatine kinase ratio) was higher with HBOC in moderate controlled HS. Alveolar/interstitial pulmonary edema was similar with HBOC and HEX, but Po_2 was higher with HBOC in severe uncontrolled HS. Jejunal villar epithelial and hepatocellular necrosis were similarly minimal to moderate in all groups. Minimal biliary changes occurred exclusively with HBOC. Aspartate aminotransferase, lactate dehydrogenase, and alkaline phosphatase were generally higher with HBOC than HEX. Mild renal papillary injury occurred more frequently with HBOC, but consistent patterns for urine output, blood urea nitrogen, and creatinine, were not seen. The 3-nitrotyrosine staining intensity was not different.**

Conclusions: **In comparison with hetastarch, HBOC-201 resuscitation of swine with HS increased survival (with severe HS), did not increase evidence of oxidative potential, and had histopathologic and/or functional effects on organs that were clinically equivocal (myocardium, lungs, hepatic parenchyma, jejunum, and renal cortex/medulla) and potentially adverse (hepatobiliary and renal papilla). The effects of HBOC-201-resuscitation in HS should be corroborated in controlled clinical trials. (Crit Care Med 2006; 34:1464–1474)**

KEY WORDS: hemoglobin-based oxygen carrier; HBOC-201, hemorrhagic shock; resuscitation; histopathology; organ function

Trauma is the leading cause of mortality among young adults and causes significant disability among survivors (1). Hemorrhage accounts for 28–30% of combat and civilian trauma deaths as well as the preponderance of salvageable deaths (2–4). While securing hemostasis, blood transfusion can be life-saving pending definitive surgical stabilization. Standard prehospital treatment of hemorrhagic shock (HS) includes resuscitation with crystalloid and colloid fluids, but packed red blood cells and whole blood have been touted as superior resuscitative agents. Unfortunately, prehospital deployment of blood transfusion capability is logistically complex, requires additional training, and is rarely

*See also p. 1566.

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available. Maximizing prehospital resuscitation is paramount for optimizing outcome. One can invest in upgrading in-hospital medical techniques, but without successful prehospital resuscitation, most efforts will be fruitless (5, 6).

Hemoglobin-based oxygen carrier (HBOC)-201 (Hemopure, Cambridge, MA) is a polymerized bovine, oxygen-transporting, room temperature stable, and universally compatible hemoglobin substitute that improves outcome in animal HS models (7–14), and it has been evaluated in nontrauma phase I–III clinical trials (15–17). However, HBOCs have been associated with vasoconstriction and secondary consequences, oxidative stress, renal dysfunction, and elevation of hepatic and pancreatic blood tests (18–20). Herein, we report HBOC-201's effects on histopathologic and associated functional variables as a prehospital resuscitation fluid in swine with HS using both controlled and uncontrolled hemorrhage models.

MATERIALS AND METHODS

Animal Studies

Animal Preparation. The experiments were conducted according to principles set forth in the *Guide for the Care and Use of Laboratory Animals*, Institute of Laboratory Animals Resources, National Research Council, National Academy Press, 1996; approved by the Walter Reed Army Institute of Research Institutional Animal Care and Use Committee; and performed in an animal facility approved by the Association for Assessment and Accreditation for Laboratory Animal Care International.

Animals were sedated and anesthesia was induced with intramuscular ketamine hydrochloride (33 mg/kg), atropine sulfate (0.05 mg/kg), and mask ventilation with isoflurane (3.0%) and 100% oxygen. Anesthesia was maintained via isoflurane (1–2.5%) in 21% oxygen. Pigs were ventilated for anesthesia-induced apnea. The right external jugular vein and carotid artery were isolated aseptically. An introducer sheath was placed in the external jugular vein and a pulmonary artery catheter inserted for hemodynamic monitoring. An angiocatheter was placed in the carotid artery, and mean arterial pressure (MAP) was transduced. Urine was collected via bladder catheterization.

Controlled Hemorrhage and Tissue Injury. Pigs were hemorrhaged 40% (moderate controlled HS) or 55% (severe controlled HS) of estimated total blood volume to induce HS; concomitantly, the left rectus abdominis muscle was mobilized and a standardized crush soft tissue injury made with a Kocher clamp. Shed blood was collected in bags containing citrate phosphate dextrose for later reinfusion. The soft tissue injury and initiation of hemorrhage denoted time 0.

Uncontrolled Hemorrhage Due to Liver Injury. In the severe uncontrolled HS model, a standardized liver injury was created by placing a ring clamp over the left lateral lobe, adjusting for hepatic size and pig weight, and lacerating through the remaining width. The liver injury denoted start of the prehospital phase (time 0). After the clamp was removed, the remaining tissue was excised, resulting in a 25% lobectomy (grade III liver injury). Bleeding was spontaneous and quantified by peritoneal suction.

"Prehospital" Resuscitation. Pigs were randomly allocated to one of three treatment groups: HBOC-201 in lactated Ringer's solution (HBOC; specifications: hemoglobin 13 g/dL, P_{50} 40 mm Hg, average molecular weight 250 kD, 290–310 mOsmM); 6% hetastarch in lactated Ringer's solution (Hextend, Abbott Laboratories, Abbot Park, IL) (HEX; specifications: 307 mOsmM); or no fluids (NON). Subsequent to controlled or uncontrolled hemorrhage (20 and 15 mins, respectively), resuscitated pigs were administered HBOC or HEX (10 mL/kg). Reinfusions (5 mL/kg) occurred between 30 and 180 mins for hypotension or tachycardia.

"Hospital Care." Hospital arrival was simulated at 4 hrs. Animals were administered shed blood or allogeneic packed red blood cells for anemia, saline for hypotension, cephazolin, and buprenorphine. Catheters were removed, a jugular vein introducer was secured for postoperative blood sampling and fluid administration, and surgical incisions were repaired. Animals were extubated and recovered from anesthesia. Clinical status was followed up to 72 hrs. Pigs continued to receive blood and saline as needed for hypotension and anemia, antibiotics, and analgesics. Wellness was assessed (moderate controlled HS) by documentation of vomiting incidence, food consumption, and activity scores. After sedation, surviving pigs were killed at 72 hrs for necropsy. Study designs have been published by Gurney et al. (14) and Philbin et al. (21).

Blood Samples

Blood urea nitrogen (BUN), creatinine, aspartate transaminase (AST), alkaline phosphatase (AP), lactate dehydrogenase (LDH), and creatine kinase (CK) were measured on a Vitros 250 (dry chemistry, Ortho-Clinical Diagnostics, Raritan, NJ), and reported only for plasma hemoglobin (B-hemoglobin method, Hemocue, Angelholm, Sweden) under interference levels (22, 23); CK-MB by fluorescence enzyme immunoassay with anti-CK-MB coated microparticles (Axsym, Abbott Laboratories, Abbott Park IL); and troponin I by chemiluminescence with Access AccuTnI immunoassay (Beckman Coulter, Brea, CA).

Histopathology

Necropsy was performed on 72-hr survivors and early deaths. Gross evaluations were performed and severity semiquantitatively scored. Standardized specimens of myocardial left ventricle (LV) free wall, lung, jejunum, liver, and kidney, and all gross lesions were collected/preserved in 10% neutral buffered formalin. Myocardial right ventricular (RV) free wall and ventricular septum (VS) sections were added midway through the study. Specimens were routinely processed, embedded in paraffin, microtomed (6 μm), stained with hematoxylin and eosin, and examined by light microscopy (LM).

LM histopathologic lesions were identified, recorded, and semiquantitatively scored. Lesion scores were based on percentage of tissue involvement and severity of cellular changes: 0, none; 1, minimal; 2, mild; 3, moderate; 4, marked; 5, severe. Myocardial necrosis/degeneration and repair scores were assigned as follows: 0, none; 1, rare foci of few affected myocytes (<1%); 2, multiple small to moderate foci involving 1–5% of myocytes; 3, multiple foci involving 6–25% of myocytes; 4, multiple and confluent foci involving 26–50% of myocytes; and 5, confluent foci involving >50% of myocytes. Scores were defined similarly for other lesions.

Standardized lung sections were also collected/fixed in 4F1G fixative (4% paraformaldehyde/1% gluteraldehyde) overnight, postfixed in 2% osmium tetroxide, and dehydrated in graded alcohols and embedded in Epon 812. Block sections (1 μ) were examined by LM, and thin (90 nm) sections stained with lead citrate and uranyl acetate and examined with a LEO 912 AB electron microscope (LEO Electron Microscopy, Thornwood, NY).

3-Nitrotyrosine

Tissues (moderate controlled HS) were covered with Tissue-Tek OCT Compound embedding medium, frozen in liquid nitrogen, and stored at −80°C. Frozen tissues were sliced (~5-μM sections), adhered to glass slides, and acetone-fixed. After washing and incubation, slides were incubated with anti-nitrotyrosine antibody solution (Cayman Chemical, Ann Arbor, MI), rinsed, reincubated in R-Phycoerythrin-conjugated Affinipure F $(ab')_2$ Fragment Goat Anti-Mouse IgG solution (Jackson ImmunoResearch Laboratories, West Grove, PA), rinsed (phosphate-buffered saline), and stored overnight at 4°C; slides were viewed using an Olympus BX51 fluorescent microscope (Olympus, Melville, NY), and relative intensity of experimental vs. control tissue ranked (1–4 scale).

Data Analysis

As there were sufficient 72-hr survivors in key groups in the two controlled HS models, reported histopathology data are for pigs necropsied at 72 hrs. As few HEX pigs survived 72 hrs in severe uncontrolled HS, reported histopathology data are for all pigs (early deaths

Table 1. Survival differences

	HS Severity/Hemorrhage Mechanism	Survival HBOC	Survival HEX	Survival NON	Sig (p) Overall (HBOC vs. HEX)
Controlled hemorrhage	Moderate/catheter withdrawal, 40% EBV (%)	8/8 (100)	7/8 (88)	5/8 (63)	.10 (>.05)
	Severe/catheter withdrawal, 55% EBV (%)	8/8 (100)	6/8 (75)	2/8 (25)	.007 (>.05)
Uncontrolled hemorrhage	Severe/grade III liver injury (%)	7/8 (88)	1/8 (13)	1/8 (13)	.002 (.004)

HS, hemorrhagic shock; HBOC, hemoglobin based oxygen carrier; HEX, Hextend; NON, not resuscitated; EBV, estimated blood volume.
Survival to 72 hrs in swine models with *moderate controlled HS*, *severe controlled HS*, and *severe uncontrolled HS*.

Table 2. Myocardium and lung changes

	Moderate Controlled HS Model, 72-Hr Survivors				Severe Controlled HS Model, 72-Hr Survivors				Severe Uncontrolled HS Model, All Pigs			
	Treatment Group			Sig (*p*) Overall	Treatment Group			Sig (*p*) Overall	Treatment Group			Sig (*p*) Overall
	HBOC n = 8	HEX n = 6–7	NON n = 4–5	(HBOC vs. HEX)	HBOC n = 8	HEX n = 6	NON n = 2	(HBOC vs. HEX)	HBOC n = 8	HEX n = 8	NON n = 8	(HBOC vs. HEX)
Myocardium												
LV												
Necrosis												
Rate, %	13	57	25	>.05 (.08)	13	17	50	>.05	13	38	50	>.05
SS	0.3 ± 0.3	1.6 ± 0.7	0.3 ± 0.3	>.05	0.1 ± 0.1	0.2 ± 0.2	1.5 ± 1.5	.07	0.3 ± 0.3	0.9 ± 0.4	0.6 ± 0.3	>.05
Fibroplasia												
Rate, %	**0**	**43**	0	>.05 **(≤.05)**	13	0	50	>.05	13	0	13	>.05
SS	**0**	**1.68 + 0.8**	**0**	**≤.05 (≤.05)**	0.1 ± 0.1	0	1.5 ± 1.5	>.05	0.3 ± 0.3	0	0.4 ± 0.4	>.05
RV												
Necrosis												
Rate, %					0	33	50	>.05	38	0	13	>.05 (.06)
SS					0	0.7 ± 0.4	1.5 ± 1.5	.09	0.8 ± 0.4	0	0.1 ± 0.1	>.05
Fibroplasia												
Rate, %					25	33	100	>.05	25	13	0	>.05
SS					0.4 ± 0.3	0.7 ± 1.0	2.5 ± 0.5	>.05	0.6 ± 0.4	0.3 ± 0.3	0	>.05
VS			ND									
Necrosis												
Rate, %					0	50	50	.08	13	0	0	>.05
SS					**0**	**0.5 ± 0.2**	**1.5 ± 1.5**	**.04** (>.05)	0.4 ± 0.4	0	0	>.05
Fibroplasia												
Rate, %					**0**	**33**	**100**	**.02** (.09)	13	0	0	>.05
SS					**0**	**0.3 ± 0.2**	**2.0 ± 1.0**	**<.001** (>.05)	0.4 ± 0.4	0	0	>.05
Lungs												
Interstitial edema												
Rate, %	37.5	43	20	>.05	50	67	100	>.05	75	63	38	>.05
SS	1.4 ± 0.4	0.9 ± 0.5	0.6 ± 0.6	>.05	1.3 ± 0.5	1.5 ± 0.5	2.5 ± 0.5	>.05	1.8 ± 0.4	1.4 ± 0.4	0.63 ± 0.3	>.05
Alveolar												
Edema												
Rate, %	25	29	20	>.05	63	17	100	.09	**50**	**0**	25	.08 **(.03)**
SS	0.8 ± 0.5	0.4 ± 0.3	0.6 ± 0.6	>.05	**1.5 ± 0.5**	**0.3 ± 0.4**	**3.5 ± 0.5**	**.02** (>.05)	**1.4 ± 0.5**	**0**	**0.25 ± 0.2**	**<.01 (<.01)**
Inflam/fibrin												
Rate, %	25	0	20	>.05	38	33	100	>.05	75	38	25	>.05
SS	0.3 ± 0.2	0	0.2 ± 0.2	>.05	1.1 ± 0.5	0.7 ± 0.5	2.5 ± 0.5	>.05	**1.6 ± 0.4**	**0.63 ± 0.3**	**0.25 ± 0.2**	**.02 (≤.05)**

HS, hemorrhagic shock; HBOC, hemoglobin based oxygen carrier; HEX, Hextend; NON, not resuscitated; SS, severity score; LV, left ventricle; RV, right ventricle; ND, not done; VS, ventricular septum.

Rates (%) and severity scores (mean ± SEM) of LM H&E findings in swine with *moderate* and *severe controlled HS* (72-hr survivors) and *severe uncontrolled HS* (all pigs—72-hr survivors and early deaths). Significance was compared using the Kruskall-Wallis test and ANOVA/Bonferroni post-test for HBOC-HEX comparisons. Significant ($p \leq .05$) interactions are in boldface.

and 72-hr survivors). Categorical data were analyzed by the Kruskal-Wallis test (www.obg.cuhk.edu.hk) or Fisher's exact test (survival) (www.graphpad.com), severity scores by analysis of variance (and Bonferroni posttest for HBOC-HEX comparisons, www.graphpad.com), and continuous data by the mixed procedure (group interactions) (SAS Institute, Cary, NC). Data represent mean ± SEM ($p \leq .05$ for significance).

RESULTS

Survival

Survival differences were insignificant in moderate controlled HS but were highly significant in the two severe models ($p < .007$); survival was higher with HBOC than HEX only in severe uncontrolled HS ($p = .004$, Table 1). Physiology and survival data were previously published for the moderate controlled HS and severe uncontrolled HS models (14, 21) and are briefly noted here for comparison with organ pathology/function data (MAP and cardiac index data following). Overall, hemodynamics were more normalized, cutaneous tissue oxygenation im-

proved, and in severe uncontrolled HS, base deficit decreased with HBOC-201.

Myocardium

With moderate controlled HS, necrotic changes were primarily subendocardial, hypercontraction type and were associated with mineralization, mononuclear cell infiltration, and fibroblast proliferation—consistent with previously described HS zonal lesions (coagulative myocytolysis) (24). LV necrosis/degeneration and fibroplasia/histiocytic infiltration (tissue response) were rarely observed in HBOC and NON pigs but occurred in ~50% of HEX pigs (Table 2, Fig. 1, $p > .05$ [overall—necrosis and fibroplasia]; $p = .08$ [HBOC-HEX—necrosis]; $p = .05$ [HBOC-HEX—fibroplasia]). LV severity scores were higher with HEX ($p > .05$ [overall—necrosis]; $p = .05$ [overall—fibroplasia]; $p < .05$ [HBOC-HEX—fibroplasia]). The CK and troponin I curves were similar ($p > .05$), but the CK-MB curve was higher for HBOC (peak 31 ± 8.1 ng/mL) than HEX (7.8 ± 1.7 ng/mL, curve and peak $p \leq .02$). MAP was restored to baseline more rapidly and was higher with HBOC than HEX, but the converse occurred for cardiac index ($p < .002$, Fig. 2A). Prehospital fluid requirements were lower with HBOC (18.8 ± 1.8 mL/kg) than HEX (29.9 ± 1.1 mL/kg, $p = .001$). Pulmonary artery occlusion pressure was similar ($p > .05$).

With severe controlled HS, LV myonecrosis was rare in HBOC and HEX (13–17%) vs. 50% of NON pigs ($p = .04$ [overall]; $p > .05$ [HBOC-HEX]). RV and VS myonecrosis rates were not different ($p > .05$). The pattern of LV, RV, and VS necrosis and fibroplasia severity scores was NON>HEX>HBOC. CK curves were similar ($p > .05$) but peak CK was higher with HBOC (11,164 ± 2758 units/L) than HEX (2344 ± 215 units/L, $p = .01$). MAP was higher ($p < .001$) and cardiac index similar ($p > .05$) between HBOC and HEX pigs (Fig. 2*B*). Prehospital fluid requirements were similar with HBOC-201 (27.5 ± 1.9 mL/kg) and HEX (28.8 ± 0.8 mL/kg, $p > .05$).

With severe uncontrolled HS, disparate survival affected group comparisons, but myonecrosis/fibroplasia rates and severity scores (≤1), CK, CK-MB, and troponin I were similar in both treatment groups ($p > .05$). MAP was higher ($p < .001$) and cardiac index similar ($p > .05$) between HBOC and HEX pigs (Fig. 2*C*). Prehospital fluid requirements were similar with HBOC-201 (24.9 ± 2.1 mL/kg) and HEX (21.4 ± 2.8 mL/kg, $p > .05$), but total fluid index (survival-based) was lower with HBOC (7.0 ± 0.80 mL/kg/survival hr) than HEX (15.5 ± 2.8 mL/kg/survival hr, $p = .01$).

Lungs

In moderate controlled HS, gross evaluation revealed pulmonary congestion in about one third of pigs ($p > .05$). On LM, interstitial and alveolar edema and alveolar inflammation/fibrin rates and severity scores (<1.5) were similar ($p > .05$, Table 2). Alveolar edema was more common with electron microscopy (EM) than LM, but group differences were not seen ($p > .05$). Surfactant was lower with HBOC ($p > .05$ [overall]; $p = .05$ [HBOC-HEX], Table 3, Fig. 3). Po_2 was similar ($p > .05$).

In severe controlled HS, on LM, interstitial and alveolar edema and alveolar inflammation rates were similar ($p > .05$). Severity scores were highest in NON pigs (Table 2). On EM, alveolar edema rates were higher than with LM but similar ($p > .05$). Fibrin deposition and alveolar red blood cells were also similar ($p > .05$), but alveolar surfactant was less common with HBOC than HEX ($p = .04$, Table 3). Po_2 was similar ($p > .05$).

In severe uncontrolled HS, gross inspection revealed similar rates of pulmonary congestion and edema ($p > .05$). Although LM rates of interstitial edema were similar ($p > .05$), alveolar edema was more common with HBOC than HEX ($p = .03$). Severity scores (≤1.75) for alveolar edema and inflammation were higher with HBOC than HEX ($p < .05$, Table 2). On EM, rates of alveolar edema were similar in the resuscitation groups ($p > .05$, Table 3). Po_2 was higher with HBOC than HEX ($p = .03$).

Small Intestine

With moderate controlled HS, gross inspection revealed only mesenteric or serosal edema in 25% of HBOC pigs. LM rates and severity scores (≤1) of villar epithelial necrosis/degeneration, mucosal neutrophilic infiltration, and mucosal and submucosal edema were similar across treatment groups in all 3 models ($p > .05$, Table 4).

Liver

In moderate controlled HS, gross pathology included hepatic congestion and common bile duct dilation in 13% of HBOC pigs. On LM, hepatic parenchymal histopathology (necrosis, congestion, sinusoidal ectasia, and random hepatitis) rates and severity scores (≤1.5) were similar ($p > .05$) except for hepatic congestion ($p < .05$). Although uncommon and of minimal severity (≤0.75), hepatobiliary changes (hyperplasia, cholestasis, and neutrophilic cholangiohepatitis) occurred exclusively with HBOC ($p > .05$, Table 4). Liver function tests usually peaked at 24 hrs. AST was higher with HBOC (peak 672 ± 135 units/mL) than HEX (peak 179 ± 51 units/mL, curve $p \leq .01$, peak $p = .007$). LDH was higher with HBOC (peak 7747 ± 1707 units/L) than HEX (peak 1977 ± 464 units/L, curve $p \leq .01$, peak $p = .004$). AP was higher with HBOC (peak 130 ± 30 units/mL) than HEX (peak 92 ± 4.4 units/mL, curve $p \leq .01$, peak $p > .05$).

With severe controlled HS, LM parenchymal histopathology rates and severity scores (≤1.5) were similar ($p > .05$). Biliary histopathology of minimal severity (≤1.1) occurred only with HBOC (38–50%, $p > .05$, Table 4). AST and AP were similar ($p > .05$), but LDH was higher with HBOC (peak 3399 ± 497 units/L)

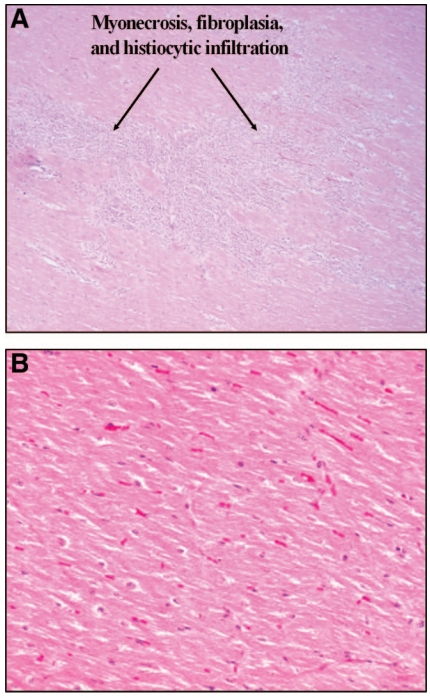

Figure 1. Cardiac histopathology. Examples of light microscopic (hematoxylin and eosin) cardiac histology in hemoglobin-based oxygen carrier (HBOC)-201-resuscitated pigs with moderate controlled hemorrhagic shock—myonecrosis/degeneration and fibroplasia/histiocytic infiltration (*A*) and normal histology (*B*).

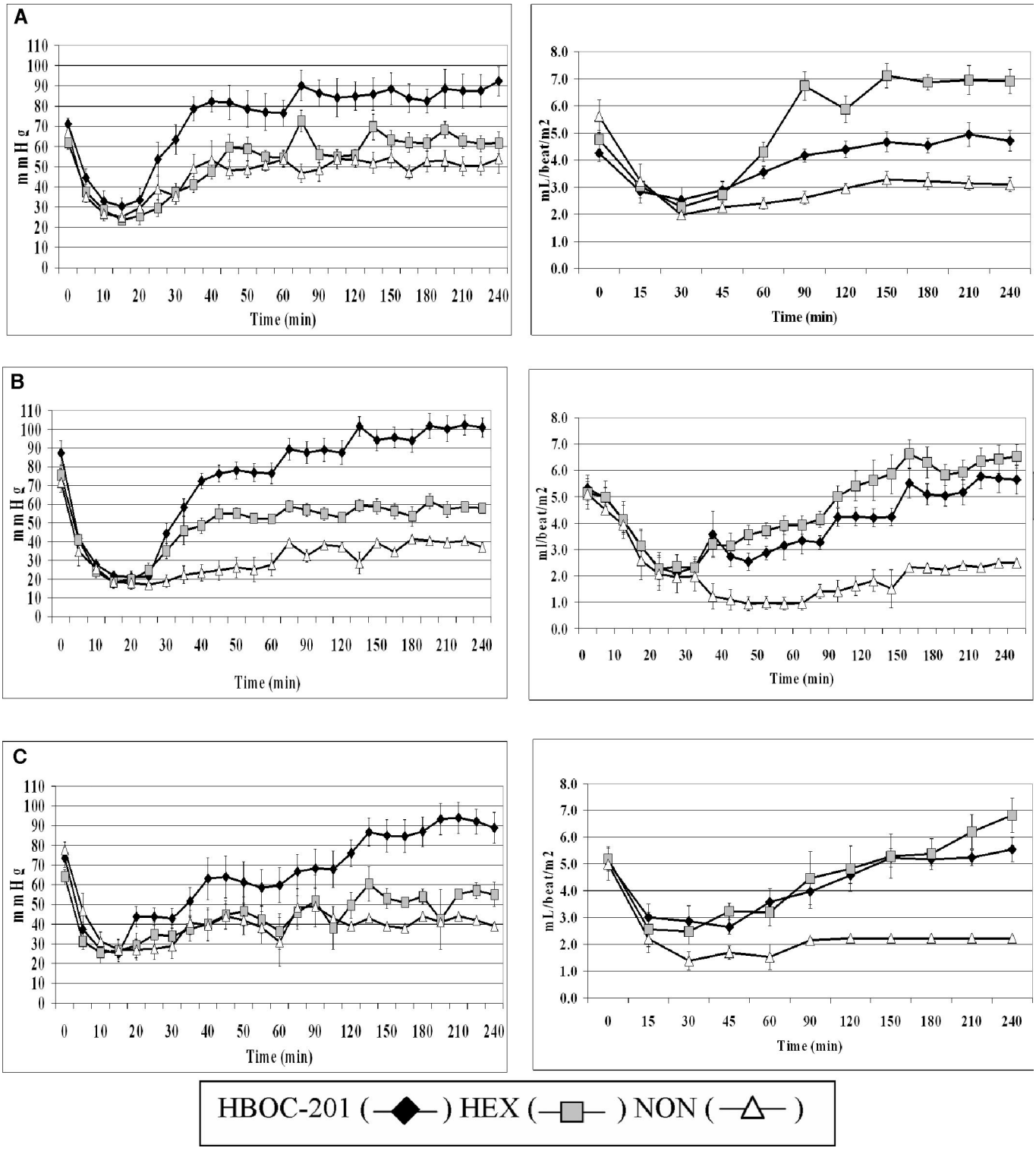

Figure 2. Hemodynamics—mean arterial pressure (MAP, *left*) and cardiac index (CI, *right*). MAP and CI curves were significantly different in moderate controlled hemorrhagic shock (*HS*, *A*), severe controlled HS (*B*), and severe uncontrolled HS (*C*). MAP was higher in hemoglobin-based oxygen carrier (HBOC)-201 than Hextend (HEX) and nonresuscitated (NON) pigs in all three models; CI was higher in HEX than HBOC pigs only in moderate controlled HS (*A*). $p \leq .05$ was considered significant.

than HEX (peak 1563 ± 433 units/L, curve $p = .002$, peak $p = .03$).

In severe uncontrolled HS, gross changes included hepatic congestion in 13–25% of HBOC and HEX pigs. LM parenchymal rates and severity scores (≤1) were similar, except sinusoidal ectasia was less common with HBOC (13% vs. 50–63% for HEX and NON, $p > .05$ [overall]; $p = .05$ [HBOC-HEX]). Biliary changes of minimal severity (≤0.75) occurred only with HBOC (13–38%, $p \leq .04$, Table 4). AST, LDH, and AP were similar in both treatment groups ($p > .05$).

Table 3. Lung electron microscopy changes

	Lesion	HBOC, % (No.)	HEX, % (No.)	NON, % (No.)
Moderate controlled HS				
All (72-hr survivors & adjusted end points)	Alveolar edema	25 (2/8)	50 (4/8)	88 (7/8)
	Fibrin	63 (5/8)	50 (4/8)	75 (6/8)
	Surfactant	**0 (0/8)**	**50 (4/8)**	**63 (5/8)**
	Alveolar RBCs	0 (0/8)	25 (2/8)	50 (4/8)
72-hr survivors	Alveolar edema	25 (2/8)	57 (4/7)	80 (4/5)
	Fibrin	63 (5/8)	43 (3/7)	60 (3/5)
	Surfactant	**0 (0/8)**	**43 (3/7)**	40 (2/5)
	Alveolar RBCs	0 (0/8)	14 (1/7)	20 (1/5)
Severe controlled HS				
All (72-hr survivors & adjusted end points)	Alveolar edema	75 (6/8)	63 (5/8)	63 (5/8)
	Fibrin	63 (5/8)	50 (4/8)	63 (5/8)
	Surfactant	13 (1/8)	50 (4/8)	25 (2/8)
	Alveolar RBCs	25 (2/8)	38 (3/8)	63 (5/8)
72-hr survivors	Alveolar edema	75 (6/8)	83 (5/6)	100 (3/3)
	Fibrin	63 (5/8)	50 (3/6)	100 (3/3)
	Surfactant	**13 (1/8)**	**67 (4/6)**	**0 (0/3)**
	Alveolar RBCs	25 (2/8)	50 (3/6)	100 (3/3)
Severe uncontrolled HS				
All (72-hr survivors & adjusted end points)	Alveolar edema	75 (6/8)	38 (3/8)	63 (5/8)
	Fibrin	**63 (5/8)**	**38 (3/8)**	**0 (0/8)**
72-hr survivors	Alveolar edema	75 (6/8)	100 (1/1)	0 (0/1)
	Fibrin	63 (5/8)	100 (1/1)	0 (0/1)

HS, hemorrhagic shock; HBOC, hemoglobin based oxygen carrier; HEX, Hextend; NON, not resuscitated; RBC, red blood cell.

Rates (%) of pulmonary EM histopathologic changes were quantified and stratified for all pigs (72-hr survivors and early deaths [adjusted endpoints]) and for pigs surviving 72 hrs. Significant ($p \leq .05$) interactions are in boldface.

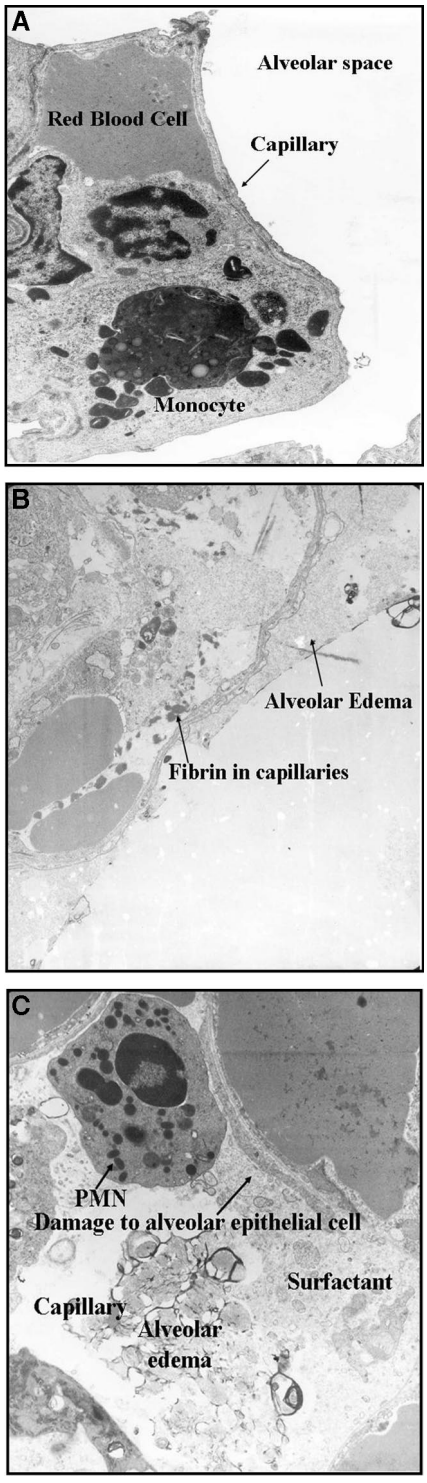

Figure 3. Pulmonary electron microscopy (EM). Examples of pulmonary EM in pigs with moderate controlled HS resuscitated with hemoglobin-based oxygen carrier-201 (*A*) or Hextend (*B*) or not resuscitated (*C*). *PMN*, neutrophil.

Kidneys

In moderate controlled HS, no cortical pathology occurred, and medullary changes were uncommon, of minimal severity (≤1), and similar ($p > .05$). Papillary changes were more common with HBOC (Fig. 4): necrosis/degeneration (71% vs. 0% for HEX and NON, $p = .01$); inflammation/edema/fibrin (86% vs. 25–40% for HEX and NON, $p > .05$); mineralization (14% vs. 0% for HEX and NON, $p > .05$); and hemorrhage (43% vs. 20–25% of HEX and NON, $p > .05$). Severity scores were all ≤1.5 but higher with HBOC for papillary necrosis ($p < .01$ overall and HBOC-HEX) and inflammation ($p = .01$ [overall]; $p < .05$ [HBOC-HEX], Table 5). Prehospital urine output (HBOC 3.2 ± 0.4 and HEX 3.5 ± 0.7 mL/kg) was similar ($p > .05$). BUN curves were similar ($p > .05$) but the 240-min peak was higher with HBOC (23 ± 2.0 g/dL) than HEX (16.9 ± 1.9 g/dL, $p = .05$). Creatinine was higher with HBOC (peak 1.59 ± 0.12 g/dL) than HEX (1.05 ± 0.05 g/dL, curve and peak [at 60–240 mins], $p \leq .001$).

In severe controlled HS, cortical/medullary histopathology included interstitial nephritis in one HEX pig. Mild severity (≤1.5) papillary changes (Table 5), fluid requirements (HBOC 27.5 ± 1.9 and HEX 28.8 ± 0.8 mL/kg), BUN, and creatinine were similar ($p > .05$).

In severe uncontrolled HS, there were no LM cortical changes and similar infrequent minimal severity (≤0.75) medullary changes ($p > .05$). Papillary pathology rates and severity (<1, Table 5), prehospital urine output (HBOC 1.1 ± 0.7 and HEX 0.6 ± 0.4 mL/kg/hr), and BUN and creatinine curves were similar ($p > .05$) in the treated groups. Urine output resumed earlier with HBOC (90 vs. 210–240 mins with HEX and NON).

Wellness

Vomiting incidence and food consumption and activity scores were not different ($p > .05$).

Nitrotyrosine Staining Intensity

Although there were no group differences ($p > .05$), there were tissue differences in relative staining intensity: liver>heart, jejunum, and kidney>lung (Fig. 5).

DISCUSSION

HBOC-201 resuscitation has been reported to stabilize hemodynamics, reverse anaerobic metabolism, decrease

Table 4. Small intestine and liver changes

	Moderate Controlled HS Model, 72-Hr Survivors				Severe Controlled HS Model, 72-Hr Survivors				Severe Uncontrolled HS Model, All Pigs			
	Treatment Group			Sig. (p) Overall (HBOC vs. HEX)	Treatment Group			Sig. (p) Overall (HBOC vs. HEX)	Treatment Group			Sig. (p) Overall (HBOC vs. HEX)
	HBOC n = 8	HEX n = 6–7	NON n = 4–5		HBOC n = 8	HEX n = 6	NON n = 2		HBOC n = 8	HEX n = 8	NON n = 8	
Jejunum												
Villar necrosis												
Rate, %	13	17	25	>.05	0	0	0	>.05	0	38	25	>.05 (.06)
SS	0.1 ± 0.1	0.2 ± 0.2	0.3 ± 0.3	>.05	0	0	0	>.05	0	0.5 ± 0.3	0.3 ± 0.2	>.05
PMN infiltration												
Rate, %	25	50	0	>.05	25	0	0	>.05	13	25	25	>.05
SS	0.3 ± 0.2	0.5 ± 0.2	0	0.05	0.6 ± 0.4	0	0	>.05	0.1 ± 0.1	0.3 ± 0.2	0.3 ± 0.2	>.05
Mucosal edema												
Rate, %	38	67	25	>.05	0	0	50	>.05	25	63	38	>.05
SS	0.5 ± 0.3	0.8 ± 0.3	0.5 ± 0.5	>.05	0	0	0.5 ± 0.5	>.05	0.4 ± 0.3	0.9 ± 0.3	0.4 ± 0.2	>.05
Submucosal edema												
Rate, %	38	50	0	>.05	0	17	50	>.05	13	50	13	>.05
SS	0.5 ± 0.3	0.7 ± 0.3	0	>.05	0	0.3 ± 0.3	1.0 ± 1.0	>.05	0.1 ± 0.1	0.6 ± 0.3	0.1 ± 0.1	>.05
Liver												
Hepatocellular necrosis												
Rate, %	0	33	0	>.05	38	0	50	>.05	13	38	13	>.05
SS	0	0.5 ± 0.3	0	>.05	0.6 ± 0.4	0	1.5 ± 1.5	>.05	0.3 ± 0.3	0.6 ± 0.4	0.3 ± 0.3	>.05
Congestion												
Rate, %	38	67	50	>.05	13	0	0	>.05	63	50	75	>.05
SS	**0.4 ± 0.2**	**1.5 ± 0.6**	0.5 ± 0.3	.10 (**≤.05**)	0.3 ± 0.3	0	0	>.05	1.0 ± 0.4	1.0 ± 0.4	1.0 ± 0.3	>.05
Sinusoidal ectasia												
Rate, %	50	33	0	>.05	50	17	0	>.05	**13**	**63**	50	>.05 (**≤.05**)
SS	0.5 ± 0.2	1.0 ± 0.6	0	>.05	0.9 ± 0.4	0.3 ± 0.3	0	>.05	0.3 ± 0.3	1.0 ± 0.3	0.9 ± 0.4	>.05
Hepatitis												
Rate, %	13	17	0	>.05	25	0	0	>.05	13	0	0	>.05
SS	0.4 ± 0.4	0.5 ± 0.5	0	>.05	0.5 ± 0.3	0	0	>.05	0.1 ± 0.1	0	0	>.05
Biliary hyperplasia												
Rate, %	13	0	0	>.05	38	0	0	>.05	13	0	0	>.05
SS	0.5 ± 0.5	0	0	>.05	0.6 ± 0.3	0	0	>.05	0.4 ± 0.4	0	0	>.05
Cholestasis												
Rate, %	25	0	0	>.05	50	0	0	.08 (.05)	**38**	**0**	**0**	**.04** (.06)
SS	0.8 ± 0.5	0	0	>.05	**1.1 ± 0.4**	**0**	0	.06 (.04)	0.8 ± 0.5	0	0	.10
PMN cholangiohepatitis												
Rate, %	13	0	0	>.05	38	0	0	>.05	**38**	**0**	**0**	**.04** (.06)
SS	0.3 ± 0.3	0	0	>.05	0.9 ± 0.4	0	0	>.05 (.08)	**0.8 ± 0.4**	**0**	**0**	**.04 (.04)**

HS, hemorrhagic shock; HBOC, hemoglobin based oxygen carrier; HEX, Hextend; NON, not resuscitated; SS, severity score; PMN, neutrophil.

Rates (%) and severity scores (mean ± SEM) of LM H&E findings in swine with *moderate* and *severe controlled HS* (72-hr survivors) and *severe uncontrolled HS* (all pigs—72-hr survivors and early deaths). Significance was compared using the Kruskall-Wallis test and ANOVA/Bonferroni post-test for HBOC-HEX comparisons. Significant ($p \leq .05$) interactions are in boldface.

fluid and transfusion requirements, and decrease mortality in swine HS models (7–14, 21), but this is the first comprehensive report on its effects on organ histology and function. In comparison with hetastarch, in swine with HS, HBOC-201 resuscitation increased survival (with severe HS), did not increase evidence of oxidative potential, and had histopathologic and/or functional effects on organs that were equivocal (myocardium, lungs, hepatic parenchyma, jejunum, and renal cortex/medulla) and potentially adverse (hepatobiliary and renal papilla). As these effects were generally of minimal to mild severity, they are unlikely to be clinically significant in the majority of patients. These data should be reassuring to clinical investigators evaluating the efficacy and safety of HBOC-201 in trauma, but they delineate the idiosyncratic side effect profile of this drug that should be expected in survivors.

As a consequence of systemic and pulmonary vasoactivity (15, 25), HBOCs have been purported to have potential for cardiac toxicity, mainly due to nitric oxide (NO) binding by tetrameric hemoglobin (26). Burhop et al. (27) reported myocardial lesions with 200 and 700 mg/kg top load infusions of diaspirin cross-linked hemoglobin in monkeys and pigs, respectively, ameliorated by hemoglobin polymerization and reduced NO affinity and mimicked by NO synthase inhibition. In contrast, an adverse dose-response was not seen in our studies, due to polymerization, non-top load, and lower dosing (~300 mg/kg) and/or difficulty distinguishing HS- from resuscitative fluid-induced myocardial toxicity. But other mechanisms, including endothelin (28) and adrenergic activation (29), and autoregulation from increased oxygen unloading may also be involved (30). In swine with HS, Fitzpatrick et al. (31) found that blood NO was not reduced post-HBOC-201-resuscitation. In any case, increased systemic and pulmonary blood pressure and vascular resistance, and secondarily decreased cardiac output, have been reported in animal models and humans (8, 11–13, 15–17, 21). Notwith-

standing, HBOC-201's beneficial effect on tissue oxygenation (14, 21, 32, 33) may be myocardially protective. Evidence for reduced myocardial infarct size, neutrophil infiltration, and CK-MB was seen in rat and canine coronary occlusion models, and a case report documented reversal of intraoperative myocardial ischemia with HBOC-201 (34–36).

HS results in two classic myocardial histopathologic changes: mural coagulative myocytolytic zonal lesions (early, sometimes reversible, associated with small intraventricular volume, catecholamine-related, and consistently reported) and subendocardial hemorrhage (late, irreversible, hypoxia-related, and inconsistently reported) (24, 37–41). We demonstrated typical zonal lesions but no subendocardial hemorrhage. HBOC-201 elicited mild to moderate systemic and pulmonary vasoconstrictive responses, but fluid requirements and cardiac output were decreased only in moderate controlled HS. These results are consistent with reports by McNeil et al. (8) (moderate HS) and Manning et al. (7) (severe HS). Most important, myocardial degenerative and secondary fibroblastic histopathologic changes were similar or lower with HBOC-201 and cardiac enzymes were not different (CK-MB was higher with HBOC-201 in moderate controlled HS, but a skeletal muscle source is likely as CK-MB/CK ratios were <2%). It appears that in very severe HS, any stabilization of blood pressure may be beneficial and some vasoactivity may, in fact, be desirable. In sum, HBOC-20s vs. hetastarch overall myocardial effects were at least equivocal and possibly myoprotective.

HBOC-201 resuscitation could theoretically increase pulmonary pathology via a number of mechanisms including pulmonary hypertension, hydrostatic and

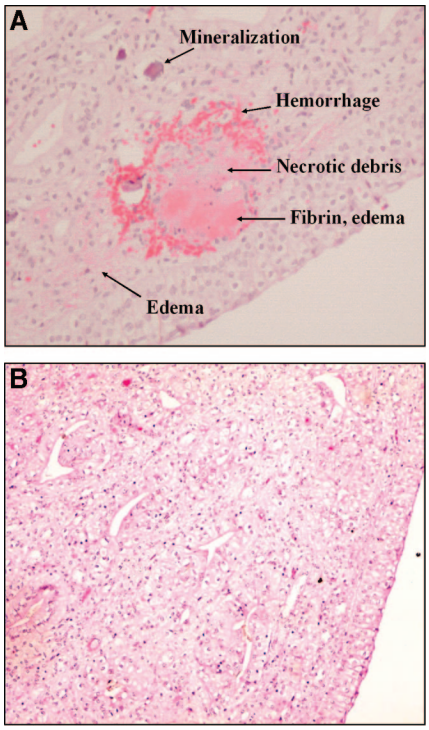

Figure 4. Renal histopathology. Examples of light microscopic (hematoxylin and eosin) renal histology in hemoglobin-based oxygen carrier-201-resuscitated pigs with moderate controlled hemorrhagic shock—papillary necrosis (*A*) and normal histology (*B*).

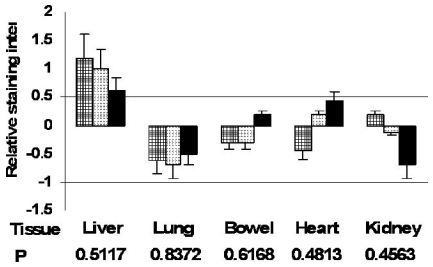

Figure 5. Organ 3-nitrotyrosine staining. Relative intensity of 3-nitrotyrosine staining in moderate controlled hemorrhagic shock. Frozen tissue slides were fixed, permeabilized, incubated in anti-nitrotyrosine antibody solution and then R-Phycoerythrin-conjugated secondary antibody solution, and rated (0–4 scale) by fluorescent microscopy. Negative values indicate intensities below normal control tissue. There were no significant differences between hemoglobin-based oxygen carrier (*hexed boxes*), Hextend (*speckled boxes*), and nonresuscitated pigs (*black boxes*) for any organ.

Table 5. Renal changes

	Moderate Controlled HS Model, 72-Hr Survivors				Severe Controlled HS Model, 72-Hr Survivors				Severe Uncontrolled HS Model, All Pigs			
	Treatment Group			Sig (*p*) Overall (HBOC vs. HEX)	Treatment Group			Sig (*p*) Overall (HBOC vs. HEX)	Treatment Group			Sig (*p*) Overall (HBOC vs. HEX)
	HBOC n = 8	HEX n = 6–7	NON n = 4–5		HBOC n = 8	HEX n = 6	NON n = 2		HBOC n = 8	HEX n = 8	NON n = 8	
Renal (papillary)												
Interstitial nephritis rate	0	0	0	>.05	0	17	0	>.05	0	0	0	>.05
Necrosis												
Rate, %	**71**	**0**	**0**	**.01**	0	0	0	>.05	38	38	25	>.05
SS	**1.3 ± 0.4**	**0**	**0**	**.01 (.01)**	0	0	0	>.05	0.4 ± 0.2	0.5 ± 0.3	0.4 ± 0.3	>.05
Inflammation												
Rate, %	86	40	25	>.05	63	33	100	>.05	38	38	50	>.05
SS	**1.4 ± 0.3**	**0.4 ± 0.2**	**0.3 ± 0.3**	**.01 (≤.05)**	1.1 ± 0.4	0.7 ± 0.4	1.5 ± 0.5	>.05	0.4 ± 0.2	0.5 ± 0.3	0.8 ± 0.4	>.05
Mineralization												
Rate, %	14	0	0	>.05	0	0	100	>.05	0	13	0	>.05
SS	0.3 ± 0.3	0	0	>.05	0	0	1.0 ± 1.0	>.05	0	0.3 ± 0.3	0	>.05
Hemorrhage												
Rate, %	43	20	25	>.05	0	33	0	>.05	13	38	13	>.05
SS	0.7 ± 0.4	0.2 ± 0.2	0.3 ± 0.3	>.05	0	0.3 ± 0.2	0	>.05	0.1 ± 0.1	0.4 ± 0.2	0.3 ± 0.3	>.05

HS, hemorrhagic shock; HBOC, hemoglobin based oxygen carrier; HEX, Hextend; NON, not resuscitated; SS, severity score.

Rates (%) and severity scores (SS) (mean ± SEM) of LM H&E findings in swine with *moderate* and *severe controlled HS* (72-hr survivors) and *severe uncontrolled HS* (all pigs—72-hr survivors and early deaths). Significance was compared using the Kruskall-Wallis test and ANOVA/Bonferroni post-test for HBOC-HEX comparisons. Significant ($p \leq .05$) interactions are in boldface.

hypoxic- and/or reperfusion injury-related noncardiogenic pulmonary alveolar edema, and pneumonia. Not surprisingly, the majority of NON pigs sustained lung injury (alveolar and interstitial edema without elevated pulmonary artery occlusion pressure) in our severe HS models, suggesting that pulmonary complications may occur with low-volume resuscitation with delayed transportation. We documented mild lung injury in the preponderance of HBOC pigs but no consistent between-group pattern with LM and EM. Clinical correlation did not reveal adverse pulmonary effects (Po_2 was higher with HBOC in severe uncontrolled HS). Overall, our results are equivocal about HBOC-201's pulmonary effects and suggest that although survival may be improved in severe HS, lung injury might be expected in survivors. Interestingly, despite concern about reperfusion injury, we did not find increased jejunal oxidative stress or injury, consistent with results of York et al. (12) and Driessen et al. (42). Thus, despite sensitivity to hypoxia and reperfusion injury (43, 44), the lungs and intestine did not appear to be adversely affected by HBOC-201 resuscitation from HS.

HBOCs have been associated with elevation of liver function tests and/or amylase/lipase (12, 17, 36, 45–48). We documented transient elevation of AST and LDH and inconsistent results regarding AP. In HS of brief duration, with or without HBOC resuscitation, hepatocellular changes predominate in hypoxia-sensitive centrilobular to midzonal regions (12, 49–51). York et al. (12) found these changes to be more common in HBOC-201-than lactated Ringer's solution/blood-resuscitated swine with HS. In contrast, and consistent with findings with HBOC-201 and other HBOCs in canine HS models (47, 48), reversible (congestion, sinusoidal ectasia, and inflammatory cell infiltration [hepatitis]) and irreversible changes (coagulative necrosis) (51–53) did not consistently differ among groups in our studies. On the other hand, we found biliary changes only with HBOC (not reported by York et al.). The significance of this finding is unclear as severity was minimal and AP was elevated only in moderate controlled HS. Taken together, these data suggest that mild and reversible hepatocellular and biliary injury occurs in HBOC-resuscitated swine with HS, could occur in humans, but is likely to be clinically irrelevant in most patients. However, elevation of liver function tests can complicate clinical care and mild hepatic changes could be clinically significant in selected populations (e.g., preexisting liver disease). The etiology of HBOC-induced hepatotoxicity has not been well elucidated but may be due to increased bilirubin/biliverdin turnover; increased delivery of heme substrate through small fenestrated sinusoids, increased heme oxygenase (HO) activity, and altered microvascular flow and cholestasis due to NO binding and CO mediators; and/or other toxic effects (12, 54, 55).

Renal histopathology was mainly papillary. These findings contrast with classic descriptions of hypovolemia-induced acute tubular necrosis (56) and less commonly described glomerular damage (57). Rates and severity of papillary changes were higher with HBOC in moderate controlled HS but were similar to other groups in severe HS. We are uncertain why papillary changes have not been reported previously (12); sampling differences may be an explanation. Nevertheless, as papillary severity scores were minimal/mild, creatinine and BUN values were generally similar, and urine output was maintained despite lower fluid requirements (in moderate controlled HS), these changes are unlikely to be clinically significant in the absence of preexisting renal disease and/or exposure to additional nephrotoxins. But other investigators found decreased urine output with HBOC-201-resuscitation in HS, possibly due to decreased fluid requirements or vasoactive effects (13). Azotemia has been documented in HBOC-201-treated surgical patients (17) due to protein-loading or renal dysfunction. Our data suggest that HBOC-201's vasoactive and fluid-sparing properties may be factors in induction of mild papillary necrosis in less severe HS, but with severe HS, these potentially adverse properties appear physiologically moderated by beneficial effects and become even more clinically insignificant. Future research should aim to elucidate mechanisms of papillary injury and whether fluid loading would be papillary protective.

HBOCs rapidly restore tissue oxygenation and, thus, may induce hypoxia-reperfusion injury via free radical formation. The free radical NO binds with superoxide (O_2^-) to form peroxynitrate ($ONOO^-$), a strong nitrating agent and oxidizer of biological substances, including tyrosine. Thus, 3-nitrotyrosine is often used as a surrogate marker for $ONOO^-$ and, indirectly, as a measure of oxidative stress. As 3-nitrotyrosine may be detected without histopathology (58), it points to oxidative damage indirectly. Although we found hepatic tissue to have the highest staining intensity, we did not detect group differences. Our results may have been confounded by differential survival times, undocumented group differences for non-$ONOO^-$ free radicals (59, 60), and HBOC-201 NO binding-related decreased 3-nitrotyrosine production (58, 61). Despite these limitations, we did not find increased $ONOO^-$ production with HBOC-201-resuscitation in HS.

Our data are limited since the HS studies were not powered to evaluate histopathology or organ function. As few non-HBOC pigs survived 72 hrs in severe uncontrolled HS, survival (and necropsy) times differed and, thus, comparisons of treatment effects may be inaccurate. As resuscitation protocols were physiology-based (blood pressure, heart rate, and anemia), infused fluid volumes differed between groups, and higher doses of Hextend may have confounded the results. As such, our conclusions relate to clinically relevant effects of the relative effects of HBOC resuscitation in HS but not necessarily to intrinsic properties of the fluids *per se*. Additionally, Hextend was used as the main comparator in these studies because it is carried by U.S. military Special Operations Forces, but results may have been different with standard crystalloid fluids.

CONCLUSIONS

This comprehensive evaluation of HBOC-201 resuscitation in three swine HS models showed that in comparison with hetastarch, HBOC-201 generally increased survival, did not increase oxidative stress, but resulted in organ histopathologic and functional effects that were equivocal (myocardium, lungs, jejunum, hepatic parenchyma, and renal cortex/medulla) and potentially adverse (hepatobiliary and renal papilla). Despite evidence of vasoactivity, myocardial necrosis, cardiac enzymes, and lung injury were generally similar with HBOC-201. Although these data add to the growing body of evidence suggesting that HBOC-201-resuscitation in HS will enhance clinical outcome, clinicians should recall

these side effects when selecting resuscitative agents. These swine data should be corroborated in controlled clinical trials.

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