Distal organ inflammation and injury after resuscitative endovascular balloon occlusion of the aorta in a porcine model of severe hemorrhagic shock

Yansong Li*, Michael A. Dubick1, Zhangsheng Yang1, Johnny L. Barr2, Brandon J. Gremmer1, Michael L. Lucas1, Corina Nescolu1, Bryan S. Jordan1, Andriy I. Batchinsky1, Leopoldo C. Cancio3

1 Department of Expeditionary Critical Care Research, US Army Institute of Surgical Research, Fort Sam Houston, Texas, United States of America, 2 Department of Damage Control Resuscitation, US Army Institute of Surgical Research, Fort Sam Houston, Texas, United States of America, 3 U.S. Army Burn Center, US Army Institute of Surgical Research, Fort Sam Houston, Texas, United States of America

* yansong.li.ctr@mail.mil

Abstract

Background and objective

Resuscitative Endovascular Balloon Occlusion of Aorta (REBOA) has emerged as a potential life-saving maneuver for the management of non-compressible torso hemorrhage in trauma patients. Complete REBOA (cREBOA) is inherently associated with the burden of ischemia reperfusion injury (IRI) and organ dysfunction. However, the distal organ inflammation and its association with organ injury have been little investigated. This study was conducted to assess these adverse effects of cREBOA following massive hemorrhage in swine.

Methods

Spontaneously breathing and consciously sedated Sinclair pigs were subjected to exponential hemorrhage of 65% total blood volume over 60 minutes. Animals were randomized into 3 groups (n = 7): (1) Positive control (PC) received immediate transfusion of shed blood after hemorrhage, (2) 30min-cREBOA (A30) received Zone 1 cREBOA for 30 minutes, and (3) 60min-cREBOA (A60) given Zone 1 cREBOA for 60 minutes. The A30 and A60 groups were followed by resuscitation with shed blood post-cREBOA and observed for 4h. Metabolic and hemodynamic effects, coagulation parameters, inflammatory and end organ consequences were monitored and assessed.

Results

Compared with 30min-cREBOA, 60min-cREBOA resulted in (1) increased IL-6, TNF-α, and IL-1β in distal organs (kidney, jejunum, and liver) (p < 0.05) and decreased reduced glutathione in kidney and liver (p < 0.05), (2) leukopenia, neutropenia, and coagulopathy (p < 0.05), (3) blood pressure decline (p < 0.05), (4) metabolic acidosis and hyperkalemia (p < 0.05),
and (5) histological injury of kidney and jejunum ($p < 0.05$) as well as higher levels of creatinine, AST, and ALT ($p < 0.05$).

**Conclusion**

30min-cREBOA seems to be a feasible and effective adjunct in supporting central perfusion during severe hemorrhage. However, prolonged cREBOA (60min) adverse effects such as distal organ inflammation and injury must be taken into serious consideration.

**Introduction**

Major torso injuries in recent wars underlined the difficulty in controlling non-compressible torso hemorrhage (NCTH) [1–3]. Deaths because of exsanguination secondary to injuries involving truncal, abdominal and pelvic vessels are considered as potentially survivable [4]. Traditionally, resuscitative thoracotomy or laparotomy are used in an effort to prevent cardiovascular collapse by increasing afterload and central aortic pressure until hemostasis may be achieved [5, 6]. These invasive steps are often undertaken after loss of pulses. Less invasive endovascular techniques such as balloon occlusion of the aorta, is an alternative to open surgery [3, 7–13]. We have shown that complete resuscitative endovascular balloon occlusion of the aorta (cREBOA), resulted in the restoration of carotid flow ($Q_{\text{carotid}}$ “cerebrovascular resuscitation”) as effectively as immediate transfusion of shed blood, with equivalent 4-hour survival [14].

The occlusion of the thoracic aorta is known to restore central aortic pressure in hemorrhagic shock [10, 14–16] and has been shown in an animal model of uncontrolled hemorrhage to increase survival in comparison with resuscitation using normal saline without hemorrhage control [8]. Deleterious effects of aortic occlusion and the magnitude of these effects are related to duration of occlusion [9, 15, 17–20]. Although the outcome of cREBOA is the same as cross clamping of the aorta through a thoracotomy, the physiologic insult by the endovascular approach has been shown to be less than with the open method [7]. However, there is a finite range of time for which the aortic balloon occlusion might be applied in one setting. A non-lethal, 35% hemorrhage in swine revealed up to 90 min as the limit of use before significant and potentially irreversible ischemia-induced organ injury occurs [9]. More severe hemorrhage (e.g, 60%), known to be fatal without resuscitation [21], is likely near the limit of physiologic tolerance for these mini-swine. Although aortic balloon occlusion restored central aortic pressure to near pre-hemorrhage values [9, 22], endpoints of resuscitation with a decisively spared circulatory volume provided by aortic occlusion are relatively unknown. We hypothesized that prolonged cREBOA (60 min) would cause distal organ inflammation and injury. Based on these premises, this study explored the effects of 30min- and 60min-cREBOA (two natural testable thresholds of cREBOA duration in human clinical use) at aortic zone 1 in a porcine model of severe hemorrhagic shock (65% total blood volume) on distal organ inflammation and injury.

**Materials and methods**

Research was conducted in compliance with the Animal Welfare Act, the implementing Animal Welfare regulations, and the principles of the Guide for the Care and Use of Laboratory Animals, National Research Council. The facility’s Institutional Animal Care and Use
Committee approved all research conducted in this study. The facility where this research was conducted is fully accredited by the AAALAC.

**Experimental design**

Sexually mature, non-castrated male Sinclair Miniature Swine (Sinclair Bio-Resources, Columbia, MO), 38 ± 9 kg, were used in this study. Animals were specially bred, socialized, vaccinated, and free from common domestic swine diseases. Total of 21 animals were randomized into three subgroups, n = 7 each. All animals underwent 65% total blood volume hemorrhage, with subsequent care as follows: 1) Positive control (PC): 65% blood volume hemorrhage and then immediate transfusion of shed blood. 2) A30: cREBOA for 30 minutes, followed by transfusion of shed blood just prior to balloon deflation. 3) A60: cREBOA for 60 minutes, followed by transfusion of shed blood after balloon deflation as described [14] (Fig 1).

**Surgical procedures and cREBOA insertion**

All surgical procedures were described previously [14]. In brief, after an overnight fast, with water available *ad libitum*, animals were transferred to a procedure room where they were received buprenorphine hydrochloride (0.05 mg/kg IM) as preemptive analgesia and anesthetized with Telazol® (Zoetis, Florham Park, NJ, 4mg/kg IM). Animals were endotracheally intubated, and inhalational anesthesia was maintained using isoflurane (1–5 vol %), delivered in 100% oxygen. A Dräger Fabius GS anesthesia machine (Dräger Medical In., Telford, PA) was used. Vascular cannulation was performed as follows. In the left neck, an 8.5 Fr introducer sheath (Teleflex, Inc., Wayne, PA) was inserted into the external jugular vein via a cut-down. An Arrow 7 Fr multilumen central venous catheter (Arrow International Inc., Reading, PA) was inserted via the introducer sheath. A 6 Fr introducer sheath (Teleflex, Inc.) was inserted into the common carotid artery, and a 5 Fr VolumeView catheter (Edwards Lifesciences Corp., Irvine, CA) was then placed. This catheter was connected via a high pressure monitoring line (Smith Medical ASD Inc., Dublin, OH) to an Infinity HemoMed Pod (Dräger Medical Inc.). In the left neck, an 8.5 Fr introducer sheath (Teleflex, Inc., Wayne, PA) was inserted into the external jugular vein via a cut-down. An Arrow 7 Fr multilumen central venous catheter (Arrow International Inc., Reading, PA) was inserted via the introducer sheath. A 6 Fr introducer sheath (Teleflex, Inc.) was inserted into the common carotid artery, and a 5 Fr VolumeView catheter (Edwards Lifesciences Corp., Irvine, CA) was then placed. This catheter was connected via a high pressure monitoring line (Smith Medical ASD Inc., Dublin, OH) to an

![Fig 1. Overview of experimental design. BL, baseline; EBV, estimated blood volume; EH, end of hemorrhage; EOS, end of study; R90, R150, R210 and R240 are 90-, 150-, 210-, and 240-min post-resuscitation, respectively; cREBOA, complete resuscitative endovascular balloon occlusion of the aorta.](https://doi.org/10.1371/journal.pone.0242450.g001)
Infinity HemoMed Pod (Dräger Medical Inc.). In the right groin, the common femoral artery was accessed via a cut-down and cannulated with a 7 Fr introducer sheath (Teleflex Inc.), for future cREBOA insertion (discussed later). In the left groin, the common femoral artery was accessed via a cut-down and cannulated with an 8 Fr introducer sheath (Teleflex Inc.), and a high-pressure tubing (ICU Medical) was advanced through the sheath. A tracheostomy was performed, and a 10-mm cuffed endotracheal tube (Lo-Pro, Mallinckrodt Inc., St. Louis, MO) was inserted.

After instrumentation, all incisions were closed with sutures or staples, and the wounds were infiltrated with 0.5% bupivacaine. Animals were transferred into a custom-built sling, which allows an animal to remain in an anatomic quadruped position. Inhalational anesthesia was discontinued, and buprenorphine continuous intravenous infusion was started at 5 mL/h (1 Kg/kg/h). Once the animal recovered sufficiently to breathe spontaneously, a midazolam bolus (2 mg/kg) was administered intravenously, and a continuous intravenous infusion of 0.1 mg/kg per hour was started. The infusion rates were decreased by half when mean arterial pressure (MAP) decreased below 80 mm Hg and were held when MAP was lower than 50 mm Hg. Analgesia was titrated to elimination of pain or distress as evident by physiological parameters (blood pressure and heart rate changes). Anesthetic depth was monitored throughout all procedures via bispectral index (to maintain a value of 80–90).

**Vital sign monitoring**

Vital function parameters including heart rate and arterial blood pressure were also periodically monitored via a high pressure monitoring line (Smith Medical ASD Inc., Dublin, OH) connected to the left common carotid artery catheter, and recorded and stored using proprietary data acquisition software (Integrated Data Exchange and Archival [IDEA] system). Arterial pO₂ and pCO₂ Arterial blood gas analysis was performed at the bedside using an iSTAT 300-G blood analyzer (Abbott Point of Care Inc., Princeton, NJ; VetScan CG4+ and CG8+ cartridges, Abaxis Inc., Union City, CA).

**Hemorrhage shock and transfusion**

Hemorrhage was performed through the left common femoral artery catheter using a computer-controlled peristaltic pump (Masterflex, Cole-Parmer, Vernon Hills, IL) as described [14]. Blood was removed via Tygon tubing (E-Lab [E3603] L/S 16, Cole-Parmer) into 500-mL Teruflex blood-collection bags containing citrate-phosphate-dextrose (CPD) with OPTISOL red/cell preservative solution (Terumo Corp., Tokyo, Japan). Estimated total blood volume (EBV) was calculated using the following formula: EBV = weight in kilogram × 65 mL/kg. After correcting for the blood volume withdrawn for baseline laboratory values (48.5 mL), animals were bled 65% of EBV over 60 minutes in an exponential manner [14]. Entirely of shed whole blood was returned at a rate of 100mL/min via the right external jugular vein using a computer-controlled peristaltic pump (Masterflex, Cole-Parmer, Vernon Hills, IL). IV calcium chloride was infused to reverse hypocalcemia induced by the citrated blood.

**REBOA device and insertion**

REBOA (ER-REBOA, Pryor Medical, Inc.) was inserted at the end of hemorrhage via the 7 Fr introducer previously placed in the right common femoral artery. The intended zone of insertion for the balloon was between the left subclavian and the celiac arteries (zone 1). Insertion depth (40cm) was guided by the centimeter marks on the catheter.
Measurement of circulating metabolic parameters
Blood samples were analyzed for pH, pCO$_2$, pO$_2$, HCO$_3$, base excess/deficit (BE/BD), lactate, and glucose levels using i-STAT cartridges (Abbott Laboratories, Abbott Park, IL).

Complete blood count (CBC) and coagulation parameter assessment
CBC and coagulation parameters (PT, prothrombin time; INR, international normalized ratio; and PTT, partial thromboplastin time) were measured using an ABX Pentra 120 hematology analyzer (ABX Diagnostics, Inc., Irvine, CA) and Start-4 (Diagnostica Stago, Rue des Freres Chausson, FranceTAGO) respectively.

Assessment of circulating end-organ damage markers
Creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and myoglobin were measured from blood samples using Dimension Xpand Plus Integrated Chemistry System (Siemens, Holliston, Mass).

Histological evaluation
Under a general anesthesia animal will be euthanized at the end of study with Fatal Plus at a dose of 10ml/10lb intravenously. Following euthanasia, tissue samples (Liver, jejunum, and kidney,) for histology were collected and fixed in neutral buffered 10% formalin for 24 hours, trimmed, embedded in paraffin, sectioned at 4 $\mu$m, and stained with hematoxylin and eosin (H&E). Histologic images were recorded with a 10x objective under a slide scanner (Axio Scan.Z1 v1.0, Zeiss, Germany). Histologic grading of injury was performed by a single pathologist blinded to the identity of the animal. For the liver, five characteristics were scored (vascular congestion, thrombosis, hepatic apoptosis, cellular degeneration, and inflammation). The severity of the injury (score 0 = normal histology, score 1 = slight, score 2 = mild, score 3 = moderate and score 4 = severe alterations). For the jejunum, each slide was scored according to the following scale: 0, normal villi; 1, villi with tip distortion; 2, villi lacking goblet cells and containing Guggenheim’s spaces; 3, villi with patch disruption of the epithelial cells; 4, villi with exposed but intact lamina propria and epithelial cell sloughing; 5, villi in which the lamina propria was exuding; and 6, hemorrhaged or denuded villi. For the kidney, each slide was scored according to this scale: 0, normal histology; 1, slight alteration (loss of brush border, mild hydropic degeneration, mild congestion); 2, mild (intensive hydropic degeneration, mild vacuolization, and interstitial edema); 3, moderate (nuclear condensation, intensive vacuolization, moderate interstitial edema); and 4, severe (necrotic/apoptotic cells, denudation/rupture of basement membrane). For each slide, Total Injury Score (TIS) was calculated as the sum of the severity and the extent of injury. The grades for changes were assigned according to the extent (score 0, 1, 2, 3 and 4 for an extent of 0%, <25%, 25–50%, 50–75%, and 75–100% respectively). For most organs, several characteristics were scored and the choice of the features was suited to a particular organ as described [14].

Measurement of tissue cytokines and oxidative stress
Liver, jejunum, and kidney tissue specimens were collected, and homogenized in 50 mM potassium phosphate buffer, pH 7.4. The cytokines (TNF-$\alpha$, IL-1$\beta$, and IL-6) were detected using the MSD multiplex system (Rockville, MD). The reduced glutathione concentrations in the tissue were measured as previously described [23]. Briefly, the tissue extract (20 $\mu$l) was incubated with 120 $\mu$l of fresh prepared 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) and glutathione reductase (GR) solution (DTNB:GR mixture: 1:1) for 30 seconds at room temperature
followed by adding 60 μl of β-NADPH. The micro-plate was immediately read the absorbance at 412 nm by the microplate reader (Molecular Devices, San Jose, CA). For internal control, the concentrations of total protein were determined with a commercial kit (Pierce, Rockford, IL).

**Statistical analysis**
Using SAS Power 9.2 and a detectable difference of at least 82%, 7 animals per group is required to achieve a power of 0.8 using an alpha = 0.05. Given that prior studies have showed that survival in aortic balloon occlusion groups at 30 and 60 minutes of occlusion ranging from 87.5% to 100%, we believe that using 7 animals per group will provide adequate power to compare survival versus the negative control group. Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA) and Excel ver. 14.0. Data were statistically analyzed by Mann-Whitney test for non-parametric and semi-quantitative data. Data were expressed as mean ± standard deviation or standard error as indicated and significance was accepted at $p < 0.05$.

**Results**

**Baseline characterization and mortality**
There was no significant difference in baseline (BL) measurements of hemodynamic and metabolic parameters among the PC, A30 and A60 groups, except that PO$_2$ were lower in both groups of A30 and A60 ($p<0.05$, vs. PC) (Table 1). Four-hour survival for the PC, A30, and A60 groups was 71% (5/7), 100 (7/7), and 100% (7/7) respectively ($p = 0.1156$, log-rank test). The death of 2 animals in the PC group occurred at 122min and 108min respectively, and was from cardiac arrest after balloon deflation. These 2 animals were similar to their cohort in all respects.

**Effect of cREBOA duration on circulating metabolism and hemodynamics**
As shown in Table 1 and Fig 2, all groups responded to resuscitation with a substantial improvement in blood pressure (SBP, systolic blood pressure; DBP, diastolic blood pressure; and MAP), heart rate (HR), and shock index (SI) throughout the resuscitation period. A30 group, but not A60 group, responded to cREBOA with a significant rise in blood pressure above the BL values from R90 to EOS (Table 1 and Fig 2, $p < 0.05$). The vital signs (SBP, DBP and MAP) of individual animals were shown in S1 Table. Compared to PC and A30 groups, A60 exhibited a higher HR and SI, which were most significant at R150, R210 and EOS after resuscitation (Fig 2A and Table 1, $p < 0.05$). However, lactate and BE concentrations in A60 group were significantly higher and lower respectively from R90 to EOS post-cREBOA indicating severe metabolic acidosis (Fig 2C and 2D, $p < 0.05$). Hemodynamic parameters in A60 such as SBP, DBP, and MAP were significantly lower than A30 values ($p < 0.05$) while hemoglobin (Hb), hematocrit (Hct), and blood potassium (K$^+$) were significantly higher than both A30 and PC values (Fig 2B and 2C, and Table 1, $p < 0.05$). There was a trend toward greater levels of blood glucose at the EOS in A60 group compared to A30 group (323 ± 64 mg/dl). Other parameters including HCO$_3^-$, PO$_2$, and PCO$_2$, also showed a significant difference in A30 and A60 when compared to PC group (Table 1, $p < 0.05$), and in A60 (Table 1, A60 vs. A30, $p < 0.05$).

**Effect of cREBOA duration on circulating CBC and coagulation**
As shown in Figs 3 and 4, there was no significant difference in white blood cells (WBCs, neutrophils, lymphocytes, platelets, INR, PT, and PTT in all groups at BL and EH ($p > 0.05$).
### Table 1. Hemodynamics and metabolic characteristics in swine.

| Parameters     | Groups | Timeline |
|----------------|--------|----------|
|                |       | BL       | EH | R90 | R150 | R210 | EOS |
| MAP (mmHg)     | PC     | 123.0±21.4 | 30.7±13.8 | 104.3±35.6 | 118.4±18.6 | 115.0±21.4 | 101.5±45.7 |
|                | A30    | 118.4±20.7 | 45.0±9.0* | 115.6±13.8 | 148.1±23.8* | 158.3±20.8* | 152.6±26.9* |
|                | A60    | 113.7±6.9  | 44.1±10.4* | 85.1±22.1† | 86.7±17.7†  | 96.4±20.0†  | 96.6±18.5† |
| SI             | PC     | 0.6±0.1   | 3.5±1.0   | 0.8±0.3   | 0.6±0.1   | 0.7±0.0   | 1.2±1.4   |
|                | A30    | 0.5±0.1   | 2.9±0.6   | 0.9±0.3   | 0.6±0.2   | 0.5±0.2   | 0.6±0.3   |
|                | A60    | 0.6±0.1   | 2.9±0.6   | 1.2±0.3   | 1.3±0.5†  | 1.2±0.3†  | 1.2±0.3†  |
| HCO3 (mmol/L)  | PC     | 30.7±0.9  | 19.2±4.1  | 25.6±6.4  | 30.6±0.9  | 30.6±1.1  | 26.2±8.3  |
|                | A30    | 30.5±2.7  | 23.9±3.4  | 20.3±2.5† | 24.3±2.9† | 25.7±3.7† | 25.5±4.0† |
|                | A60    | 30.8±1.8  | 22.4±5.3  | 11.6±2.4† | 14.1±2.9† | 14.7±2.5† | 17.6±4.0† |
| PO2 (mmHg)     | PC     | 88.1±9.8  | 85.9±37.7 | 83.3±11.0 | 85.2±3.0  | 77.5±3.7  | 66.8±22.4 |
|                | A30    | 73.3±11.3* | 97.3±7.4  | 87.4±5.3  | 92.6±4.0* | 88.7±4.3* | 85.3±9.3* |
|                | A60    | 78.1±6.9  | 98.9±18.9 | 106.4±9.1† | 105.0±7.6† | 110.6±15.7† | 100.4±10.1† |
| PCO2 (mmHg)    | PC     | 47.3±1.8  | 44.0±14.0 | 48.1±17.1 | 44.6±1.2  | 44.2±1.6  | 61.0±25.0 |
|                | A30    | 47.1±5.2  | 28.9±4.5  | 35.9±4.1† | 39.7±3.1† | 40.6±3.3  | 39.9±3.9† |
|                | A60    | 48.0±2.3  | 31.9±10.9 | 31.0±4.7  | 30.2±6.0† | 38.8±5.6† | 35.5±6.5† |
| Hb (mmol/L)    | PC     | 22.0±1.6  | 19.2±4.5  | n/a       | n/a       | n/a       | 24.3±3.2  |
|                | A30    | 22.0±1.5  | 21.7±1.5  | 23.4±3.7  | 24.3±2.9  | 25.0±3.2  | 25.0±3.0  |
|                | A60    | 22.0±1.1  | 20.2±1.6  | 26.2±2.0† | n/a       | n/a       | 32.7±18.4† |
| Hct (%)        | PC     | 32.9±2.6  | 29.6±4.1  | n/a       | n/a       | n/a       | 36.5±5.9  |
|                | A30    | 32.7±1.5  | 31.7±3.3  | 34.8±5.0  | 35.2±4.7  | 35.4±4.8  | 35.3±5.0  |
|                | A60    | 32.7±2.5  | 31.4±2.2  | n/a       | n/a       | n/a       | 46.9±2.7† |
| Gluc (mmol/L)  | PC     | 7.0±1.4   | 21.1±8.8  | n/a       | n/a       | n/a       | 13.9±16.1 |
|                | A30    | 6.5±0.8   | 11.8±2.5* | n/a       | 14.9±1.9  | n/a       | 12.4±3.5  |
|                | A60    | 6.5±0.7   | 13.2±3.5  | n/a       | n/a       | n/a       | 17.9±8.9  |
| K⁺ (mmol/L)    | PC     | 3.8±0.2   | 4.9±1.4   | n/a       | n/a       | n/a       | 4.3±0.7   |
|                | A30    | 4.0±0.2   | 4.0±0.2*  | n/a       | 4.5±0.7   | n/a       | 4.2±0.4*  |
|                | A60    | 4.0±0.2   | 4.3±0.3   | n/a       | n/a       | n/a       | 7.1±1.2*  |

Abbreviations: BL, baseline; EH, end of hemorrhage; EOS, end of study; MAP, mean arterial pressure; N/A, not available; R, post-resuscitation; SI, shock index. Data was shown as Mean ± SD; *p<0.05, A30 vs. PC; †p<0.05, A60 vs. PC; ‡p<0.05, A60 vs. A30. Data were analyzed by Mann-Whitney test.

Circulating number of WBCs (Fig 3A) and neutrophils (Fig 3B) in A60 at EOS was significantly lower by 3- and 2-fold than PC and A30 respectively (p < 0.05). Lymphocyte counts were less in A60 at EOS compared to PC, but no significant differences were noted (Fig 3C) p > 0.05). The platelet count decreased in both A30 and A60 groups compared with PC, but did not reach statistical difference (Fig 4A p > 0.05). Significantly prolonged INR, PT and PTT in A60 at EOS were observed when compared to PC and A30 (Fig 4B–4D, p < 0.05).

**Effect of cREBOA duration on tissue inflammatory cytokines and oxidative stress**

Both A30 and A60 showed significant increase in renal IL-6, intestinal TNF-α, and hepatic IL-6 (vs. PC, p < 0.05, Fig 5A–5C), while higher levels of IL-6 in both kidney and liver tissues.
were observed in A60 group when compared to the A30 group ($p < 0.05$, Fig 5A and 5C). There were significant elevated tissue levels of TNF-α and IL-1β in liver compared with the PC ($p < 0.05$, Fig 5C). No significant difference was detected in renal TNF-α and IL-1β, and intestinal hepatic IL-1β and IL-6 within the 3 groups ($p > 0.05$, Fig 5A–5C). No significant change of renal, hepatic and intestinal IL-10 was observed among the 3 groups.
Next we analyzed reduced glutathione in different tissue isolated from the animals of the cREBOA and PC groups. Our analysis revealed significant decrease in renal (Fig 5A) and hepatic (Fig 4C) reduced glutathione in A60 when compared with PC or A30 respectively ($p < 0.05$), but there were no statistical differences of intestinal reduced glutathione in both A30 and A60 when compared with PC ($p > 0.05$, Fig 5B).

**Effect of cREBOA duration on circulating end organ damage markers**

As shown in Fig 6, the organ damage profile across the 3 groups was very similar at BL and EH ($p > 0.05$). Significant increases in plasma creatinine concentrations were detected at EOS in A60 compared with PC or A30 respectively ($p < 0.05$), but there were no statistical differences of intestinal reduced glutathione in both A30 and A60 when compared with PC ($p > 0.05$, Fig 5B).
Effect of cREBOA duration on histopathological alterations

Histopathology evaluation of the distal organ injury was performed at the EOS (Fig 7). Renal injury characterized by dilated Bowman's space filled with hyaline materials, intensive vacuolization and necrotic/apoptotic cells in renal tubules, interstitial edema and inflammatory cell infiltration, and thrombosis/congestion, and semi-quantitative histopathological evaluation showed significantly higher injury score in A60 than PC (p < 0.05). Intestinal tissue of the A60 group presented with significant damage featured by villus hemorrhage and denudation, lamina propria inflammatory cell infiltration, and congestion and exudation along with an increased injury score when compared with A30 (p < 0.05). There was no significant difference in histological injury score in the liver tissue among the 3 groups (p > 0.05), but greater congestion, dilation, and leukocyte sequestration within hepatic sinusoids were observed in the A60 group compared to the A30 and PC. Semi-quantitative histopathology evaluation of brain showed a trend of improved injury scores in the cerebral cortex (A30: 0.68 ± 0.44, A60: 0.82 ± 0.32) and hypothalamus (A30: 1 ± 0.37, A60: 1.23 ± 0.4) after the cREBOA when compared with the changes in the cerebral cortex (1.32 ± 0.55) and hypothalamus (1.59 ± 0.53) of the animals in the PC group (p > 0.05).

Discussion

Previously, several studies addressed cREBOA-associated ischemia reperfusion injury (IRI) sequelae including systemic inflammation, metabolic acidosis, and organ dysfunction after a
prolonged cREBOA use in hemorrhagic shock models in anesthetized pigs [24–27], baboons [28], and sheep [29]. However, there were unanswered questions in terms of cREBOA balloon inflation time and effects on distal organ inflammation, function and injury. In this current study, we sought to evaluate these questions regarding aortic balloon inflation times in a conscious and sedated porcine severe hemorrhagic shock model. The principal findings were as follows: compared with 30min-cREBOA group, 60min-cREBOA demonstrated (1) significantly greater systemic and distal organ inflammatory response, and reduction in reduced glutathione in kidney, jejunum and liver; (2) significant multiple-end organ dysfunction and injury; and (3) disturbances of hemodynamics and metabolism as well as coagulation.

Rasmussen and his colleagues [10, 22, 30–32] demonstrated that cREBOA has emerged as an effective life-saving maneuver for the management of non-compressible torso hemorrhage in trauma patients. cREBOA is inherently associated with the burden of lower body IRI and possible organ dysfunction. Therefore, the use of cREBOA is limited by the burden of IRI to distal organs. Indeed, as mentioned others have addressed cREBOA-associated IRI sequelae including systemic inflammation, metabolic acidosis, and organ dysfunction after in several

Fig 6. Changes of end-organ damage in plasma from swine undergoing cREBOA. Data are presented as mean ± SEM and were statistically analyzed using two-tailed Mann-Whitney test. * p<0.05, PC vs. A60 group; † p<0.05, A60 vs. A30 group. ALT, alanine aminotransferase; AST, aspartate aminotransferase.

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Consistent with these findings, the current data revealed that prolonged cREBOA (60min balloon inflation) after hemorrhage led to distal tissue hypoperfusion reflected by hypotension from R90 to the EOS and the higher lactate and lower BE from 30min to the EOS after the occlusion indicating a longer ischemia time. The hypoperfusion after the prolonged cREBOA appears to contribute to distal multiple-organ dysfunction (kidney, liver, and muscle) and damage (kidney and jejunum), hyperkalemia, and coagulation alterations. The hypotension in A60 group may be caused by reduced cardiac output as a result of hyperkalemia and/or preload reduction due to IRI-induced hyperemia reflected by increased tissue congestion in the distal organs (kidney, jejunum and liver). Although previous studies demonstrated that prolonged cREBOA time resulted in a systemic inflammatory response in swine [24] and non-human primate [28], tissue inflammatory response to prolonged cREBOA after hemorrhage in the distal organs has not been investigated. The enhanced release of the inflammatory cytokines TNF-α, IL-1β, and IL-6 are the biomarkers of IRI [33]. The most noteworthy finding in the current study was tissue inflammatory response in tissues as evidenced by greater levels of IL-6 in kidney, TNF-α in jejunum, and TNF-α, IL-1β and IL-6 in liver in the 60min-cREBOA group when compared to the PC or the 30min-cREBOA group. Our findings of highly elevated pro-inflammatory cytokines in the organs below the aortic occlusion indicate that these tissues underwent IRI. Recent studies illustrated glutathione derangement and stress hyperglycemia in the settings of tissue injury/hemorrhage and critical illness [34, 35]. Reduced glutathione is a powerful intracellular antioxidant that can modulate NF-kB and AP-1 activation influencing transcription of cytokines and adhesion molecules [36]. In this study, decreased levels of reduced glutathione in kidney and liver after 60min-cREBOA may at least partially contribute to the tissue cytokine production and organ damage observed.

In contrast to a previous report [28], the 60min cREBOA group in this current study had acute leukopenia, neutropenia, and platelet decline at the EOS. Acute leukopenia is a recognized condition in critically ill septic and cancer patients [37, 38]. The exact mechanism of leukopenia and platelet reduction after cREBOA and hemorrhage is unknown, but there are...
interesting speculations. According to previous studies [39, 40], we generally assume that the acute leukopenia/neutropenia and platelet reduction after 60min-cREBOA in this study are probably due to IRI-induced (1) leukocyte/platelet activation and sequestration, and/or (2) overwhelming bacterial translocation from the damaged intestine. Indeed, upregulation of neutrophil integrin CD11b after supraceliac aortic clamping in patients was shown as the primary adhesive glycoprotein responsible for neutrophil tissue entrapment and neutrophil-mediated reperfusion injury [41]. Moreover, histological evidence of liver, jejunum, and kidney injury reflected by vascular congestion and inflammatory cell infiltration indicate that leukocyte/platelet activation and sequestration are the most likely mechanism for leukopenia and platelet reduction in this study. The leukocyte sequestration in the injured tissues may contribute to the increase in levels of TNF-\(\alpha\), IL-1\(\beta\), and IL-6 in liver, kidney and small intestine. Our finding is consistent with the previous report that neutrophil infiltration into IRI tissues is involved in upregulation of hepatic inflammatory proteins, the complement system, chemokine, oxidative stress, and increased capillary permeability [42, 43]. Uncontrolled inflammation is often accompanied by microvascular thrombosis. Several inflammatory cytokines such as TNF-\(\alpha\) may activate tissue factor and coagulation [44] as we have shown in our study with the 60min cREBOA treatment. Unlike previous reports [24, 28], lack of clear changes in the blood cytokines (IL-1\(\beta\), IL-6, IL-8, IL-10, and TNF-\(\alpha\)) in this study may simply reflect different experimental conditions as species, preoperative status, differences in surgical extensity, the methods of aortic occlusion and subject management, and a short follow-up.

Below the aortic occlusion, IRI primarily affects endothelium-dependent vasodilation other than vasoconstriction, a state known as “reactive hyperemia” contributing to reduction in afterload that may result in distributive shock [45, 46]. In accordance with it, the 60min cREBOA time in the current study led to increased tissue congestion and edema in the distal organs (kidney, jejunum and liver) compared with the 30min-cREBOA group. These alterations may explain, at least in part, why the 60min-cREBOA group had significantly higher levels of Hb and Hct, and lower blood pressures post-cREBOA deflation.

Numerous studies in critically ill patients have demonstrated that stress hyperglycemia is associated with poor clinical outcomes. Although the acute treatment of mild-moderate hyperglycemia with intensive glucose control in acutely ill patients lacks biologic plausibility [47], persistent severe stress hyperglycemia may lead to its effects on osmolarity-induced fluid shift, diuresis and volume depletion [35]. In this study, severe hyperglycemia (60min-cREBOA vs. 30min-cREBOA: 323 mg/dl vs. 224 mg/dl) may be harmful, which may partially contribute to the organ dysfunction and damage. Therefore, persistent severe stress hyperglycemia may benefit from moderate glycemic control in our future cREBOA studies.

It is becoming evident that multiple-organ crosstalk after IRI plays an important role in multiple-organ dysfunction [48]. Recent insights in basic and clinical research have been gained into organ-organ crosstalk, which contributes to metabolic homeostasis, inflammatory response, endothelial injury, oxidative stress and reactive oxygen species production, apoptosis, and coagulopathy during sepsis and acute kidney injury (AKI) [49–51]. Although liver tissue is relatively resistant to IRI itself, this condition triggers the generation of many factors including cytokines and chemokines produced by Kupffer cells (KCs), anaerobic metabolism, mitochondrial damage, oxidative stress, and intracellular Ca\(^{2+}\) overload, which cause acute liver failure (ALF) as well as commonly lead to extrahepatic multiple-organ dysfunction (MOD) [52, 53]. Indeed, liver tissue generation of cytokines (IL-1\(\beta\) and TNF-\(\alpha\)) produced by KCs precedes the late, cellular phase of reperfusion injury [54, 55]. Generally, AKI occurs often in patients with acute liver injury [56]. Hypotension leads to up-regulation of renin-angiotensin system that can severely reduce glomerular filtration rate, urinary sodium excretion, and free water excretion. KCs are activated after liver IRI and predominantly contribute
to increased circulating levels of pro-inflammatory cytokines, including IL-6, TNF-α, and high-mobility group box 1 protein and their release from the liver tissue can initiate inflammation in the kidney tissue [54]. In the current study, the plasma myoglobin concentration was higher in A60 than in the A30 group, indicating A60 as a stronger rhabdomyolysis case. Acute renal injury appears as a complication of myoglobinuria in the setting of any trauma [57, 58]. Rhabdomyolysis that is associated with the A60 group could contribute to the pathogenesis of AKI observed in our study.

Intestinal IRI disrupts mucosal integrity and increases intestinal permeability resulting in dysbiosis, and translocation of bowel bacteria and their endotoxins into the circulation that lead to systemic inflammation, and immune, metabolic and coagulation alteration [59]. This altered homeostasis may mediate and potentiate the development of MOD including AKI and ALF [49, 60, 61]. In turn, the development of AKI and ALF decreases the clearance of inflammatory mediators, metabolic products, and translocated bacteria and their endotoxins, leading to a vicious cycle and further gut injury. It may also have a role in the pathogenesis of coagulopathy, along with hepatic ischemia. Increased plasma concentrations of cytokines (e.g. TNF-α, IL-6, IL-8, and IL-10) are often observed in patients with aortic cross-clamping/ischemia longer than 40 minutes [62, 63]. In our porcine study, cREBOA treatment for 60 minutes did induce significant damage to the intestinal tissue, but displayed no significant changes of IL-1β, IL-6, and oxidative stress unlike kidney and liver tissue. Plausible explanations are that these contradictory data may be due to a different kinetics of the pro-inflammatory cytokines/glutathione production in jejunum in response to IRI, and/or loss of villi where are heavily populated with activated leukocytes after IRI. Similarly, liver enzymes (AST and ALT) increased to abnormal concentrations with no significant differences in liver tissue damage even though oxidative stress was severe. These results demonstrate that reduced glutathione or AST and ALT may be not reliable indicators of intestinal and liver damage, respectively. High-mobility group box 1 protein, matrix metalloproteinases, neutrophil gelatinase-associated lipocalin, diamine oxidase, fatty acid-binding proteins, and citrulline could be used as new biomarkers of IRI of intestine and liver [52, 64].

Therefore, in order to mitigate the prolonged cREBOA-induced IRI, It will be worthwhile to consider different treatment approaches as follows: (1) immunomodulation, (2) metabolic therapeutic interventions, (3) intermittent infusion of oxygenated fluid/blood, and (4) self-regulated partial REBOA.

Limitations

Our study does not include spinal cord effects that may yield new challenges. Because interaction among damage-associated molecular patterns, complement cascade, and inflammasome plays a pivotal role in tissue IRI [65], the study of these cascades deserves attention in highlighting their importance. Since different organs may have different time windows of the inflammatory response to IRI, future study will be needed to determine the kinetics of the tissue inflammation during cREBOA. Finally, the observation period of this study is too short to determine a long-term impact on survival, inflammatory response and distal organ damage, longer follow-up should be carried out in future.

Conclusions

Although life-saving in 100% of animals, cREBOA treatment of severe hemorrhage should be shorter than 60 minutes due to its impact on pathophysiological sequelae, specifically on distal organ inflammation and damage. In all, cREBOA application deserves further research that
would include mitigation of inflammation, coagulopathy, distal organ IRI, and longer follow-up.

Supporting information
S1 Table. Vital signs of individual animals.
(DOCX)

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Author Contributions
Conceptualization: Yansong Li, Michael A. Dubick, Andriy I. Batchinsky, Leopoldo C. Cancio.
Data curation: Johnny L. Barr, Corina Necsoiu.
Formal analysis: Yansong Li, Zhangsheng Yang, Johnny L. Barr.
Funding acquisition: Michael A. Dubick, Andriy I. Batchinsky, Leopoldo C. Cancio.
Investigation: Yansong Li, Michael A. Dubick, Zhangsheng Yang, Johnny L. Barr, Brandon J. Gremmer, Michael L. Lucas, Corina Necsoiu, Bryan S. Jordan, Andriy I. Batchinsky, Leopoldo C. Cancio.
Methodology: Yansong Li, Michael A. Dubick, Zhangsheng Yang, Johnny L. Barr, Brandon J. Gremmer, Michael L. Lucas, Corina Necsoiu, Bryan S. Jordan, Andriy I. Batchinsky, Leopoldo C. Cancio.
Project administration: Yansong Li, Michael A. Dubick, Andriy I. Batchinsky, Leopoldo C. Cancio.
Supervision: Yansong Li, Michael A. Dubick, Andriy I. Batchinsky, Leopoldo C. Cancio.
Validation: Yansong Li, Michael A. Dubick, Leopoldo C. Cancio.
Visualization: Yansong Li, Zhangsheng Yang.
Writing – original draft: Zhangsheng Yang, Brandon J. Gremmer.
Writing – review & editing: Yansong Li, Michael A. Dubick, Zhangsheng Yang, Corina Necsoiu, Andriy I. Batchinsky, Leopoldo C. Cancio.

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