RESEARCH ARTICLE

Monitoring Organs Susceptible to Ischemia/Reperfusion Injury after Prolonged Resuscitative Endovascular Balloon Occlusion of the Aorta in a Hemorrhagic Shock Swine Model

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Abstract

Background: Despite advancements in critical care, hemorrhage remains a leading cause of potentially survivable deaths in civilian and military settings. Resuscitative endovascular balloon occlusion of the aorta (REBOA) has emerged as a viable technology that could be used in the prehospital setting. However, the potential complications of prolonged REBOA use, especially in the military setting where the prehospital phase could exceed 2 hours, are not completely understood with regards to organ damage susceptibility to prolonged REBOA use.

Materials and methods: Fifteen male Yorkshire swine underwent a 40% volume-controlled hemorrhage over 20 minutes. Animals were then randomly assigned (n = 5/group) to REBOA inflation times of 120, 180, and 240 minutes, followed by 1 hour of resuscitation with shed whole blood and crystalloid before euthanasia. Samples were collected for blood gas analysis, chemistry, Luminex, enzyme-linked immunosorbent assays (ELISAs), and histology.

Results: Metabolic acidosis increased with prolonged REBOA inflation times along with inflammation as shown by increases in interleukin (IL)-6 and neutrophil levels. Organs most susceptible to prolonged REBOA inflation times were the liver and intestines as demonstrated by histology.

Conclusion: While REBOA has been shown to effectively staunch hemorrhage and improves survival, complications exist for prolonged REBOA inflation times. The results of this study demonstrate that the liver and intestines are particularly susceptible to prolonged REBOA inflation out to 4 hours, in addition to increased metabolic acidosis and systemic inflammation. These findings should help guide clinicians while using REBOA over a prolonged period of time to improve survival and mitigate potential REBOA-associated ischemic organ damage.

Keywords: Experimental hemorrhagic shock, Noncompressible torso hemorrhage REBOA, Shock, Swine, Trauma.

Resumen

Antecedentes: A pesar de los avances en cuidados intensivos, la hemorragia sigue siendo una de las principales causas de muertes potencialmente que podrían sobrevivir en entornos civiles y militares. La oclusión de reanimación endovascular con balón de la aorta (REBOA) ha surgido como una tecnología viable que podría utilizarse en el entorno prehospitalario. Sin embargo, las posibles complicaciones del uso prolongado de REBOA, especialmente en el entorno militar donde la fase prehospitalaria podría exceder las 2 horas, no se comprende completamente con respecto a la susceptibilidad al daño orgánico por el uso prolongado de REBOA.

Materiales y métodos: Quince cerdos machos de Yorkshire sufrieron una hemorragia controlada en volumen del 40% durante 20 minutos. A continuación, a los animales se asignaron aleatoriamente (n = 5 / grupo) a tiempos de inflado de REBOA de 120, 180 y 240 minutos, seguidos de 1 hora de reanimación con sangre entera derramada y cristaloides antes de la eutanasia. Se recolectaron muestras para análisis de gases en sangre, química, Luminex, ELISA e histología.

Resultados: La acidosis metabólica aumentó con tiempos prolongados de inflación de REBOA junto con la inflamación, como lo demuestran los aumentos en los niveles de interleucina (IL) -6 y neutrófilos. Los órganos más susceptibles a tiempos prolongados de inflación de REBOA fueron el hígado y los intestinos, como lo demuestra la histología.

Conclusión: Si bien se ha demostrado que REBOA detiene eficazmente la hemorragia y mejora la supervivencia, existen complicaciones para tiempos prolongados de inflación de REBOA. Los resultados de este estudio demuestran que el hígado y los intestinos son particularmente susceptibles a una inflación prolongada de REBOA hasta 4 horas, además de un aumento de la acidosis metabólica y la inflamación sistémica. Estos hallazgos deberían ayudar a guiar a los médicos mientras usan REBOA durante un período prolongado de tiempo para mejorar la supervivencia y mitigar el daño orgánico isquémico potencial asociado con REBOA.

Palabras clave: REBOA, hemorragia torso no compresible, trauma, shock, shock hemorrágico experimental, porcino.

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Introduction

Hemorrhage remains the leading cause of potentially survivable death following traumatic injury. A large study of combat-related deaths over 10 years found that of 4,574 prehospital deaths, 24% of them were potentially survivable with 91% of those deaths from uncontrolled hemorrhage.[1] The concept of the "golden hour" following hemorrhage has remained a guiding principle for

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clinicians over the last several decades emphasizing the importance of rapid goal-directed care. Recent research efforts have focused on not only extending but also improving the care during this time to improve patient survival and outcome following traumatic injury. Advancements in this area have largely been led by the military, where providers face unique and challenging situations with injured warfighters. Rapid control of noncompressible hemorrhage may not be feasible during evacuation or transport, mandating novel strategies for temporary hemorrhage control.

While prehospital measures, such as, tourniquets have led to improvements in outcomes from compressible hemorrhage, noncompressible hemorrhage remains a particular challenge with between 50% and 86% of preventable death caused by exsanguination from a noncompressible source.[1,2] Resuscitative endovascular balloon occlusion of the aorta (REBOA) is a technology currently in use in both military and civilian trauma centers to help control noncompressible torso hemorrhage. Studies demonstrate effective restoration of proximal blood pressure, but resulting complications from distal ischemia remain a challenge and are poorly studied.[3–5] Complications can be related to distal vascular complications due primarily to mismanagement of the intra-arterial sheath, balloon over-inflation, improper balloon placement, and sheath size relative to small arteries.[6] However, distal organ ischemia represents the larger challenge. Animal studies demonstrate acute kidney injury, liver failure, intestinal ischemia, limb loss, myocardial damage, spinal cord infarction, and distal arterial thrombosis following REBOA.[5,7–9]

Previous investigations of these complications demonstrated mild distal organ ischemia, but inflation times were carried out to only 90 minutes of complete REBOA occlusion in a 25% hemorrhage model, finding there were signs of necrosis and disruption of the duodenal mucosa.[10] Another study in a swine hemorrhage model with complete occlusion of 60 minutes compared with partial occlusion found kidney and small bowel injury in both complete and partial occlusion groups, with more injury seen in the complete occlusion group.[11]

In this study, we evaluated more prolonged REBOA inflation times, up to 4 hours, in a porcine hemorrhagic shock (HS) model utilizing a resuscitation-limited strategy, as would be expected in a prolonged prehospital transport scenario. We hypothesized that REBOA inflation beyond 2 hours would lead to significant ischemic organ damage. Our primary objective was to determine which organs were most susceptible to ischemic damage from prolonged REBOA occlusion, as well as assess how systemic inflammation responds to prolonged REBOA use. This information could be used to determine the immediate post-deflation resuscitation needs following prolonged aortic occlusion (AO), to inform further both resource utilization and triage decisions in both military and civilian trauma centers.

Materials and Methods

Study Overview

The study protocol was reviewed and approved by the University of Maryland, Baltimore, as well as the Animal Care and Use Review Office in compliance with all applicable Federal regulations governing the protection of animals in research. The experiments reported herein were conducted in compliance with the Animal Welfare Act and per the principles outlined in the "Guide for Care and Use of Laboratory Animals", Institute of Laboratory Animals Resources, National Research Council, National Academy Press, 2011.

This is an *in vivo* study designed to characterize the effect of HS and subsequent AO by REBOA on the circulating markers of ischemia. Fifteen male Yorkshire swine (*Sus scrofa;* 70–90 kg) were acquired from a herd of animals raised for research purposes by the same commercial provider. The study consisted of four phases, which included preparation, induction of HS, AO, and reperfusion/critical care. After animal preparation and induction of HS, groups were divided into three groups of five animals each undergoing increasing lengths of AO for 120, 180, and 240 minutes. The AO phase represents the time necessary for prehospital transport from the point of injury to definitive care with varying lengths of time to simulate different environments. The resuscitation/reperfusion phase represented the last phase and the time point at which the patient arrived at definitive care with surgical capabilities and comprehensive resuscitation modalities.

Animal Preparation and Hemorrhage

Animals were induced with general anesthesia consisting of intramuscular ketamine at 10–15 mg/kg and xylocaine 1–2.2 mg/kg followed by sedation using propofol infusion at 0.2–0.6 mg/kg/minute to effect and isoflurane with a range of 1–4% by snout mask. Using a midline neck incision, animals then underwent tracheostomy then were preferentially ventilated using volume control at 6 mL/kg, positive end-expiratory pressure (PEEP) of 5 cm H_2O, and FiO_2 40–100% to maintain a SpO_2 >92%.

An arterial catheter was placed in the right common carotid artery. The right jugular vein was cannulated with a large-bore venous resuscitation catheter as well as a 9-F sheath to accommodate the pulmonary arterial (PA) pressure transducer. Given the baseline excitability of swine myocardium, a dose of lidocaine (2–4 mg/kg) was administered before the insertion of the pulmonary artery catheter. This was followed by a prophylactic lidocaine infusion (30–80 µg/kg/minute) to treat arrhythmia. To minimize the exposure and dissection of the left carotid artery, the left jugular vein was not cannulated.

Bilateral incisions were made in the groin with exposure of underlying vasculature at the level of the common femoral vessels. A large-bore venous resuscitation catheter was inserted in the left common femoral vein for controlled hemorrhage and resuscitation.

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Conflict of interest: None

An arterial transducer was placed in the left common femoral artery (CFA) to ensure adequate AO. The right CFA was cannulated with a 7-Fr sheath for insertion of an ER-REBOA™ catheter (Prytime Medical Inc., Boerne, Texas, USA). An open cystostomy was performed, and urine was drained continuously.

Controlled Hemorrhage

Hemorrhagic shock was induced using a volume-controlled method designed to accurately achieve a severe physiologic response seen in patients with hemorrhage.[12] During HS phase, 40% of the animal's estimated blood volume was removed over the course of 20 minutes from the left common femoral vein catheter. The first 20% of blood volume was removed by withdrawing 2.15 mL/kg/minute over 7 minutes, and the remaining 20% of blood volume removed over 13 minutes at a rate of 1.15 mL/kg/minute.12 Shed blood was collected in citrated collection bags and kept at room temperature. Animals then underwent a 10-minute period where no intervention or resuscitation was performed, besides limited crystalloid administration.

Aortic Occlusion

Zone 1 aortic occlusion (AO) was performed at the end of the HS phase. The balloon was advanced to zone 1 of the aorta, inflated, and confirmed by fluoroscopy. Animals were then divided into three groups of five animals each undergoing either 120, 180, or 240 minutes of occlusion. During this period, a veterinary physician with extensive resuscitation experience followed a strict resuscitation protocol based on systolic blood pressure (SBP) and was designed to simulate the real-world resuscitation capabilities of a single prehospital provider with limited resources. The animals were preferentially resuscitated with an IV crystalloid bolus of 1 L if both heart rate (HR) >105 and SBP <90. Resuscitation with shed whole blood was limited to 1 unit (~300 mL) per hour if any three of the following criteria were met: HR >105, SBP <90, hematocrit (Hct) <32, and pH <7.25. No inotropic or vasopressor support was allowed during the AO phase.

Resuscitation/Critical Care

The intensive care unit (ICU) phase began after the deflation of the balloon. This phase represents the arrival of the patient to a full-resource resuscitation facility using standard-of-care practices. Shed blood was preferentially reinfused along with intravenous calcium. If all shed blood was given, then intravenous 0.9% saline was administered to achieve a mean arterial pressure (MAP) >60 mm Hg, until the animal was no longer fluid responsive as guided by real-time transesophageal echocardiography (TEE). At this point, an infusion of norepinephrine commenced, followed by inotropic support using epinephrine and dobutamine as guided by TEE. Further resuscitation was guided by arterial and venous blood gas analysis, pulmonary artery pressures, invasive blood pressure monitoring, and bedside point-of-care laboratory evaluations. Transesophageal echocardiography was performed by a credentialed cardiac-trained anesthetist with extensive resuscitation experience.

Euthanasia

At the end of the 1-hour ICU phase, isoflurane was increased to 5%, and a lethal injection of potassium chloride (2 mEq/kg IV) was administered using the right internal jugular venous resuscitation catheter.

Arterial Blood Gas Analysis

Arterial blood samples were acquired in sodium heparin blood collection tubes every 30 minutes starting with a baseline (BL) measurement immediately before induction of HS. These samples were used for arterial blood gas analysis including lactate, pH, potassium, and phosphorus on a blood gas analyzer.

Blood Chemistry Markers of Organ Function

Arterial blood samples for blood chemistry were acquired in lithium heparin blood collection tubes every 30 minutes starting with a BL measurement immediately before induction of HS. Blood chemistry markers analyzed included creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), creatine phosphokinase (CPK), and lactate dehydrogenase (LDH).

Complete Blood Counts

Arterial blood samples for complete blood counts were acquired in EDTA blood collection tubes every 30 minutes starting with a BL measurement immediately before induction of HS. Complete blood counts' analysis included white blood counts (WBC), neutrophils, hemoglobin, and platelets.

Circulating Markers of Ischemia

Samples for cytokine analysis were drawn at BL immediately prior to hemorrhage, after HS phase immediately prior to and at the end of AO, and euthanasia in EDTA tubes. Plasma samples for Luminex were analyzed using the porcine custom 5-plex for interleukin (IL)-1α, IL-1 receptor antagonist (IL-1ra), IL-6, IL-8, and IL-10 (Millipore, Burlington, Massachusetts, USA). The Luminex assay was run according to the manufacturer's instructions using undiluted plasma samples and analyzed on the Bio-plex® 200 Luminex system with the Bio-plex Manager™ software. Enzyme-linked immunosorbent assays (ELISAs) for porcine-specific syndecan-1, cytokeratin 18 M30 (CK18 M30), and cytokeratin 18 M65 (CK18 M65) fragments were obtained from Bluegene (Shanghai, China) and performed according to the manufacturer's instructions using undiluted plasma samples. Absorbances for ELISAs were read at 450 nm on a plate reader (BioTek Instruments Inc., Vermont, USA).

Histology

Organs collected after euthanasia included liver, kidney, and intestines. Organ samples were fixed with 10% formalin, embedded, sectioned, and stained with hematoxylin and eosin (H&E) for analysis by a blinded board-certified pathologist. Liver injury was scored using the Suzuki scoring system. This ranking system scores from 0 to 4 going from none [0], to minimal [1], mild [2], moderate [3], and severe [4]. The components that are ranked include congestion, vacuolization, and necrosis.[13] The small and large bowels including the proximal and distal ends of each were scored using the Park/Chiu scoring scale which included normal mucosa [0], subepithelial space at villus tips [1], an extension of subepithelial space with moderate lifting [2], massive lifting down the sides of the villi, some denuded tips [3], denuded villi, dilated capillaries [4], the disintegration of lamina propria [5], crypt layer injury [6], transmucosal infarction [7], and transmural infarction [8].[14]

Outcomes

The primary outcomes were changes in circulating marker concentrations between the end of HS and the end of AO. Secondary endpoints included variance in marker levels between baseline (BL), end of HS, end of AO, and euthanasia.

Statistical Analysis

Data were organized using Microsoft Excel (Redmond, Washington, USA) and analyzed using GraphPad Prism (La Jolla, California, USA).

Data were log-transformed, and a one-way ANOVA with multiple comparisons using the Bonferroni correction was employed to compare within-group time differences. Histological scoring was analyzed using the Kruskal–Wallis test with Dunn's multiple comparisons. Pearson's correlation coefficient was used to assess correlations. Statistical significance was defined as a p value ≤ 0.05. Results are reported as mean $\pm$ SD.

Results

General Markers of Ischemia

In evaluating the effect of prolonged REBOA, circulating markers of ischemia were assessed to monitor organ damage and inflammation. Lactate levels began significantly increasing in all groups by the end of hemorrhage and continuing until the time of euthanasia (Fig. 1A). The pH fell significantly in all groups by 30 minutes following AO and continued to decrease throughout inflation and resuscitation until the final 30 minutes (Fig. 1B). No group returned to baseline levels; however, the pH of the 120-minute REBOA group returned to its predeflation value. Potassium levels did not significantly change from baseline except for the 180-minute group, which demonstrated elevated potassium at 60 minutes after hemorrhage (Fig. 1C). Phosphorus levels became significantly elevated for all groups during the resuscitation period, with the 180-minute group significantly elevated at the beginning of resuscitation and within the first 30 minutes for the 120- and 240-minute groups (Fig. 1D). Overall, no significant correlations were demonstrated between lactate, pH, potassium, or phosphorus and REBOA inflation times.

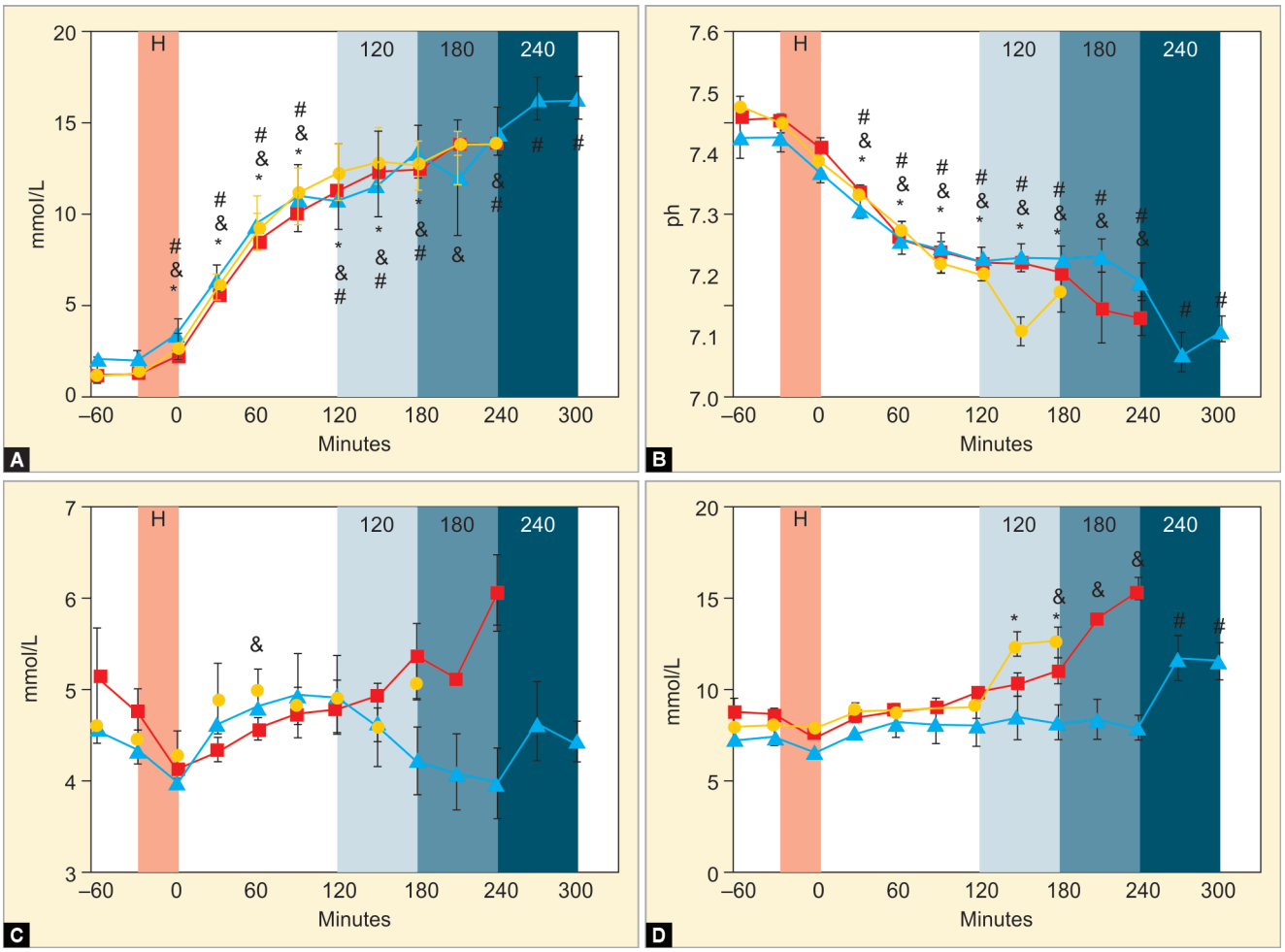
Organ-specific Markers

Kidney

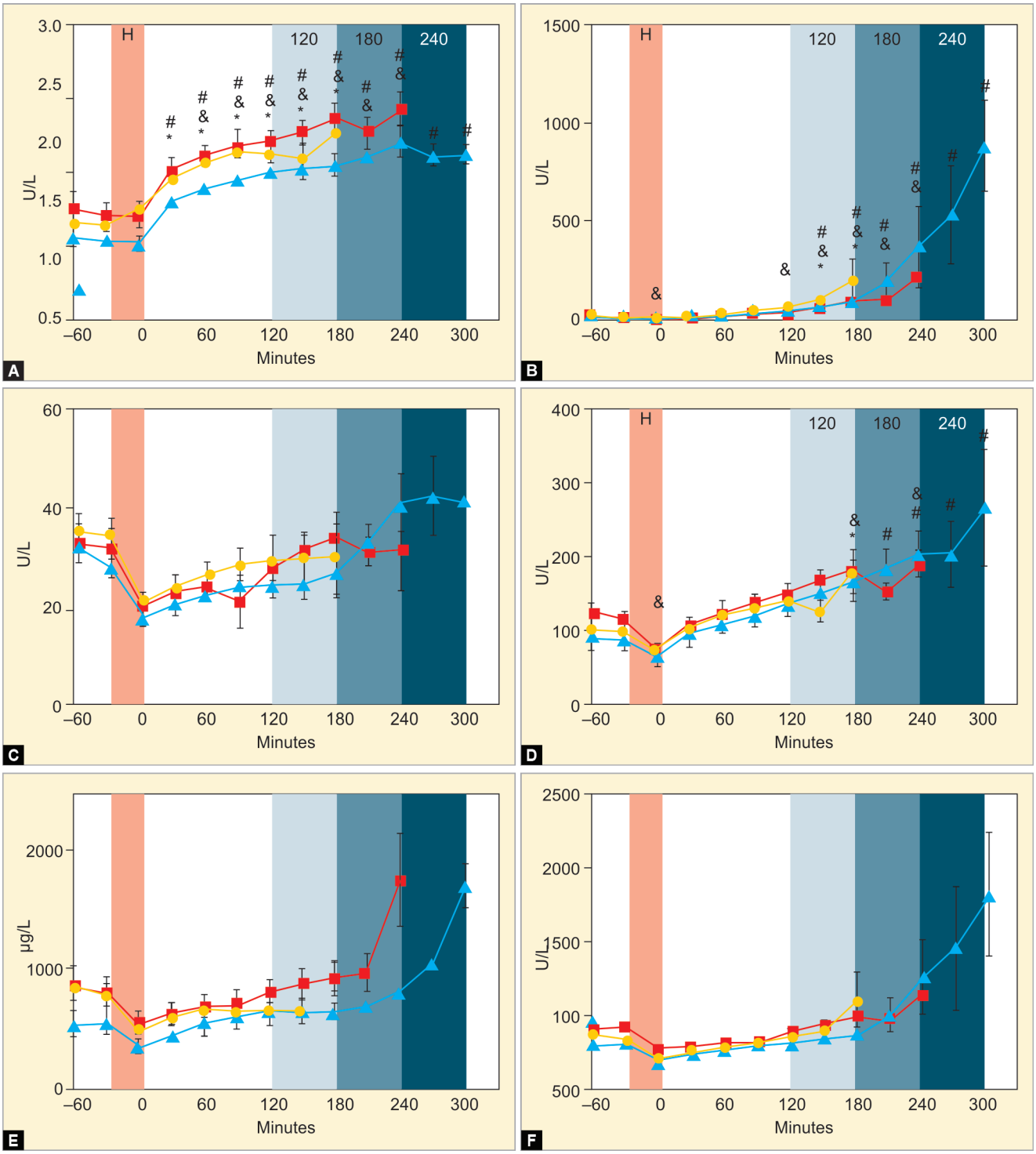
Creatinine increased significantly compared with baseline 30 minutes after the end of hemorrhage in the 120- and 240-minute groups and all groups showed significant increases from baseline by 60 minutes after the end of the hemorrhage and continued throughout the study (Fig. 2A). By the end of resuscitation, levels of creatinine increased 0.76, 0.84, and 0.70 mg/dL from baseline levels. No measurable urine was made during any experiment.

Liver

Levels of AST were significantly increased from baseline until the study end starting at 30 minutes into resuscitation, 60 minutes before resuscitation, and 120 minutes before resuscitation, in the 120-, 180-, and 240-minute groups, respectively (Fig. 2B). The



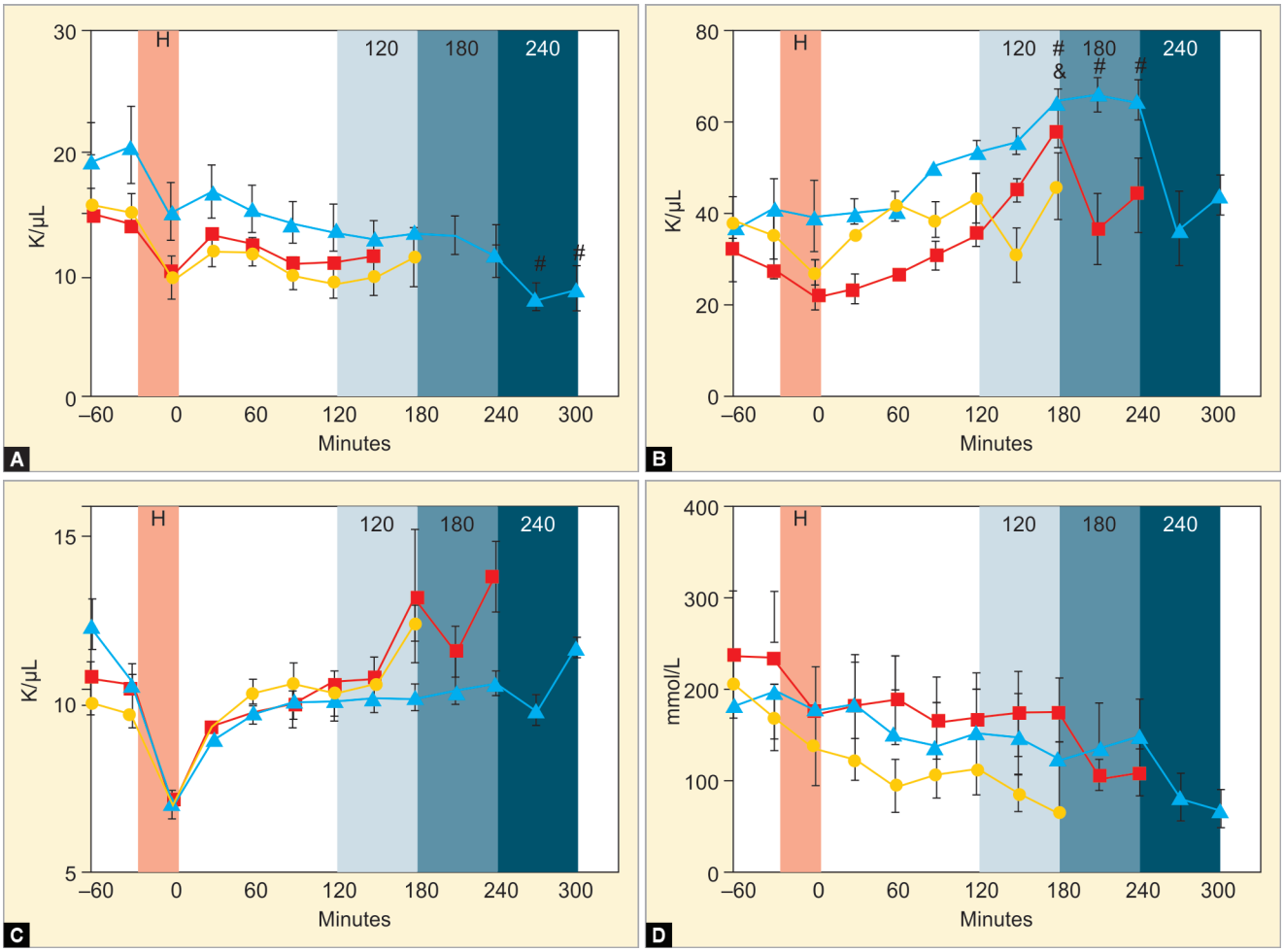
Figs 1A to D: Arterial blood gas (ABG) analysis. Results of the ABG analysis for lactate (A), pH (B), potassium (C), and phosphorus (D) are shown in the Figures. "H" is the hemorrhage period, columns labeled 120, 180, or 240 represent the hospital arrival resuscitation phase. Significance is shown by "*" (120 minutes), "&" (180 minutes), and "#" (240 minutes) REBOA groups at $p > 0.05$ significance



Figs 2A to F: Blood chemistries. Blood chemistry markers are shown in the figure for creatinine (A), aspartate aminotransferase (AST) (B), alanine aminotransferase (ALT) (C), alkaline phosphatase (ALP) (D), creatine phosphokinase (CPK) (E), and lactate dehydrogenase (LDH) (F). "H" is the hemorrhage period, columns labeled 120, 180, or 240 represent the hospital arrival resuscitation phase. Significance is shown by "*" (120 minutes), "&" (180 minutes), and "#" (240 minutes) REBOA groups at $p>0.05$ significance

change in AST levels were 183, 206, and 868 U/L, respectively. There were no significant changes in ALT (Fig. 2C).

Alkaline phosphatase steadily increased for all groups becoming significantly increased from baseline to the study end for the 120-minute REBOA group, at the beginning of resuscitation for the 180-minute REBOA group, and from 30 minutes before resuscitation for the 240-minute REBOA group (Fig. 2D). Alkaline phosphatase levels increased from baseline to the study end for the 120-, 180-, and 240-minute REBOA groups was 79, 68, and 174 U/L.



Figs 3A to D: Complete blood count (CBC) analysis. CBC results are shown in the figure for white blood counts (WBC) (A), neutrophils (B), hemoglobin (C), and platelet levels (D). The *y*-axis shows the concentration units and the *x*-axis shows the time-points. "H" is the hemorrhage period, columns labeled 120, 180, or 240 represent the hospital arrival resuscitation phase. Significance is shown by "*" (120 minutes), "&" (180 minutes), and "#" (240 minutes) REBOA groups at $p > 0.05$ significance

Heart

Creatine phosphokinase concentrations significantly increased only in the 240-minute REBOA group compared with baseline at 30 minutes into the resuscitation phase and remained significantly elevated through study end (Fig. 2E). The increase in CPK levels between baseline and study end was 99, 890, and 1178 µg/L, respectively, and a significant correlation between REBOA inflation time and CPK levels was found ($r = 0.99$; $p < 0.05$).

Levels of LDH increased in all groups after initially falling during hemorrhage, with significantly elevated levels seen at study end in the 180-minute group and the start of resuscitation in the 240 minutes (Fig. 2F). Changes in LDH levels between baseline and study end were 294, 301, and 1280 U/L for the 120-, 180-, and 240-minute REBOA groups.

Blood Counts

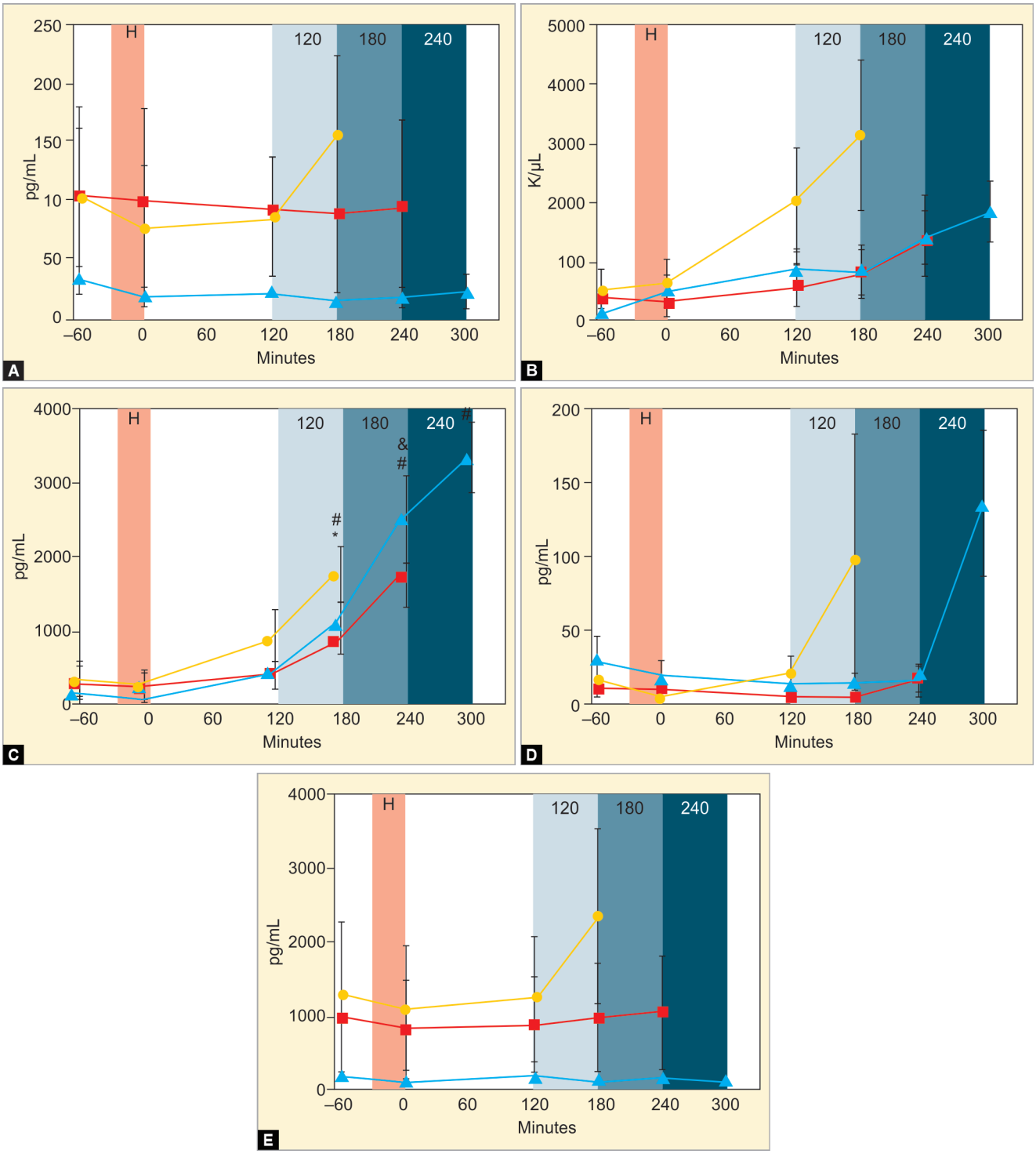
White blood counts (WBC) steadily decreased during hemorrhage, REBOA inflation, and resuscitation. Neutrophil levels began increasing following REBOA inflation with significant elevations seen in the 180-minute REBOA group at the beginning of resuscitation and in the 240-minute REBOA group 60 minutes before and up until the start of resuscitation. By the study end, neutrophil levels again dropped, resulting in an overall small change from baseline in the 120-minute (7K/µL), 180-minute (12K/µL), and 240-minute (7K/µL) groups (Fig. 3B).

Hemoglobin levels decreased significantly in all groups following hemorrhage, with improving levels as blood was infused according to the resuscitation-limited strategy. Compared to baseline, platelet counts continuously dropped in all three groups. No significant differences were observed between baseline and any time-point in any group (Fig. 3D).

While trends were identified, no significant positive or negative correlations were seen between REBOA inflation time and levels of WBCs, neutrophils, hemoglobin, or platelets.

Cytokine/Chemokine Analysis

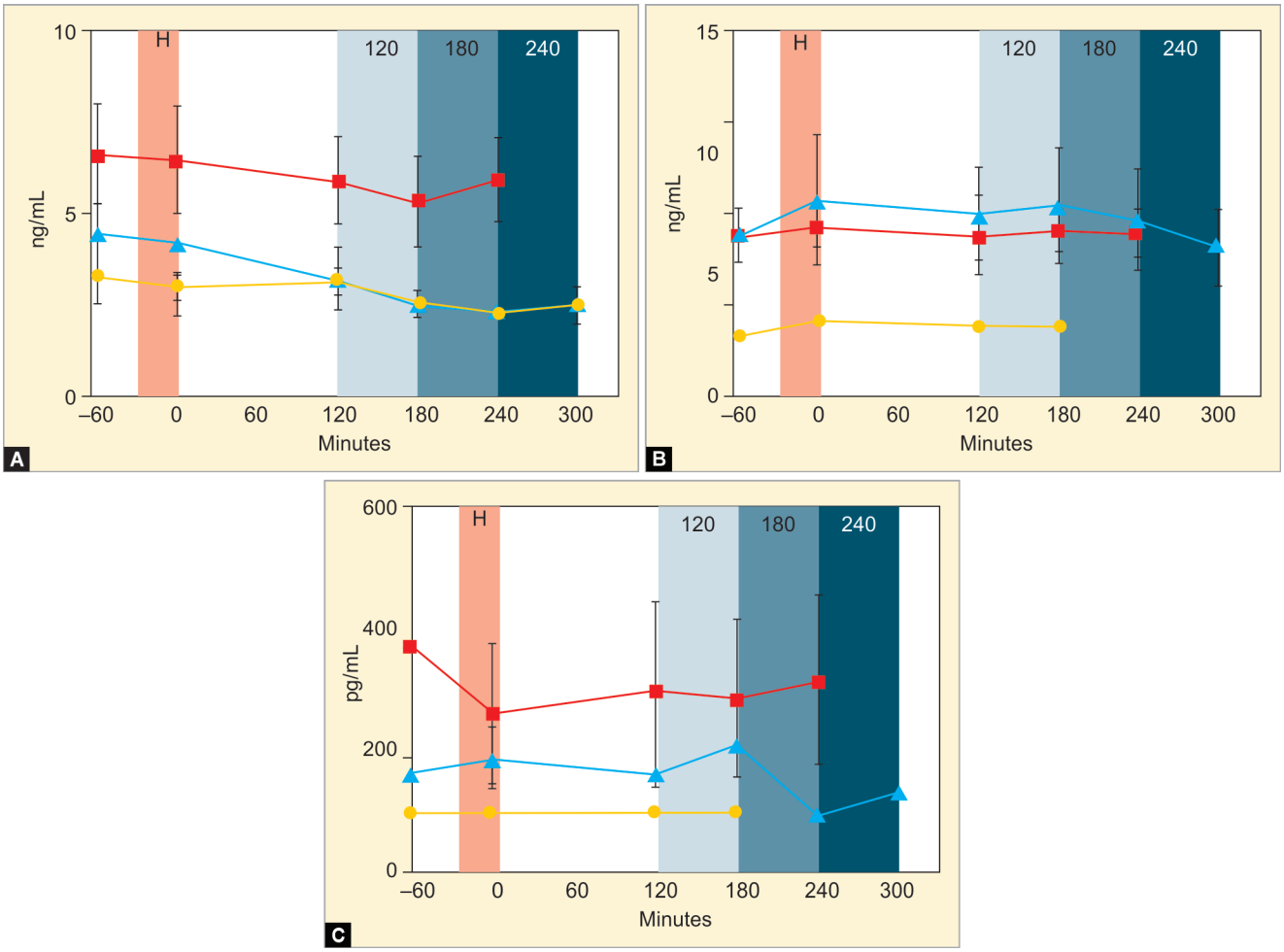
Selected cytokine/chemokine levels were also monitored. No significant changes from baseline in IL-1α levels were noted for any group (Fig. 4A). For anti-inflammatory IL-1ra, only the 240-minute REBOA group exhibited a significant increase in the study end compared to baseline (Fig. 4B). Levels of IL-6, an acute phase pro-inflammatory cytokine showed significant



Figs 4A to E: Cytokine/chemokine levels of the three groups. Cytokine/chemokine concentration are shown in the figure for plasma levels of interleukin (IL)-1α (A), IL-1ra (receptor antagonist) (B), IL-6 (C), IL-8 (D), and IL-10 (E). The *y*-axis shows the concentration units and the *x*-axis shows the time-points. "H" is the hemorrhage period, columns labeled 120, 180, or 240 represent the hospital arrival resuscitation phase. Significance is shown by "*" (120 minutes), "&" (180 minutes), and "#" (240 minutes) REBOA groups at $p > 0.05$ significance

increases compared to baseline at the study end for the 120- and 180-minute groups, but were seen starting at 180 minutes of inflation for the 240-minute REBOA group (Fig. 4C). The overall change in IL-6 concentration between baseline and study end for the 120-, 180-, and 240-minute groups were 1420, 1439, and 3158 pg/mL. No significant changes from baseline were noted for chemokine IL-8 and the anti-inflammatory cytokine IL-10 (Figs 4D and E).

We also assessed for overall cell damage and endotheliopathy with respect to REBOA inflation times. However, no significant



Figs 5A to C: Protein analysis for endotheliopathy and cell death. Endotheliopathy (Syndecan-1 [A]) and cell death (cytokeratin M18 [B] and M30 [C]) were monitored. The *y*-axis shows the concentration units and the *x*-axis shows the time-points. "H" is the hemorrhage period, columns labeled 120, 180, or 240 represent the hospital arrival resuscitation phase. Significance is shown by "*" (120 minutes), "&" (180 minutes), and "#" (240 minutes) REBOA groups at $p > 0.05$ significance

changes from baseline were noted for syndecan-1, CK18 M30, or CK18 M65 (Fig. 5).

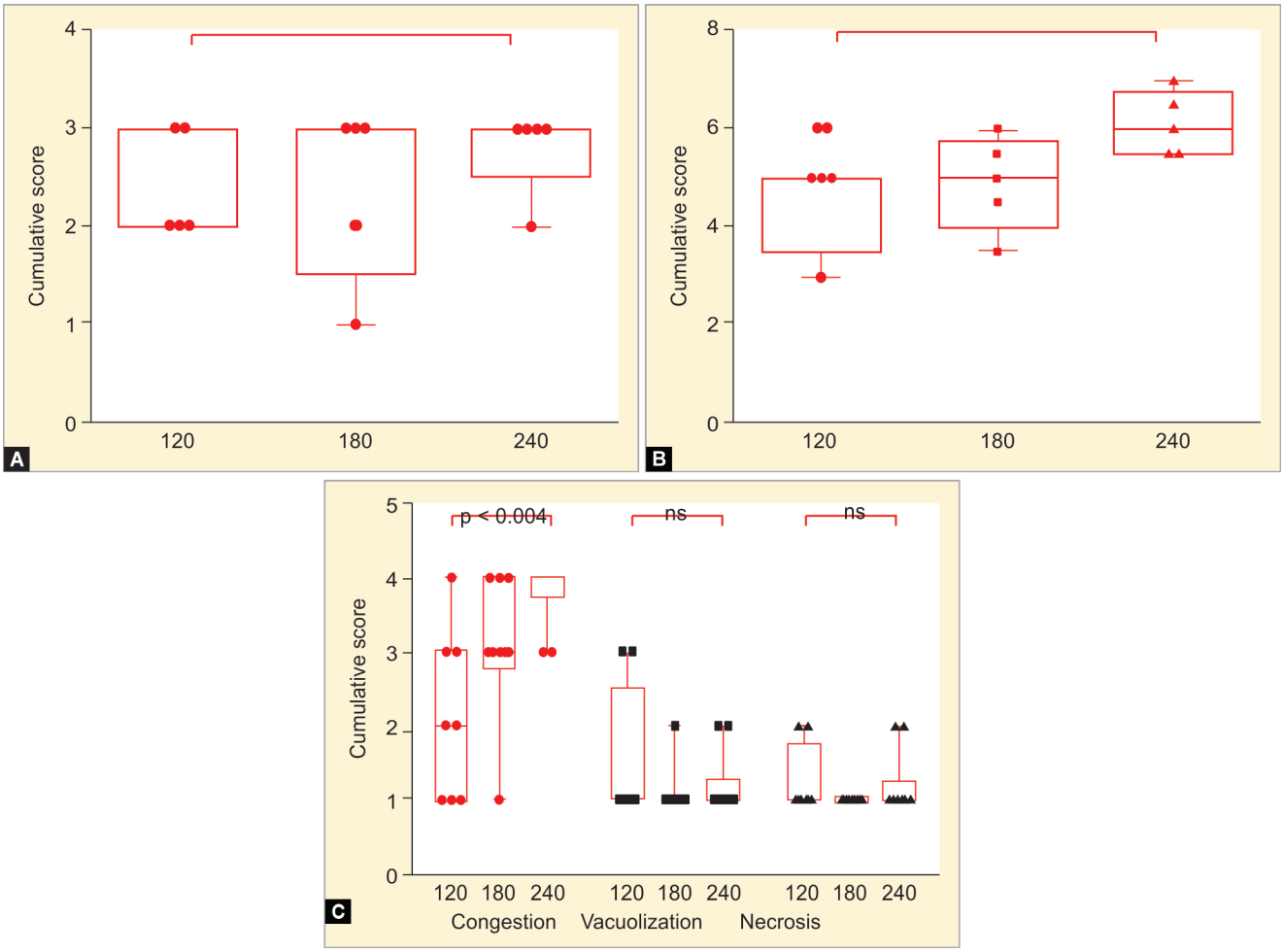
Histological Analysis

Lastly, the effect of REBOA inflation time on organ damage by histopathology was assessed. Resuscitative endovascular balloon occlusion of the aorta inflation times had no detectable detrimental effect on the kidney (Fig. 6A). However, the liver demonstrated a significantly increased injury in the 240-minute REBOA group compared to the 120-minute REBOA group (Fig. 6B), with a significant increase in sinusoidal congestion (Fig. 6C). While no significant differences were noted between groups for the small bowel, at either the proximal or distal end (Figs 7A and B), the large bowel demonstrated an increased and significant difference in histological scoring between the 120- and 240-minute groups, particularly at the distal end of the large bowel (Figs 7A and C).

Discussion

Prehospital REBOA in the civilian and military settings has continued to gain momentum and use, with reports of improves survival with prehospital REBOA use.[15–18] However, their complications associated with prolonged REBOA use remain poorly understood and are not mutually agreed upon.[7,19–21] Specifically, data are lacking as to how organs respond to prolonged REBOA and which organs are most affected as most animal studies have limited REBOA inflation times to <2 hours.[10,11,22–24] The current study aimed to assess which organs and metabolic and cellular processes are affected first and most severely with increasing REBOA inflation times using a porcine model of HS. The results revealed that the predominant complications of prolonged REBOA use include increasing lactic acidosis, inflammation associated with increasing interleukin and neutrophil levels, as well as liver and intestinal damage.

In assessing the metabolic derangements of prolonged REBOA use, pH and lactate levels continued to worsen as REBOA inflation times increased. Even at the shortest REBOA inflation time of 120 minutes, 1 hour of aggressive resuscitation did not result in correction of pH and lactate levels, although the levels of both markers began to stabilize over time in all groups. Regardless of the amount of REBOA inflation, there was no significant difference between groups for either pH or lactate or significant correlations with REBOA inflation times. However, the small sample size and variance amongst groups contributed to these findings.

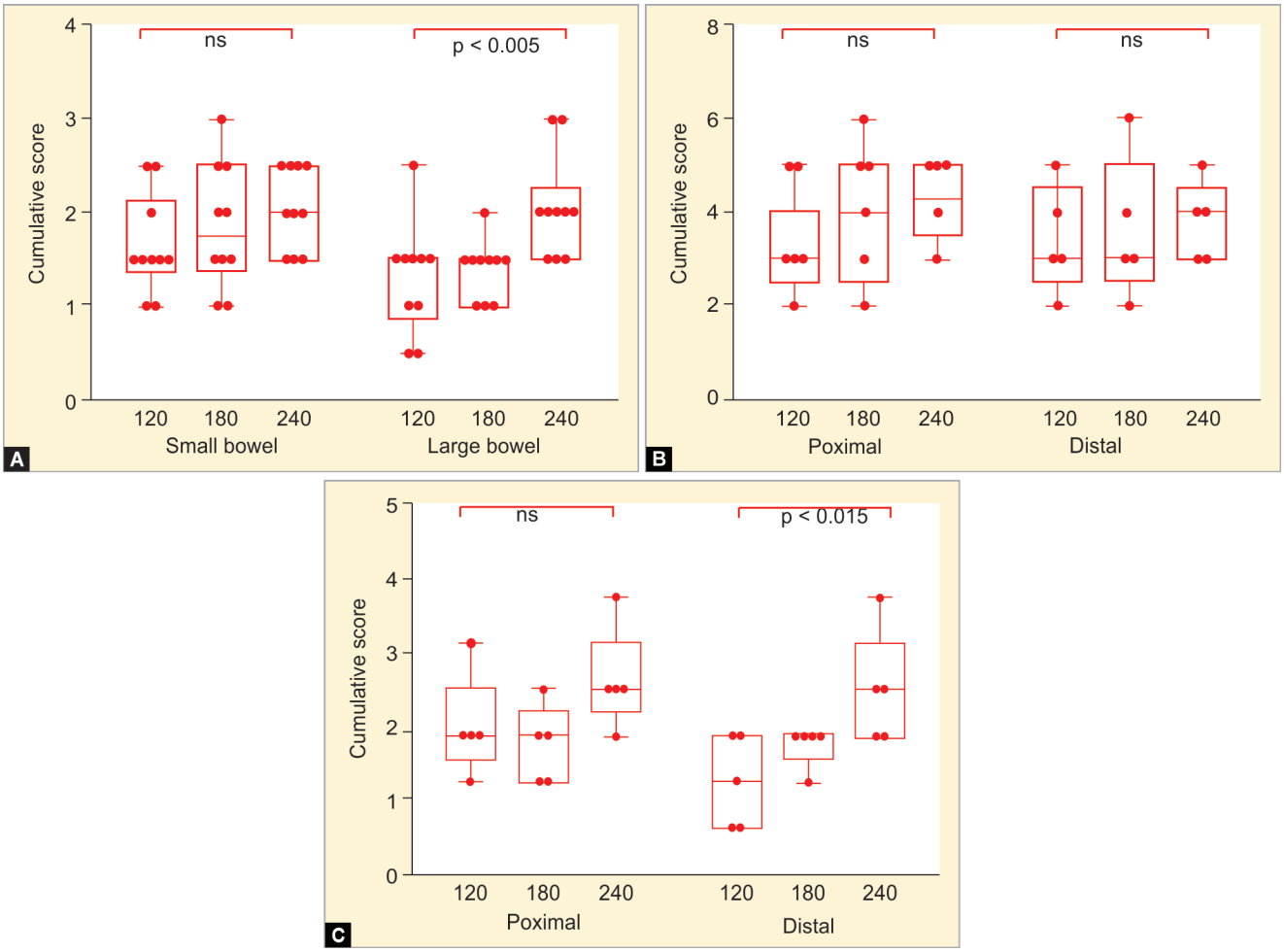


Figs 6A to C: Liver and kidney histological analysis. Kidney injury was scored using the tubular injury scoring system and liver injury was scored by the Suzuki scoring system. The *y*-axis shows the scoring and the *x*-axis shows the REBOA group of 120, 180, or 240 minutes of REBOA inflation. Overall kidney injury scoring (A), overall liver injury scoring (B), and breakdown of each liver injury component scoring (C). Significance between groups is noted by the horizontal bars and the *p* value at significance is < 0.05

With respect to organ injury, all three groups had significant decreases in kidney and liver function. Although elevated creatinine indicates kidney dysfunction, the histological analysis did not reveal any significant renal histopathology between groups. Similarly, elevated AST levels indicate liver damage, although the ALT levels remained unchanged in all groups. Histological analysis revealed a significant increase in liver congestion with the 240-minute REBOA group. Interestingly, hepatic congestion can result from cardiac damage, and a previous study from our group found that increasing REBOA inflation times can lead to cardiac damage.[25,26] Previous animal models of intestinal ischemia have found elevated serum levels of biomarkers of organ injury including ALP, CPK, LDH, and AST.[27] Increases of these markers seen in the current study correspond with the histological injury seen the following necropsy with significant large bowel ischemia apparent even after 1 hour of resuscitation. These data present the expected time-dependent relationship between REBOA inflation times and end-organ injury and suggest the liver and intestine are two organs that should be closely monitored in REBOA patients.

Following the HS, only ischemia from 240 minutes of REBOA significantly increased cytokines including IL-1ra and IL-6. In a human study, ischemia/reperfusion (I/R) caused by myocardial infarction also demonstrated a correlation between injury severity and cytokine concentration.[28] IL-1 receptor antagonist is an anti-inflammatory cytokine that suppresses IL-1 signaling by binding to the IL-1 receptor without inducing a response. Interleukin-1 mediates the acute inflammatory response during I/R injury and is known to reduce the expression of calcium regulatory genes as well as increasing apoptosis and tissue damage during I/R injury.[29,30] Furthermore, a separate study demonstrated in a rat model of I/R that overexpression of IL-1ra provided cardioprotection which was associated with a reduction in apoptosis, supporting its important role in regulating inflammation following I/R injury.[31]

Interleukin-6 levels were significantly increased at the 180-minute time-point in the REBOA 240-minute group with a similar, yet not significant, the trend at the same 180-minute time-point in the REBOA 180-minute group when compared to baseline. However, subgroup analysis demonstrated that measuring IL-6 concentrations from baseline to 180 minutes for all animals in the 180- and 240-minute groups showed a significant increase in IL-6 at the 180-minute time-point compared to baseline. Similar to the current study, human patients with I/R injury due to myocardial



Figs 7A to C: Intestine histological analysis. The small and large bowels of the intestine were scored for injury using the Park/Chui scoring scale. The *y*-axis shows the scoring and the *x*-axis shows the REBOA group of 120, 180, or 240 minutes of REBOA inflation, which is further divided into either the small and large bowel (A), the proximal and distal ends of the small bowel (B), or the proximal and distal ends of the large bowel (C). Significance between groups is noted by the horizontal bars and the *p* value at significance is < 0.05

infarction have demonstrated increased levels of IL-6, particularly during the reperfusion period.[32] However, several studies have shown contradictory information for IL-6 in animal models of I/R injury. For example, IL-6 knockout mice subjected to cerebral I/R injury have reduced survival, while IL-6 knockout mice exposed to gut I/R injury have reduced inflammation.[33,34] In mice subjected to I/R induced renal injury, an IL-6 antibody reduced renal injury, inflammatory cytokine levels, and infiltrating neutrophils suggesting a deleterious effect of IL-6 in I/R.[35] While it remains to be defined in what scenarios or models IL-6 is protective or detrimental to I/R injury, our results show that increasing IL-6 levels correlate with increasing ischemia from prolonged REBOA use and that 180 to 240 minutes of REBOA use could result in deleterious IL-6 driven consequences.

All groups in this study demonstrated significantly increasing cytokine/chemokine levels from baseline during the reperfusion period. During ischemia, mitochondrial complexes become disrupted, creating an excessive amount of reactive oxygen species (ROS) beyond the capacity that antioxidant systems can manage. Upon reperfusion, this mechanism is further exacerbated.[36] The damaging effects of ROS can result in cell damage and death, leading to the release of damage-associated molecular patterns from cells and increasing inflammation.[37] Furthermore, the endothelium is also sensitive to the interruption of blood flow, which can trigger endothelial dysfunction, thus promoting leukocyte adhesion and rolling.[38] Thus, extensive cellular damage could continue to occur long after the initiation of reperfusion. Although only one time-point was taken after reperfusion in this study, further research should focus on the effects of prolonged REBOA use over time following reperfusion, including whether this leads to sustained high levels of cytokine/chemokine expression. For example, a hind limb I/R study in rats demonstrated that plasma IL-6 levels continued to increase over time following reperfusion after a 3-hour ischemic insult.[39] Monitoring cytokine/chemokine levels over multiple time-points after reperfusion could determine if longer REBOA use times lead to more severe reperfusion injury.

In the current study, full REBOA inflation was used for all three time-points, which resulted in liver and intestinal damage and inflammation with increasing REBOA inflation times. However, partial REBOA or intermittent REBOA inflation are modified REBOA techniques that may prevent and/or mitigate the organ damage and inflammation that are otherwise observed with prolonged, constant

REBOA inflation times. A study in a porcine model of high-grade liver injury found reduced organ damage with intermittent REBOA use out to 1 hour, as compared to continuous REBOA.[11] Another study using a porcine model of hemorrhage found that complete REBOA inflation for 90 minutes, as compared to partial REBOA inflation for the same time period led to increased serum levels of lactate and histological necrosis and disruption of the duodenal mucosa.[10] Thus, in prolonged field care, scenarios where REBOA inflation may exceed 1 hour, intermittent, or partial REBOA inflation may be preferable for mitigation of the potential effects and complications of prolonged REBOA use.

There are several limitations to the current study. First, the animal was euthanized 1 hour after resuscitation. Studies with a prolonged resuscitation period would be useful for determining any long-term effects of prolonged REBOA use, especially following their initial resuscitation. Second, while the pig is an excellent trauma model that has been extensively studied in trauma, the organs identified to be most susceptible to prolonged REBOA use may not directly correlate to *in vivo* effects in humans. Third, the resuscitation strategy used during balloon inflation was based on a protocol used by the US military in a relatively austere battle environment. This may limit applicability to the civilian environment. Furthermore, while limited blood products were used during inflation as a result of this resuscitation strategy, these products were shed whole blood, which remains relatively unavailable to most trauma patients but is increasingly being used in the civilian setting.

In conclusion, while REBOA use out to 4 hours in a prehospital model is a viable option, it is not without potential complications as organs, such as, the liver and intestines may be more susceptible than others to I/R damage associated with prolonged REBOA inflation. Thus, with the use of REBOA circulating markers of organ damage can help guide the use of REBOA inflation and deflation times to ensure survival from a hemorrhage while mitigating the potential side effects of prolonged REBOA use.

Disclaimers

The views expressed in this manuscript are those of the authors and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, nor the U.S. Government.

Ethics Approval

This protocol was approved by the Animal Use and Care Committees of the University of Maryland and the US Army.

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