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Intraosseous Erythropoietin for Acute Tissue Protection in Battlefield Casualties Suffering Hypovolemic Shock

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14. ABSTRACT

The original project was designed to determining whether erythropoietin (EPO) administered intraosseously (1,200 U/kg) during hemorrhagic shock in a swine model could provide tissue protection and promote survival. Several experimental series were conducted during the 4 year duration of the project. Initially, controlled removal of 50% of blood volume yielded 88% resuscitability and 60% survival at 72 hours, unaffected by EPO. Controlled removal of 65% of blood volume yielded 25% resuscitability, but again unaffected by EPO. The model was then modified incorporating early and sustained infusion of vasopressin, also intraosseously (0.04 U/kg·min^{-1}) to improve survival for the same 65% blood volume removal. Resuscitability markedly increased to 92% leading to 83% survival at 72 hour with EPO showing a possible beneficial effect on organ function but without a survival effect. While conducting these experiments, we adopted restrictive fluid resuscitation reducing the 0.9% NaCl volume given during resuscitation from threefold the blood volume removed in the initial two series to half the blood volume removed in the third series observing that vasopressin infusion promoted remarkable hemodynamic stability and enabled restrictive fluid resuscitation. Next, we used a factorial design to simultaneously examine the effects of EPO, 0.9% NaCl (low-volume), vasopressin infusion, and percentage of blood volume removed (65% or 75%), showing a positive impact on 72-h survival associated with vasopressin and 0.9% NaCl but not with EPO or % of blood volume removed. Finally, we examined the effects of vasopressin infusion and restrictive fluid resuscitation in a model of uncontrolled bleeding produced by liver laceration, observing that neither vasopressin infusion nor 0.9% NaCl accentuated bleeding from liver lacerations; yet, vasopressin improved 240 min survival. Accordingly, the project failed to demonstrate a survival benefit elicited by EPO; yet, it found that early and sustained vasopressin infusion to be a highly effective intervention to promote hemodynamic stability and enable restrictive fluid resuscitation under conditions of controlled and uncontrolled hemorrhagic shock. Vasopressin infusion also produced a sustained increase in arterial blood pressure, which combined with restrictive fluid resuscitation and no accentuation of bleeding from liver laceration made us proposed its further investigation for the concurrent treatment of hemorrhagic shock and traumatic brain injury.

15. SUBJECT TERMS

Shock, Hemorrhagic; Resuscitation; Erythropoietin; Vasopressin; Swine; Polytrauma and Blast Injury; Maintain Tissue Viability; Hemodynamic stability; Survival; Uncontrolled Hemorrhage; Liver Laceration; Low-Volume Fluid Resuscitation

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INTRODUCTION

The project explored in a swine model approaches to improve outcome from hemorrhagic shock in the battlefield. We initially hypothesized that erythropoietin (EPO) – a hormone best known for its role in erythropoiesis but recently shown to also activate potent cell survival mechanisms prompting organs to resist better ischemia and reperfusion injury – could reduce organ injury during hemorrhagic shock and improve initial resuscitability and 72-hour survival. Adjusting the hemorrhagic shock severity of the swine model, we incorporated vasopressin infusion and observed a marked hemodynamic benefit the promoted high initial resuscitability and subsequent survival, while simultaneously failing to observe a consistent organ protective effect of EPO and improvement in survival. With approval of the U.S. Army Medical Research & Materiel Command (Dr. John Carney) we shifted the focus of the project from EPO to vasopressin infusion and explored the effects of vasopressin infusion and restrictive fluid resuscitation in a liver laceration model of hemorrhagic shock, again demonstrating a marked effect on hemodynamic function and resuscitability. Most of the work has been published or is undergoing peer review as described below and has also been presented at various national/international scientific venues. The project also led us to hypothesized that vasopressin (and more specifically, selective vasopressin 1A receptor agonists) could play an important role in the concurrent acute management of hemorrhagic shock and traumatic brain injury and have been invited to submit a full proposal for a Fiscal Year 2016 Prolonged Field Care Research Award under Funding Opportunity W81XWH-16-DMRDP-CCCRP-PFCRA.

BODY

Given that most of the work has been already published or is being peer reviewed, this section is structured to briefly describe the work performed with the details available in the attached publications (please see appendices). The work follows the initial Statement of Work subsequently modified and approved refocusing on the effects of vasopressin infusion.

1. Effects of Intraosseous Erythropoietin during Hemorrhagic Shock in Swine

The objective was to determine whether EPO given during hemorrhagic shock could ameliorate organ injury while improving resuscitation and survival. Three series of 24 pigs each were studied using a controlled model of hemorrhagic shock induced by withdrawing blood through a right atrial cannula using a computer-driven peristaltic pump. In an initial series, 50% of the blood volume was removed in 30 minutes and 0.9% NaCl (threefold the blood volume removed) given starting at minute 90 infusing each third in 30, 60, and 150 minutes. Following 0.9% NaCl, shed blood was reinfused at minute 330 (*HS-50$_{BV}$*). In a second series, the same *HS-50$_{BV}$* protocol was used but removing an additional 15% of blood volume from minute 30 to 60 (*HS-65$_{BV}$*). In a final series, blood was removed as in *HS-65$_{BV}$* and intraosseous vasopressin given from minute 30 (0.04 U/kg·min^{-1}) until the start of shed blood reinfusion at minute 150 (*HS65$_{BV+VP}$*). 0.9% NaCl was reduced to half the blood removed and given from minute 90 to 120 in half of the animals. In each series, animals were randomized 1:1 to receive EPO (1,200 U/kg) or control solution intraosseously after removing 10% of the BV. In *HS-50$_{BV}$*, O_2 consumption remained near baseline yielding minimal lactate increase, 88% resuscitability, and 60% survival at 72 hours. In *HS-65$_{BV}$*, O_2 consumption was reduced and lactate increased yielding 25% resuscitability. In *HS65$_{BV+VP}$*, vasopressin promoted hemodynamic stability yielding 92% resuscitability and 83% survival at 72 hours. EPO did not affect resuscitability or subsequent survival in any of the series but increased interleukin-10, attenuated lactate increases, and ameliorated organ injury based on lesser troponin I, AST, and ALT increases and lesser neurological deficits in the *HS-65$_{BV+VP}$* series. We concluded that EPO given during HS in swine failed to alter resuscitability and 72 hour survival regardless of hemorrhagic shock severity and concomitant treatment with fluids and vasopressin but attenuated acute organ injury. The studies also showed the efficacy of vasopressin and restrictive fluid resuscitation for hemodynamic stabilization and survival. (Please see Appendix 1; Borovnik-Lesjak V, et al. Effects of intraosseous erythropoietin during hemorrhagic shock in swine. PLOS ONE 2014;9[11]:e110908).

2. Vasopressin Infusion with Small-Volume Fluid Resuscitation during Hemorrhagic Shock Promotes Hemodynamic Stability and Survival in Swine

The objective was to determine using a factorial design the effects of vasopressin infusion along with small-volume fluid resuscitation while concomitantly assessing the effects of EPO and hemorrhagic shock severity. Hemorrhagic shock was induced in 24 male domestic pigs (36 to 41 kg) by withdrawing blood through a right atrial cannula according to a mono-exponential decay function to model spontaneous bleeding. The initial 12 pigs received no fluids whereas the last 12 pigs received 0.9% NaCl half the blood volume removed. Pigs were randomized 2:1 to receive intraosseously vasopressin (0.04 U/kg·min^{-1}) or vehicle control from minute 7 to minute 210. Pigs assigned to vasopressin were further randomized 1:1 to receive EPO (1,200 U/kg) or vehicle control and 1:1 to have 65% or 75% of their blood volume removed. Shed blood was reinfused at 210 minutes and the pigs recovered from anesthesia. Survival at 72 hours was influenced by vasopressin and 0.9% NaCl but not by EPO or % of blood volume removed. Vasopressin with 0.9% NaCl promoted the highest survival (8/8) followed by vasopressin without 0.9% NaCl (3/8), 0.9% NaCl without vasopressin (1/4), and neither treatment (0/4) with overall statistical significance (log-rank test, p = 0.009) and each subset different from vasopressin with 0.9% NaCl by the Holm-Sidak test. The survival effect was associated with vasopressin infusion increasing systemic

vascular resistance and 0.9% NaCl increasing cardiac output. EPO failed to confirm previously reported beneficial effects on acute organ injury. In fact, pigs that received EPO had a lower mean aortic pressure, a blunted inotropic response, a higher systemic oxygen extraction ratio, and higher levels of aspartate aminotransferase and alkaline phosphatase during hemorrhagic shock. Their neurological deficit score was higher and overall performance category worse at 24 hours returning to baseline by 72hours. We conclude that vasopressin infusion with small-volume fluid resuscitation was highly effective during severe hemorrhagic shock enabling critical hemodynamic stability and improved 72 hour survival. There was no additional effect of EPO observed in this series. (Please see Appendix 2; Gazmuri RJ *et al*. Vasopressin infusion with small-volume fluid resuscitation during hemorrhagic shock promotes hemodynamic stability and survival in swine. PLOS ONE 2015;10[6]:e0130134).

3. Early and Sustained Vasopressin Infusion Augments the Hemodynamic Efficacy of Restrictive Fluid Resuscitation and Improves Survival in a Liver Laceration Model of Hemorrhagic Shock

The objective was to investigate the effects of early and sustained vasopressin infusion with and without restrictive fluid resuscitation in a swine model of uncontrolled hemorrhagic shock produced by liver laceration. Forty male domestic pigs (32 to 40 kg) had a liver laceration inflicted with an X-shaped blade clamp, 32 received a second laceration at minute 7.5, and 24 received two additional lacerations at minute 15. Using a two-by-two factorial design, animals were randomized 1:1 to receive vasopressin infusion (0.04 U/kg·min^{-1}) or vehicle control given intraosseously from minute 7 until minute 240 and 1:1 to receive 0.9% NaCl (12 ml/kg) intravenously at minute 30 or no fluids. Results: Kaplan-Meier curves showed overall survival differences (log-rank test, p=0.095) favoring vasopressin with 0.9% NaCl (8/10) over vasopressin without 0.9% NaCl (4/10), 0.9% NaCl without vasopressin (3/10), and no intervention (3/10). Logistic regression showed that vasopressin improved survival at 240 minutes (p = 0.042). Vasopressin augmented mean aortic pressure between 10 and 20 mm Hg without intensifying the rate of bleeding from liver laceration, which was virtually identical to that of control animals (33.9 ± 5.1 and 33.8 ± 4.8 ml/kg). Vasopressin increased systemic vascular resistance and reduced transcapillary fluid extravasation augmenting the volume of 0.9% NaCl retained (6.5 ± 2.7 vs 2.4 ± 2.0 ml/kg by minute 60). The cardiac output and blood flow to the myocardium, liver, spleen, kidney, small bowel, and skeletal muscle at minute 120 and minute 180 were comparable or higher in the vasopressin group. We concluded that early and sustained vasopressin infusion provided critical hemodynamic stability during hemorrhagic shock induced by liver laceration and increased the hemodynamic efficacy of restrictive fluid resuscitation without intensifying bleeding or compromising organ blood flow resulting in improved 240 minute survival. (Please see Appendix 3; Gazmuri RJ *et al*. Early and sustained vasopressin infusion augments the hemodynamic efficacy of restrictive fluid resuscitation and improves survival in a liver laceration model of hemorrhagic shock).

KEY RESEARCH ACCOMPLISHMENTS

- Erythropoietin in dose of 1,200 U/kg given intraosseously early during hemorrhagic shock did not improve initial resuscitability or subsequent 72-h survival in our swine model of controlled blood removal.
- Early and sustained vasopressin infusion during hemorrhagic shock was remarkably effective in promoting hemodynamic stability and 72-h survival in our swine model of controlled blood removal.
- Vasopressin infusion favorably interacted with restrictive fluid resuscitation enabling sustained hemodynamic stability; with vasopressin increasing peripheral vascular resistance and fluid increasing cardiac index.
- Vasopressin infusion augmented the amount of fluid retained intravascularly after fluid resuscitation, an effect recently observed by other investigators and attributed to attenuation of vascular leak through vasopressin 1A receptors.
- Vasopressin infusion was also effective in promoting hemodynamic stability and survival in a model of uncontrolled hemorrhagic shock produced by liver laceration without accentuating the rate of bleeding; an effect worth of subsequent research for the concurrent management of hemorrhagic shock and traumatic brain injury aimed at generating higher systolic blood pressure without accentuating bleeding while minimizing fluid requirement and edema formation.

REPORTABLE OUTCOMES

Peer Reviewed Original Scientific Articles

1. Borovnik-Lesjak V, Whitehouse K, Baetiong A, Miao Y, Currie BM, Velmurugan S, Radhakrishnan J, **Gazmuri RJ**. Effects of intraosseous erythropoietin during hemorrhagic shock in swine. *PLOS ONE* 2014 Nov 3;9(11):e110908. doi: 10.1371/journal.pone.0110908. eCollection 2014.
2. **Gazmuri RJ**, Whitehouse K, Whittinghill K, Baetiong A, Radhakrishnan J. Vasopressin infusion with small-volume fluid resuscitation during hemorrhagic shock promotes hemodynamic stability and survival in swine. *PLOS ONE* 2015 2015 Jun 24;10(6):e0130134. doi: 10.1371/journal.pone.0130134. eCollection 2015.

Abstracts

1. Whitehouse K, Borovnik-Lesjak V, Miao Y, Baetiong A, Velmurugan S, Currie B, Radhakrishnan J, **Gazmuri RJ**. Effects of erythropoietin during hemorrhagic shock in a swine model. *Circulation* 2012;126:A18674.
2. Borovnik-Lesjak V, Whitehouse K, Baetiong V, Currie B, Radhakrishnan J, **Gazmuri RJ**. Identification of critical level of blood volume reduction in a swine model of hemorrhagic shock. *Circulation* 2012;126:A12073.
3. **Gazmuri RJ**, Whitehouse K, Borovnik-Lesjak V, Baetiong A, Radhakrishnan J. Vasopressin infusion during severe hemorrhagic shock increases systemic blood flow and markedly improves survival in a swine model. *Circulation* 2012;126:A15905.
4. **Gazmuri RJ**, Whitehouse K, Whittinghill KL, Baetiong A, Radhakrishnan J. Vasopressin with low-volume resuscitation is highly effective for resuscitation and survival from severe hemorrhagic shock. *Circulation* 2013;128:A341.
5. Whitehouse HK, Baetiong A, Whittinghill KL, Radhakrishnan J, **Gazmuri RJ**. Vasopressin and restrictive fluid resuscitation improves survival from hemorrhagic shock after liver laceration in swine. *Circulation* 2014;130:A7.
6. **Gazmuri RJ**, Whitehouse HK, Baetiong A, Whittinghill KL, Radhakrishnan J. Vasopressin maintains robust organ blood flow during resuscitation from hemorrhagic shock. *Circulation* 2014;130:A175.

Invited Lectures

2012 Targeting Venous Capacitance during Resuscitation from Hemorrhagic Shock. Grand Rounds, Captain James A. Lovell Federal Health Care Center North Chicago, IL, August 2.

2013 Novel Experimental Approaches to Resuscitation from Hemorrhagic Shock. Department of Medicine Grand Rounds. Rosalind Franklin University of Medicine and Science/The Chicago Medical School, North Chicago, IL, January 9.

2013 Venous Tone Augmentation with Vasopressin for Hemodynamic Stabilization during Hemorrhagic Shock (presentation, May 31). The Wolf Creek XII Conference. Westin Mission Hills Resort & Spa, Rancho Mirage, CA, May 30–June 2.

2013 Options for Hemodynamic Stabilization during Severe Hemorrhagic Shock in a Swine Model; Lawson Health Research Institute, Critical Illness Research Seminar Series (lecture, December 3). London, Ontario, Canada.

2014 High Dose EPO for Cardiac Arrest and Traumatic Injury (Invited Speaker, November 15). Resuscitation Science Symposium 2014 (ReSS) organized by the American Heart Association. Chicago, IL.

2015 Beneficial Effects of Vasopressin Infusion during Hemorrhagic Shock (Invited Speaker, March 4). Department of Pharmacology, Loyola University Health System. Maywood, IL.

2015 Vasopressin infusion increases the efficacy of small-volumen fluid resuscitation and improves survival in a liver laceration model of Hemorrhagic Shock in swine (presentation, April 16). The Wolf Creek XIII Conference. Lan Tian Hotel, Shanghai, China, April 16-18.

Awards (Senior Author)

2014 Young Investigator Award. American Heart Association 2014 Resuscitation Science Symposium. Vasopressin and Restrictive Fluid Resuscitation Improves Survival from Hemorrhagic Shock after Liver Laceration in Swine. Whitehouse K, Baetiong A, Radhakrishnan J, **Gazmuri RJ**.

CONCLUSION

The present study supports early and sustained vasopressin infusion for severe hemorrhagic shock given to rapidly achieve critical hemodynamic stability while enhancing the hemodynamic efficacy of restrictive fluid resuscitation until control of the source of bleeding is achieved.

REFERENCES

Please see above under reportable outcomes and in appendices.

APPENDICES and SUPPORTING DATA

Below as appendices are three original articles reporting the findings from this award; two already published and the third undergoing peer -review.

OPEN ACCESS Freely available online

PLOS ONE

Effects of Intraosseous Erythropoietin during Hemorrhagic Shock in Swine

Vesna Borovnik-Lesjak[1], Kasen Whitehouse[1], Alvin Baetiong[1], Yang Miao[1], Brian M. Currie[1], Sathya Velmurugan[1], Jeejabai Radhakrishnan[2], Raúl J. Gazmuri[3]*

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Abstract

Objective: To determine whether erythropoietin given during hemorrhagic shock (*HS*) ameliorates organ injury while improving resuscitation and survival.

Methods: Three series of 24 pigs each were studied. In an initial series, 50% of the blood volume (BV) was removed in 30 minutes and normal saline (threefold the blood removed) started at minute 90 infusing each third in 30, 60, and 150 minutes with shed blood reinfused at minute 330 (*HS-50$_{BV}$*). In a second series, the same *HS-50$_{BV}$* protocol was used but removing an additional 15% of BV from minute 30 to 60 (*HS-65$_{BV}$*). In a final series, blood was removed as in *HS-65$_{BV}$* and intraosseous vasopressin given from minute 30 (0.04 U/kg min^{-1}) until start of shed blood reinfusion at minute 150 (*HS-65$_{BV}$+VP*). Normal saline was reduced to half the blood removed and given from minute 90 to 120 in half of the animals. In each series, animals were randomized 1:1 to receive erythropoietin (1,200 U/kg) or control solution intraosseously after removing 10% of the BV.

Results: In *HS-50$_{BV}$*, O_2 consumption remained near baseline yielding minimal lactate increases, 88% resuscitability, and 60% survival at 72 hours. In *HS-65$_{BV}$*, O_2 consumption was reduced and lactate increased yielding 25% resuscitability. In *HS-65$_{BV}$+VP*, vasopressin promoted hemodynamic stability yielding 92% resuscitability and 83% survival at 72 hours. Erythropoietin did not affect resuscitability or subsequent survival in any of the series but increased interleukin-10, attenuated lactate increases, and ameliorated organ injury based on lesser troponin I, AST, and ALT increases and lesser neurological deficits in the *HS-65$_{BV}$+VP* series.

Conclusions: Erythropoietin given during HS in swine failed to alter resuscitability and 72 hour survival regardless of HS severity and concomitant treatment with fluids and vasopressin but attenuated acute organ injury. The studies also showed the efficacy of vasopressin and restrictive fluid resuscitation for hemodynamic stabilization and survival.

Citation: Borovnik-Lesjak V, Whitehouse K, Baetiong A, Miao Y, Currie BM, et al. (2014) Effects of Intraosseous Erythropoietin during Hemorrhagic Shock in Swine. PLoS ONE 9(11): e110908. doi:10.1371/journal.pone.0110908

Editor: Raghavan Raju, Georgia Regents University, United States of America

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

Funding: This research was supported by the Telemedicine and Advanced Technology Research Center (TATRC) at the U.S. Army Medical Research and Materiel Command (USAMRMC) Fort Detrick, MD under contract number: W81XWH-11-2-0019. Funding was received by RJG. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Acute hemorrhage resulting from traumatic injury is responsible for a high percentage of death in military personnel engaged in combat operations [1]. A recent report including 4,596 battlefield fatalities from Operation Iraqi Freedom and Operation Enduring Freedom between October 2001 and June 2011 showed that 87.3% of all injury related deaths occurred before arriving to a medical treatment facility [2]. Of these deaths, 24.3% were deemed potentially survivable with acute mortality associated with hemorrhage in 90.9%. The current acute management of hemorrhage focuses on hemostasis, hemodynamic stabilization, and rapid transfer to a medical treatment facility.

Erythropoietin (EPO) – a hormone best known for its effect on red blood cell production – has been shown to protect organs and tissues from ischemia and reperfusion injury including the heart [3–7], brain [8,9], spinal cord [10,11], kidney [12–14], liver [13–15], and skin [16,17]. We have reported beneficial effects of EPO for resuscitation from cardiac arrest in animal models [18–20] and in human victims of sudden cardiac arrest [21]. These effects were in part associated with non-genomic activation of mitochondrial protective pathways (e.g., Akt and PKC$_{\varepsilon}$) leading to lesser myocardial injury and dysfunction during and after resuscitation

[20]. We hypothesized that similar benefits could be elicited in other low-flow states such as hemorrhagic shock (HS) and ameliorate organ injury improving resuscitability and survival. This hypothesis was supported by rat models of HS in which pretreatment with EPO improved survival associated with lesser reductions in mean aortic pressure and lesser increases in lactic acid, tumor necrosis factor (TNF)-α, and interleukin (IL)-6 [14] along with lesser injury to the liver and kidneys [13,14], and by studies – also in rats – showing that EPO given during HS attenuated intestinal mucosal injury and bacterial translocation [22] along with maintaining intestinal microcirculatory blood flow [23]. Although – to the best of our knowledge – the effects of EPO during HS have not been investigated in large animal models (i.e., swine, sheep, and dog), EPO has been shown to exert tissue protection in swine models of liver [15] and spinal cord [11] ischemia.

We developed a model of HS in swine – an animal higher in the phylogenic scale and thus of greater translational relevance – and investigated the effects of EPO incorporating logistic constraints expected to limit care in far forward combat operations. We used a protocol of controlled bleeding as the initial approach in a multi-year project to first characterize the effects of the proposed interventions without the confounding elements of uncontrolled bleeding and tissue injury (to be incorporated in future series). We conducted three successive series of 24 animals each in which animals were randomized 1:1 to receive EPO (1,200 U/kg) or control solution. The series had in common (a) removal of blood to a target percentage of the estimated blood volume (simulating bleeding and hemostasis in the field); (b) delivery of EPO through the intraosseous route upon removal of 10% of the animal's blood volume (simulating early drug delivery using a low-skill technique); (c) fluid resuscitation with 0.9% NaCl (normal saline) initiated after a period of untreated HS (simulating delayed access to rescuers); (d) shed blood reinfusion at the end of HS (simulating arrival to a medical post), and (e) contingent on the series, recovery from anesthesia and 72 hour observation. The first series modeled low severity HS; the second series modeled high severity HS; and the third series modeled high severity HS with use of vasopressin to augment resuscitability while examining the role of limited fluid resuscitation.

Materials and Methods

The studies were approved by the Institutional Animal Care and Use Committee (IACUC) at Rosalind Franklin University of Medicine and Science (approval number 12–23) and by the United States Army Medical Research and Materiel Command Animal Care and Use Review Office (ACURO) and were conducted according to institutional guidelines.

Animal Housing and Husbandry

Animals were group housed in pens at the Biological Resource Facility (AAALAC accredited facility) at the Rosalind Franklin University of Medicine and Science in which lights are set at the recommended illumination levels of a 12/12-hour cycle controlled via automatic timers. Temperature was maintained at 61–81°F. Resting mats were provided and Aspen Sani-Chip bedding from a certified vendor (Harlan Laboratories, Indiana) was used. Health assessment for general health and well-being, possible injuries, or death was performed daily by animal care technicians and the day before/during/after the experiment by the investigator.

Animal Preparation

Basic Preparation. Male domestic pigs (32–48 kg, age ~11 weeks) were sedated with ketamine hydrochloride (30 $mg \cdot kg^{-1}$ intramuscularly). Anesthesia was induced with propofol (2 $mg \cdot kg^{-1}$ through an ear vein) and the animal intubated with a size 8 tracheal tube initiating positive pressure ventilation with a volume controlled ventilator (840 Ventilator System, Nellcor Puritan Bennett, Boulder, CO) set to deliver a tidal volume of 10 $mL \cdot kg^{-1}$, peak flow of 60 $l \cdot min^{-1}$, and FiO_2 of 0.5. Respiratory rate was adjusted at baseline to maintain the end-expired PCO_2 ($P_{ET}CO$) between 35 and 45 mmHg (Capnogard, Novometrix Medical Systems, Wallingford, CT). Anesthesia was continued using isoflurane (1.75% to 2.75%) and a 1:1 mixture of nitrous oxide and oxygen. The electrocardiogram was recorded through defibrillator adhesive skin pads. All procedures were performed using sterile technique. A 7 F high-fidelity micro-tip catheter transducer (Millar Instruments, Houston, TX) was advanced through the right femoral artery into the descending thoracic aorta for pressure measurement (Figure 1). A 7 F thermodilution balloon-tipped pulmonary artery catheter was advanced through the left cephalic vein or through the left internal jugular vein (when the cephalic vein was used to access the great cardiac vein as described under *Experimental Series*) into the pulmonary artery for measuring core temperature and thermodilution cardiac output along with pressures in the right atrium and pulmonary artery. A 6 F high-fidelity micro-tip pressure transducer pigtail catheter (Millar Instruments, Houston, TX) was advanced through the surgically exposed left carotid artery for measuring left ventricular pressure. A 23 F cannula (Bio-Medicus, Medtronic, Minneapolis, MN) was advanced through the left external jugular vein into the right atrium and used for blood withdrawal into a blood transfer bag. Core temperature was maintained between 37.5°C and 38.5°C with water-circulated blanket (Blanketrol II, Cincinnati SubZero, Cincinnati, OH).

Hemorrhagic Shock Protocol

The animal's blood volume was estimated at 60 ml/kg-body weight and a predetermined percentage was withdrawn into a heparinized transfer bag (heparin 10 $U \cdot ml^{-1}$ of blood) using a roller pump (model 313S, Watson Marlow, Inc., Wilmington, MA) controlled by a custom-developed software in LabVIEW 6.0. The heparinized transfer bag was placed on an electronic scale (model 2200, Doran Scales, Inc., Batavia, IL) connected to the LabVIEW

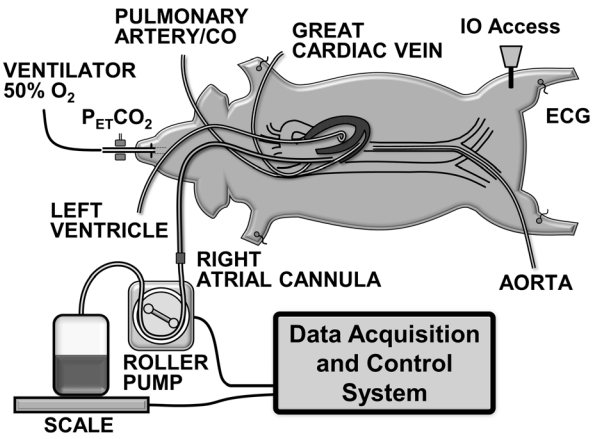

Figure 1. Swine model of hemorrhagic shock. CO, cardiac output; IO, intraosseous; $P_{ET}CO_2$, end-tidal PCO_2; ECG, electrocardiogram.
doi:10.1371/journal.pone.0110908.g001

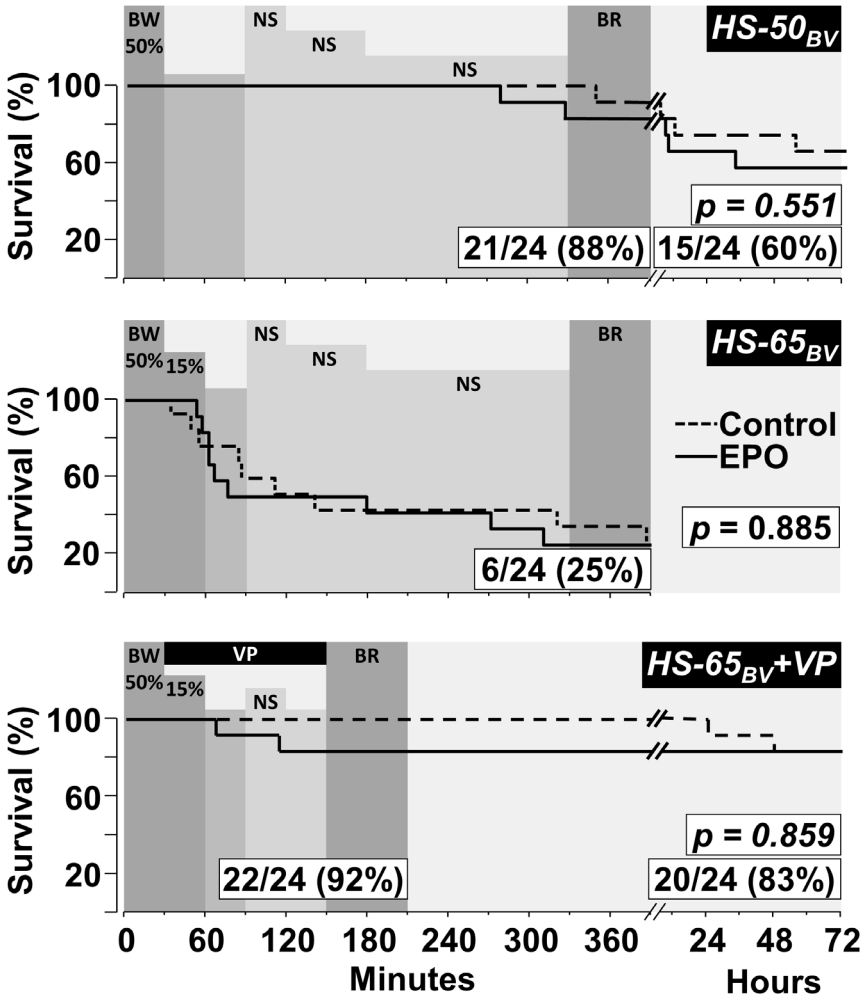

Figure 2. Survival curves comparing pigs treated with EPO and controls. Series *HS-50$_{BV}$* and *HS-65$_{BV}$+VP* include 72 hour survival. The *p*-values for survival differences between groups were calculated using the Gehan-Breslow test and are shown within each graph along with the resuscitation and survival rates for the combined EPO and control groups. The shaded horizontal bars successively represent; the percentage of blood withdrawn (BW), the interval of hemorrhagic shock after blood withdrawal without fluid administration, the administration of normal saline (NS) as described in the Method, and blood reinfusion (BR). Shown in *65$_{BV}$+VP* is the vasopressin infusion (VP).
doi:10.1371/journal.pone.0110908.g002

software to gravimetrically monitor the rate of blood withdrawal (blood density = 1.06 g/ml) and automatically adjust the pump rate as needed (Figure 1). The withdrawn blood was kept in a water bath at 37.5°C until reinfusion. Resuscitation was subsequently attempted by administration of normal saline followed by reinfusion of the shed blood using a blood transfusion filter (PALL Biomedical, Port Washington, NY). The volume, timing, and use of additional drugs varied as described under *Experimental Series*. In each series, pigs were randomized 1:1 to receive a 1,200 U/kg bolus of erythropoietin (Epogen [epoetin alpha]; 20,000 U/ml, Amgen) or normal saline vehicle (control) into the left tibia upon 10% removal of the blood volume (6 minutes from the start of blood removal). The investigators were blind to the treatment assignment and the group identification was revealed only after completion of the data analysis in each series.

At the completion of resuscitation in the first and third series, all catheters were removed, vessels ligated, and the skin wounds stapled, all under sterile conditions. The animal was allowed to recover from anesthesia and the endotracheal tube removed after resumption of spontaneous breathing and returned to its pen. The animal was then monitored every 60 minutes until it was able to right itself to sternal recumbency and thereafter every 4 hours for the initial 24 hours and at a minimum interval of 8 hours until completion of the 72 hours. A fentanyl dermal patch was used for analgesia throughout the 72 hour post-resuscitation period. If additional analgesia was needed, 2.2 mg/kg of flunixin meglumine was administered intramuscularly. The neurological status was evaluated at 24, 48, and 72 hours post-resuscitation using a neurological deficit score (0 = best; 420 = worst) [24]. The pig was euthanized at 72 hours by intravenous injection of euthanasia solution (pentobarbital sodium and phenytoin sodium; 5 ml, Vedco Inc., St Joseph, MO), or earlier – for humanitarian reason – in the event of moderate to severe of pain and distress unalleviated by analgesic agents, inability to eat or drink unassisted after 24 hours post-surgery, non-weight bearing or paralysis after 24 hours, depression or lethargy after 48 hours, profuse diarrhea, infection not resolved with antimicrobial therapy, lack of righting reflex, or cyanosis with difficulty breathing. The choice of drugs, route of administration, surgical preparation, and method of euthanasia were based on the recommendations by ACLAM board certified DVMs.

Table 1. Baseline Characteristics.

	$HS\text{-}50_{BV}$		$HS\text{-}65_{BV}$		$HS\text{-}65_{BV}$+VP	
Variable	**CTR**	**EPO**	**CTR**	**EPO**	**CTR**	**EPO**
n	12	12	12	12	12	12
Weight (kg)	39.6±3.7	37.5±4.2	34.6±1.5	35.4±1.2	39.4±2.4	38.1±2.5
Preparation Time (min)	170±36	174±41	123±59	114±21	119±22	113±21
Temperature (°C)	38.1±0.4	38.0±0.4	38.0±0.3	38.0±0.2	38.2±0.3	38.2±0.2
Respiration Rate (bpm)	31±5	31±4	35±2	36±1	36±2	35±2
End-tidal CO_2 (mmHg)	38±2	38±2	40±2	42±2	38±3	37±3
Mean Arterial Pressure (mmHg)	62±8	62±8	59±6	63±9	61±7	68±9
Cardiac Index (ml/min·m^{-2})	4.6±0.6	4.4±1.1	3.9±0.6	4.2±1.1	4.8±0.8	4.9±0.8
Heart Rate (bpm)	98±20	92±9	98±10	106±16	105±20	100±12
Blood Withdrawal Index (ml/m^2)	1398±70	1370±43	1909±162	1972±316	1814±39	1796±39

Values are mean ± SD. $HS\text{-}50_{BV}$, blood volume withdrawal 50%; $HS\text{-}65_{BV}$, blood volume withdrawal 65%; $HS\text{-}65_{BV}$+VP, blood volume withdrawal 65% and vasopressin infusion. CTR, control; EPO, erythropoietin. There were no statistically significant differences between groups within each series.
doi:10.1371/journal.pone.0110908.t001

After euthanasia, the whole left lung was weighed before and after drying in the oven at 70°C for at least 72 hours for calculations of the wet/dry ratio in the last series.

Sample Size

The sample size of 12 pigs per group was based on extrapolation from work in similar animal models intended to identify biologically robust differences in survival effects and continuous variables with a power>0.60 at an α level of 0.05.

Experimental Series

Experiments were performed between 9 AM to 5 PM in a large animal surgical suite located inside the university Biological Resource Facility. Three consecutive series of 24 experiments each were conducted. The sequence of interventions are described below and depicted in Figure 2. In the first series, 50% of the estimated blood volume was withdrawn in 30 minutes ($HS\text{-}50_{BV}$). Animals remained untreated for 60 minutes after which normal saline – threefold the blood volume removed – was infused delivering sequentially a third of each in 30, 60, and 150 minutes followed by infusion of the shed blood in 60 minutes. The $HS\text{-}50_{BV}$ protocol triggered a vigorous adaptive response that enabled maintaining oxygen consumption close to baseline resulting in minimal lactate increases and high resuscitability and survival without differences between EPO and control. To test EPO under greater HS severity, a second series was conducted withdrawing an additional 15% of the blood volume in 30 minutes after completing the initial 50% of blood volume removal for a total of 65% of blood volume removed ($HS\text{-}65_{BV}$). Animals remained untreated for 30 minutes after which the same $HS\text{-}50_{BV}$ protocol for fluid resuscitation and blood reinfusion was applied. In this series, an additional 7 F angiographic catheter was advanced with the aid of fluoroscopy from the left cephalic vein into the great cardiac vein to assess effect on myocardial metabolism [25]. The $HS\text{-}65_{BV}$ protocol was indeed severe, reducing resuscitability to only 25%, but again showing no difference between EPO and control. A third series was then conducted using the same $HS\text{-}65_{BV}$ protocol for blood withdrawal but infusing arginine vasopressin to prevent death by maintaining a higher coronary perfusion ($HS\text{-}65_{BV}$+VP). Vasopressin (Pitressin, JHP Pharmaceuticals, Rochester, MI) was given intraosseously as a bolus (0.04 U·kg^{-1}) upon completion of the initial 50% of blood volume removal followed by a continuous infusion (0.04 U·kg^{-1}·min^{-1}) using a syringe pump (PHD 2000 Syringe Pump Series, Harvard Apparatus, Holliston, MA) until start of blood reinfusion.

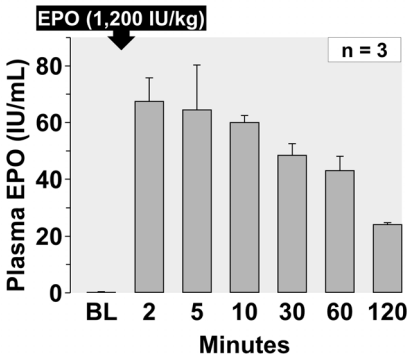

Figure 3. Plasma levels of EPO measured in 3 representative experiments from the $HS\text{-}50_{BV}$ series. Values are mean ± SEM.
doi:10.1371/journal.pone.0110908.g003

In $HS\text{-}65_{BV}$+VP, we also assessed the effect of less or no fluid resuscitation [26] under conditions of shorter HS duration. Thus, animals were also randomized 1:1 to receive either normal saline infusion – half of the blood volume withdrawn in 30 minutes – or no fluid at all. Blood was reinfused starting at 150 minutes (Figure 2). The addition of vasopressin dramatically improved resuscitability, allowing examination of survival and impact on organ function by blood sampling every 24 hours from the superior vena cava after sedation with ketamine hydrochloride (30 mg·kg^{-1} intramuscularly). Pigs were euthanized at 72 hours.

Experimental Outcomes

The primary outcome was survival at 390 minutes in series $HS\text{-}65_{BV}$ (without recovery from anesthesia) and survival at 72 hours in series $HS\text{-}50_{BV}$ and in series $HS\text{-}65_{BV}$+VP (with recovery from anesthesia). Secondary outcomes included: (1) hemodynamic and metabolic function, (2) myocardial function, (3) organ injury including the heart, brain, lung, liver, and kidney, (4) plasma cytokines, and (5) blood cell count.

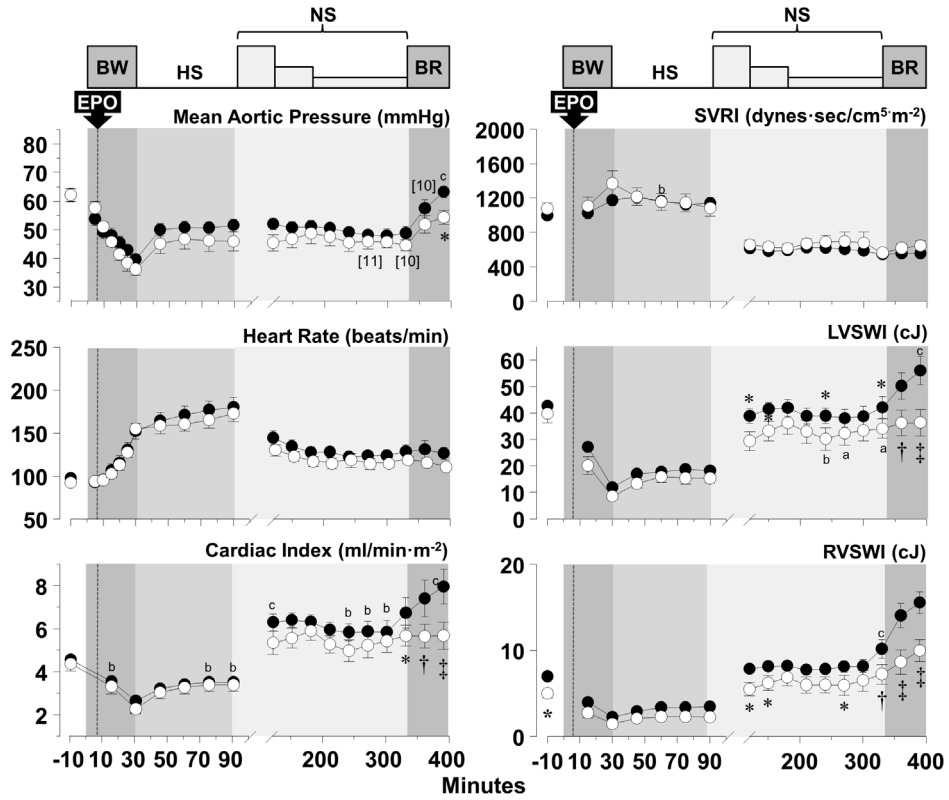

Figure 4. Hemodynamic and myocardial effects of EPO (open circles, n = 12) and vehicle control (closed circles, n = 12) in series *HS-50$_{BV}$*. Numbers in brackets indicate when the number of animals decreased from the preceding time point consequent to death of the animal. BL, baseline; BW, blood withdrawal; HS, hemorrhagic shock; NS, normal saline; BR, blood reinfusion; Ao, aortic pressure; SVRI, systemic vascular resistance index; LVSWI, left ventricular stroke work index; RVWI, right ventricular stroke work index. Values are shown as mean ± SEM. Differences between groups were analyzed by two-way repeated measures ANOVA. There were no overall significant treatment effects. However, there were overall statistically significant interactions between treatment and time for Ao mean ($p = 0.033$), cardiac index ($p < 0.001$), LVSWI ($p = 0.001$), and RVSWI ($p < 0.001$). *$p \leq 0.05$, †$p \leq 0.01$, and ‡$p \leq 0.001$ denote statistically significant differences between groups at the specified time points. [a]$p \leq 0.05$, [b]$p \leq 0.01$, and [c]$p \leq 0.001$ denote significant differences *vs* baseline using the Holm-Sidak test for multiple comparisons showing the differences only when they occurred in one of the two groups.
doi:10.1371/journal.pone.0110908.g004

Measurements

Blood analysis. Blood samples were collected from the aorta and pulmonary artery in all three series with the addition of great cardiac vein in *HS-65$_{BV}$*. Blood samples were processed on site for pH, PO_2, PCO_2, hemoglobin, and lactate using a cartridge based device (OPTI CCA-TS Blood Gas and Electrolyte Analyzer, OPTI Medical Systems, Roswell, GA) and for common hemoglobin types (oxy-, met-, carboxy-, and reduced-) using a co-oximeter (AVOXimeter 4000, AVOX systems Inc., San Antonio, TX). O_2 content in the aorta (CaO_2), pulmonary artery (CvO_2), and great cardiac vein ($CgcvO_2$) was calculated according to the following equation:

$$O_2Content\left(\frac{ml}{dl}\right) = Hemoglobin\left(\frac{g}{dl}\right) \times 1.39\left(\frac{ml}{g}\right) \times S_FO_2 + 0.003\left(\frac{ml}{dl}\cdot mmHg^{-1}\right) \times PO_2(mmHg)$$

where 1.39 denotes ml of O_2 bound to 1 g of hemoglobin (Hufner's number), S_FO_2 the fraction of oxyhemoglobin relative to the four hemoglobin types, and 0.003 the O_2 solubility coefficient. Aortic blood samples were also taken and processed for complete blood count and chemistry (blood urea nitrogen [BUN], creatinine, alanine aminotransferase [ALT], aspartate aminotransferase [AST], and troponin I) at the Captain James A. Lovell Federal Health Care Center, North Chicago, IL.

Plasma EPO. In series *HS-50$_{BV}$*, the serum level of EPO was measured in three animals that received EPO and in one control using a double-antibody "sandwich" enzyme-linked immunosorbent assay kit (MD Bioproducts, St Paul, MN) targeted to human EPO according to the manufacturer instructions. The EPO level in serum samples (diluted 100 times) was calculated using a standard curve generated with EPO calibrators included in the kit (0, 10.3, 24.8, 48, 156, and 523 mU/ml). The final plasma concentration was determined by applying the dilution factors and a conversion factor whereby one U/ml of EPO [Epogen (epoetin alpha), Amgen] equaled 0.798 U/ml of the calibrator.

Hemodynamic Measurements. Thermodilution cardiac output was measured in duplicate after bolus injection of normal saline (5 ml) into the right atrium (HP-Philips M012AT cardiac output module, Amsterdam, The Netherlands). Cardiac output was normalized to body surface area using the Kelley equation (body surface area [m^2] = 0.073·body-weight$^{2/3}$ [kg]) [27]. Aortic and left ventricular pressure signals were calibrated with a built-in calibration system (PCU-2000, Millar). Other pressure signals were zeroed to mid-cavity level. All signals were sampled and digitized at 250 Hz using a 16-bit data acquisition board

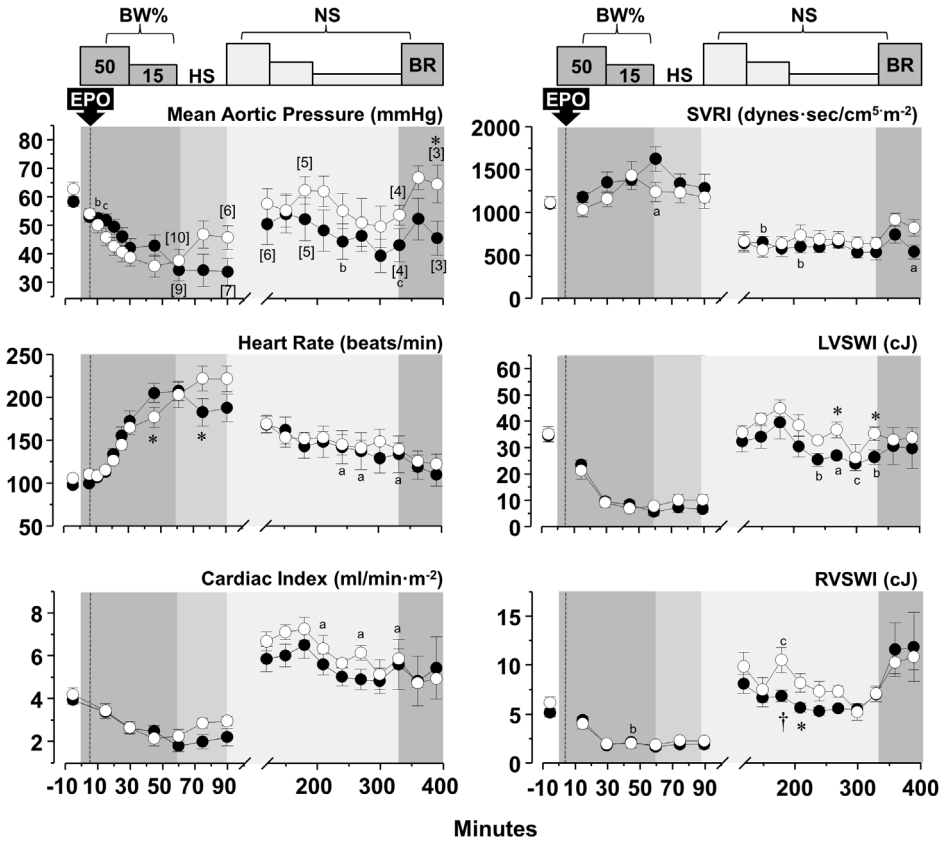

Figure 5. Hemodynamic and myocardial effects of EPO (open circles, n = 12) compared with vehicle control (closed circles, n = 12) in series *HS-65$_{BV}$*. Numbers in brackets indicate when the number of animals decreased from the preceding time point consequent to death of the animal. BL, baseline; BW, blood withdrawal; HS, hemorrhagic shock; NS, normal saline; BR, blood reinfusion; Ao, aortic pressure; SVRI, systemic vascular resistance index; LVSWI, left ventricular stroke work index; RVWI, right ventricular stroke work index. Values are shown as mean ± SEM. Differences between groups were analyzed by two-way repeated measures ANOVA. There were no overall significant treatment effects. However, there was an overall statistically significant interaction between treatment and time for Ao mean ($p = 0.002$). *$p \leq 0.05$, †$p \leq 0.01$, and ‡$p \leq 0.001$ denote statistically significant differences between groups at the specified time points. [a]$p \leq 0.05$, [b]$p \leq 0.01$, and [c]$p \leq 0.001$ denote significant differences *vs* baseline using the Holm-Sidak test for multiple comparisons showing the differences only when they occurred in one of the two groups.
doi:10.1371/journal.pone.0110908.g005

(AT-MIO-16XE-50; National Instruments, Austin, TX) and analyzed using custom developed software (Labview 6.0, National Instruments).

Cytokine Measurements. In the *HS-65$_{BV}$+VP* series, plasma levels of IL-6, IL-8, IL-10, and TNF-α were measured by a prototype 4-plex porcine cytokine electrochemiluminescence assay kit (Lot# Z00X2801, Meso Scale Discovery) using a QuickPlex SQ 120 multiplex imager (Meso Scale Discovery). The assay was performed as recommended by the manufacturer. Standards were prepared using IL-6, IL-8, IL-10, and TNF-α calibrators provided in the assay kit after a series of dilutions representing concentrations of 10,000 pg/ml, 2500 pg/ml, 625 pg/ml, 156.3 pg/ml, 39.1 pg/ml, 9.8 pg/ml, 2.4 pg/ml, and 0 pg/ml. Plasma collected at baseline, end of blood withdrawal, 24 hours after resuscitation, and 72 hours after resuscitation previously stored at −80°C was thawed in ice and centrifuged at 2,320 *g* for 10 minutes. Twenty five microliters of the plasma was used for the assay. All standards and samples were run in duplicates. Concentrations were calculated from a 4-parameter logistic equation standard curve using Discovery Workbench software (Meso Scale Discovery). Lower limit of detection (LLOD) of the assay was 2 pg/ml for IL-6, 5 pg/ml for IL-8, 1 pg/ml for IL-10, and 4 pg/ml for TNF-α. The coefficient of variation between the duplicate samples was <5%.

Cardiac Function. Indices of cardiac function were derived from left ventricular pressures, reporting the maximal rate of left ventricular pressure increase (dP/dt_{max}) and pressure decrease (dP/dt_{min}), the stroke volume index (SVI), and the left and right ventricular stroke work (LVSWI and RVSWI), corresponding to SVI times the difference between the systolic and end-diastolic left ventricular pressures (LVSWI) and SVI times the difference between the mean pulmonary and right atrial pressures (RVSWI) expressed in centijoules (cJ) by multiplying by 0.013332 [28].

Statistical Analysis

SigmaPlot 11.0 (Systat Software, Point Richmond, CA) was used for statistical analysis. For all repetitive variables, two-way repeated measures ANOVA was used to test for the treatment effect between groups and their interaction over time identifying differences at specified time points when present. For clarity, statistical results are presented only at time points shown in tables and figures, but reflect analysis of all available time points. Kaplan-Meier survival curves were plotted and statistical differences assessed using the Gehan-Breslow test. The hematological data from survivors in the *HS-50$_{BV}$* and *HS-65$_{BV}$+VP series* were pooled; analyzing changes from baseline to 72 hours post-resuscitation within each treatment group by paired *t*-test and differences between groups by unpaired *t*-test. The data were

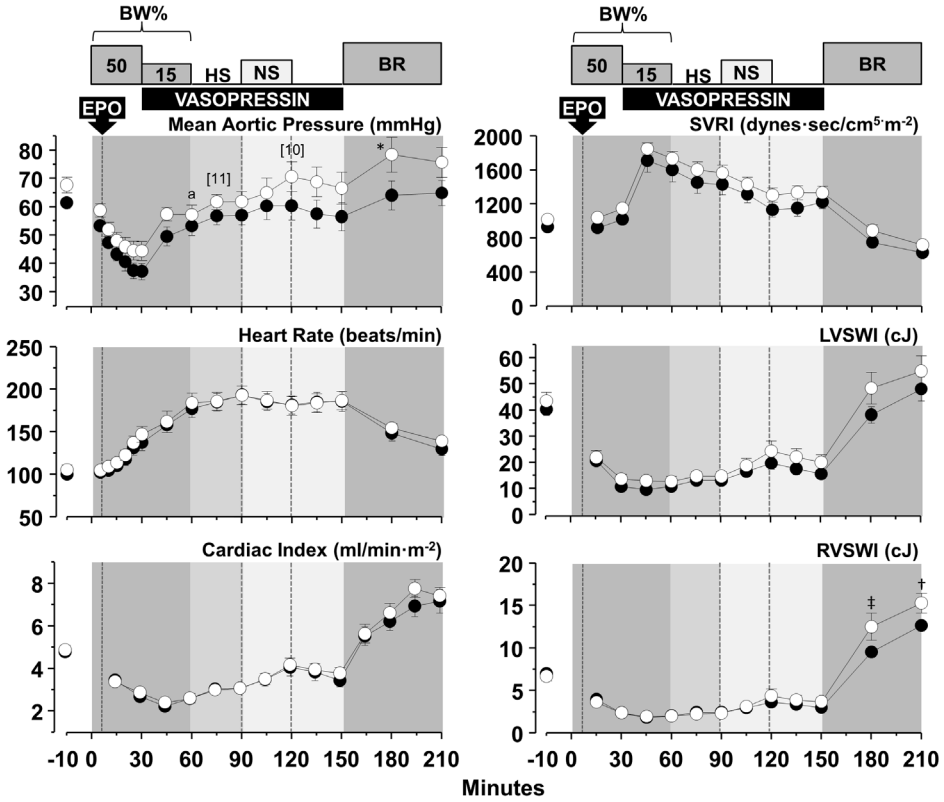

Figure 6. Hemodynamic and myocardial effects of EPO (open circles, n = 12) compared with vehicle control (closed circles, n = 12) in *HS-65$_{BV}$+VP*. Numbers in brackets indicate when the number of animals decreased from the preceding time point consequent to death of the animal. BL, baseline; BW, blood withdrawal; HS, hemorrhagic shock; NS, normal saline; BR, blood reinfusion; Ao, aortic pressure; SVRI, systemic vascular resistance index; LVSWI, left ventricular stroke work index; RVWI, right ventricular stroke work index. Values are shown as mean ± SEM. Differences between groups were analyzed by two-way repeated measures ANOVA. There was an overall statistically significant treatment effect for LVSWI ($p = 0.035$) and an overall statistically significant interaction between treatment and time for Ao mean ($p = 0.002$). *$p \leq 0.05$, †$p \leq 0.01$, and ‡$p \leq 0.001$ denote statistically significant differences between groups at the specified time points. [a]$p \leq 0.05$, [b]$p \leq 0.01$, and [c]$p \leq 0.001$ denote significant differences *vs* baseline using the Holm-Sidak test for multiple comparisons showing the differences only when they occurred in one of the two groups.
doi:10.1371/journal.pone.0110908.g006

presented as means ± SD unless otherwise stated. A two-tailed probability value of $p<0.05$ was considered significant.

Results

No unexpected adverse events occurred. Demise occurred attributed to hemorrhagic shock consequent to hemodynamic compromise during the acute phase and to organ dysfunction during the 72 hour observation interval.

Baseline

No significant differences between EPO and control groups were observed at baseline within each series as shown on Table 1 and on each successive tables and figures, except for RVSWI in *HS-50$_{BV}$*.

EPO plasma levels

Plasma levels of EPO averaging 3 representative experiments from series *HS-50$_{BV}$* are shown in Figure 3 confirming the adequacy of the intraosseous route yielding levels in excess of 20 U/ml for at least 120 minutes after administering 1,200 U/kg.

Resuscitation and survival

The initial resuscitation rate for all three series and subsequent 72 hour survival for *HS-50$_{BV}$* and *HS-65$_{BV}$ +VP* are shown in Figure 2. For the EPO and the control group combined; *HS-50$_{BV}$* resulted in 88% initial resuscitation and 60% survival; *HS-65$_{BV}$* resulted in only 25% initial resuscitation (noticing that demise started after removing more than 50% of the blood volume); and *HS-65$_{BV}$ +VP* resulted in 92% initial resuscitation (preventing demise after exceeding 50% of blood volume withdrawal) and 83% survival. EPO had no effect on initial resuscitation or subsequent survival in any of the three series.

Hemodynamic and myocardial function

Blood removal triggered a chronotropic response that attenuated reductions in cardiac index and aortic blood pressure yielding a relatively stable hemodynamic state after completion of blood removal despite marked reduction in left and right ventricular work indexes, attributed mainly to reduced preload (Figures 4–6). Administration of normal saline, three-fold the volume of blood removed in series *HS-50$_{BV}$* and *HS-65$_{BV}$*, markedly increased cardiac index (to levels higher than baseline), normalized left and right ventricular work indexes, and reduced the chronotropic response without substantial change in aortic pressure (Figures 4

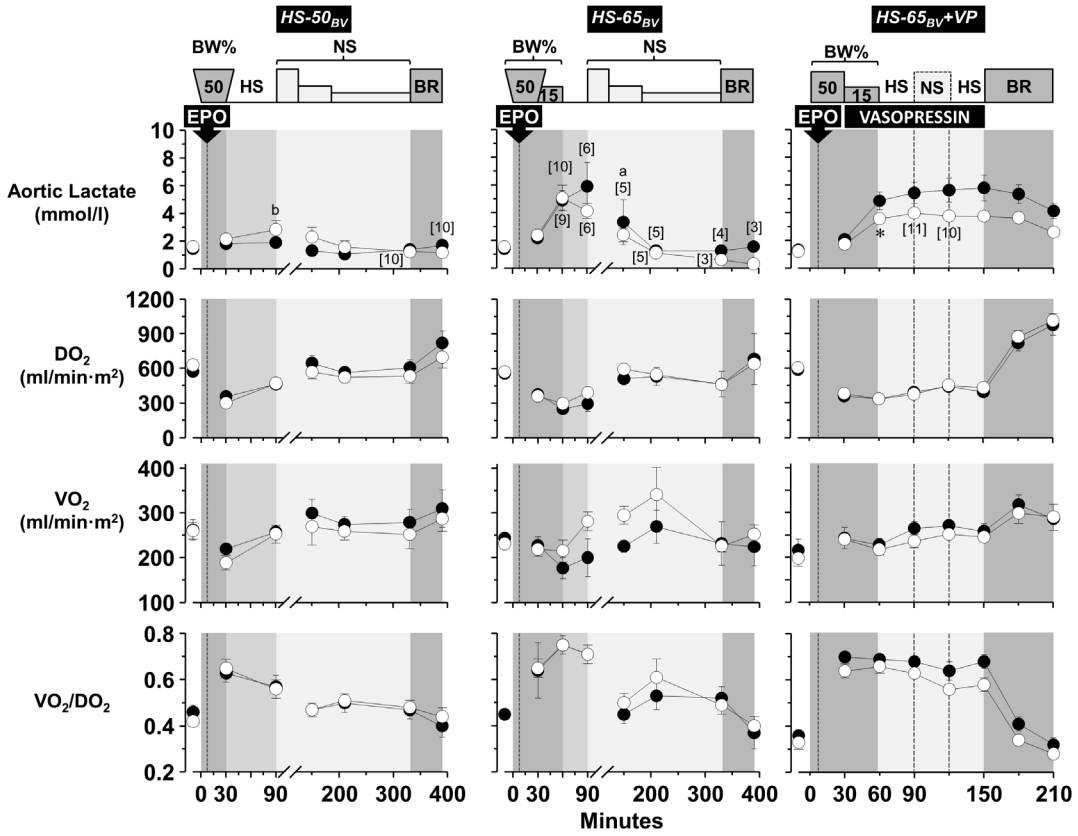

Figure 7. Metabolic effects of EPO (open circles) compared with vehicle control (closed circles) in series *HS-50$_{BV}$*, *HS-65$_{BV}$*, and *HS-65$_{BV}$+VP*. Numbers in brackets indicate when the number of animals decreased from the preceding time point. BW, blood withdrawal; HS, hemorrhagic shock; NS, normal saline; BR, blood reinfusion. Values are shown as mean ± SEM. Differences between groups were analyzed by two-way repeated measures ANOVA for each series separately. There were no overall significant treatment effects. However, there was an overall statistically significant interaction between treatment and time for lactate in series *HS-65$_{BV}$+VP* ($p = 0.007$). *$p \leq 0.05$ denotes statistically significant differences between groups at the specified time points. [a]$p \leq 0.05$ and [b]$p \leq 0.01$ denote significant differences *vs* baseline using the Holm-Sidak test for multiple comparisons showing the differences only when they occurred in one of the two groups.
doi:10.1371/journal.pone.0110908.g007

and 5). Administration of vasopressin in *HS-65$_{BV}$+VP* with or without normal saline (equal to half the volume of blood removed) increased systemic vascular resistance and aortic pressure with a modest increase in cardiac index and left and right stroke work indexes (Figure 6). Blood reinfusion maintained or increased cardiac index, aortic pressure, and work indexes in *HS-50$_{BV}$* and *HS-65$_{BV}$*; whereas in *HS-65$_{BV}$+VP* the predominant effect was increase in cardiac index and work indices (Figure 4–6). Relatively minor effects that varied contingent on the series were observed in relation to EPO. In *HS-50$_{BV}$*, EPO appeared to blunt the hemodynamic and myocardial response to HS and the subsequent fluid resuscitation and blood reinfusion interval (Figure 4). The opposite effect was observed in *HS-65$_{BV}$* and *HS-65$_{BV}$+VP* in which favorable hemodynamic and myocardial response were more prominent in the EPO group during HS and the subsequent fluid resuscitation and blood reinfusion intervals (Figure 5 and 6).

Oxygen metabolism and lactatemia

The chronotropic response along with the expected increase in systemic oxygen extraction in *HS-50$_{BV}$* allowed adequate adaptation to HS evidenced by minimal lactatemia and high resuscitation and survival rates (Figure 7 and Figure 2, *HS-50$_{BV}$*). Greater blood volume removal in *HS-65$_{BV}$* exhausted the adaptive response evidenced by higher systemic oxygen extraction, higher levels of lactic acid, and substantial demise after removing more than 50% of the blood volume (Figure 7 and Figure 2, *HS-65$_{BV}$*). Use of vasopressin in *HS-65$_{BV}$+VP* enabled survival despite similar exhaustion of the adaptive response and was attributed to maintaining a higher aortic pressure required for coronary perfusion (Figure 7 and Figure 2, *HS-65$_{BV}$+VP*). Relatively minor metabolic effects related to EPO were observed, highlighting an attenuation of lactate increase in *HS-65$_{BV}$+VP* (Figure 7).

Myocardial metabolism

In *HS-65$_{BV}$*, potential myocardial metabolic effects produced by severe HS were assessed measuring myocardial oxygen, lactate, and pCO_2 differences across the coronary circuit. As shown in Table 2, HS was not associated with myocardial ischemia (despite ischemia in other organs evidenced by lactatemia), with EPO and control groups behaving similarly.

Effects of fluid resuscitation

The effect of low-volume fluid administration was assessed in the *HS-65$_{BV}$+VP series* and shown in Figure 8 and Table 3. Fluid administration promoted an increase in cardiac index, mean aortic pressure, and left ventricular dP/dt_{max} (Figure 8) accompanied by attenuation of systemic oxygen extraction and faster normalization of lactic acidosis (Table 3). Of the 12 animals that received fluids, 11 were resuscitated and remained alive at 72 hours. Of the 12

Table 2. Myocardial Metabolic Effects of EPO in *HS-65$_{BV}$*.

	Baseline	End BW 50%	End BW 15%	End HS	NS	End NS	End BR
	−10 min	30 min	60 min	90 min	210 min	330 min	390 min
Ao O_2 Content (ml/dl)							
CTR	14.1±1.2	13.9±1.9	13.7±1.3[9]	12.6±2.7[6]	9.3±1.4[5]	8.1±1.3[4]	12.2±1.8[3]
EPO	13.6±1.3	13.6±1.4	13.3±1.4[10]	13.3±1.4[6]	8.8±1.0[5]	7.9±0.9[3]	13.0±1.0
GCV O_2 Content (ml/dl)							
CTR	3.6±1.6	3.5±1.5	2.5±0.6[c]	2.7±0.6[c]	3.0±0.4[c]	1.3±1.5	4.01±1.4
EPO	2.8±0.8	2.8±0.8	2.6±1.3	2.6±0.3	2.9±0.9	2.9±0.3	4.04±1.3
O_2 Extraction Ratio ([Ao-GCV]/Ao)							
CTR	0.75±0.10	0.76±0.09	0.81±0.05	0.78±0.04	0.67±0.03	0.66±0.07	0.67±0.08
EPO	0.80±0.05	0.79±0.05	0.80±0.09	0.80±0.02	0.66±0.11	0.63±0.04	0.69±0.11
GCV-Ao Lactate Gradient (mmol/l)							
CTR	−0.8±0.5	−1.2±0.7	−0.9±0.7	−1.1±0.6	0.1±0.3	−0.0±0.7	−0.5±0.5
EPO	−0.9±0.5	−1.5±0.5	−1.6±0.4	−1.3±0.8	0.0±0.4	−0.2±0.1	0.0±0.0
GCV-Ao pCO_2 Gradient (mmHg)							
CTR	13±4	15±4	19±4[a]	16±4	12±6	11±9	10±3
EPO	15±3	14±2	16±10	18±3	13±4	9±2	12±3

Numbers in brackets indicate when the sample size decreased from the initial twelve animals. BW, blood withdrawal; HS, hemorrhagic shock; NS, normal saline; BR, blood reinfusion; EPO, erythropoietin; CTR, control; Ao, aorta; GCV, great cardiac vein. Values are mean ± SD. The data was analyzed using two-way repeated measures ANOVA. There were no overall significant treatment effects and no overall statistically significant interactions between treatment and time. [a]$p \leq 0.05$; [c]$p \leq 0.001$ denote significant differences *vs* baseline using the Holm-Sidak test for multiple comparisons showing the differences only when they occurred in one of the two groups.

doi:10.1371/journal.pone.0110908.t002

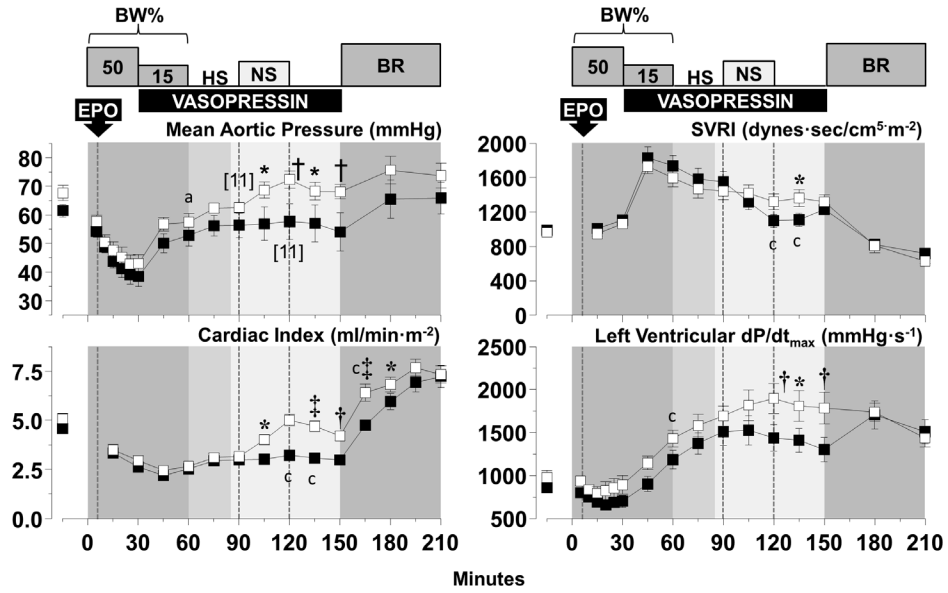

Figure 8. Hemodynamic effects of fluid resuscitation (open symbols, n = 12) and no fluid resuscitation (closed symbols, n = 12) in series *HS-65$_{BV}$+VP*. Numbers in brackets indicate when the number of animals decreased from the preceding time point consequent to death of the animal. BL, baseline; BW, blood withdrawal; HS, hemorrhagic shock; NS, normal saline; BR, blood reinfusion; Ao, aortic pressure; SVRI, systemic vascular resistance index. Values are shown as mean ± SEM. Differences between groups were analyzed by two-way repeated measures ANOVA. There was an overall statistically significant treatment effect for cardiac index ($p = 0.021$). There were also overall statistically significant interactions between treatment and time for cardiac index ($p < 0.001$) and SVRI ($p < 0.001$). *$p \leq 0.05$, †$p \leq 0.01$, and ‡$p \leq 0.001$ denote statistically significant differences between groups at the specified time points. [a]$p \leq 0.05$, and [c]$p \leq 0.001$ denote significant differences *vs* baseline using the Holm-Sidak test for multiple comparisons showing the differences only when they occurred in one of the two groups.
doi:10.1371/journal.pone.0110908.g008

Table 3. Metabolic Effect of Fluid Resuscitation in *HS-65$_{BV}$+VP*.

	Baseline	End BW 65%	HS/NS	End HS	End BR
	−10 min	60 min	120 min	150 min	210 min
Aortic Lactate (mmol/l)					
NS	1.3±0.7	3.9±1.0	4.0±1.0[11] *	3.9±0.9*	2.7±1.0*
No NS	1.3±0.5	4.0±2.4	5.6±3.1[11]	5.8±3.3	4.1±1.9
Aortic pH					
NS	7.48±0.05	7.41±0.06	7.33±0.03	7.37±0.04	7.40±0.03
No NS	7.47±0.04	7.38±0.06	7.33±0.06	7.34±0.02	7.37±0.02
Aortic O_2 Content (ml/dl)					
NS	12.7±1.3	13.3±1.3	10.1±1.1‡	10.9±1.0*[c]	13.0±0.6
No NS	12.1±2.1	12.8±1.8[a]	12.4±1.7	12.4±1.5	14.1±1.9[c]
Venous O_2 Content (ml/dl)					
NS	8.1±1.2	4.6±1.1	4.7±0.7	4.7±0.9	9.2±0.7
No NS	8.0±1.4	4.0±1.3	4.2±1.6	3.8±1.6	9.8±2.3
VO_2/DO_2 (ratio)					
NS	0.36±0.10	0.66±0.08	0.54±0.04*	0.57±0.07*	0.29±0.04*[a]
No NS	0.33±0.09	0.69±0.08	0.67±0.11	0.70±0.11	0.31±0.12

Numbers in brackets indicate when the sample size decreased from the initial twelve animals. Values are mean ± SD. BW, blood withdrawal; HS, hemorrhagic shock; NS, normal saline; BR, blood reinfusion; VO_2/DO_2, oxygen consumption divided by oxygen delivery. The data was analyzed using two-way repeated measures ANOVA. There were overall statistically significant interactions between treatment and time for aortic pH ($p = 0.043$), aortic O_2 content ($p \leq 0.001$), and VO_2/DO_2 ratio ($p \leq 0.001$). There was no overall statistically significant treatment effect. *$p \leq 0.05$ and ‡$p \leq 0.001$ denote statistically significant differences between groups at the specified time point. [a]$p \leq 0.05$ and [c]$p \leq 0.001$ denote statistically significant differences *vs* baseline using the Holm-Sidak test for multiple comparisons showing the differences only when they occurred in one of the two groups.
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Table 4. Effect of EPO on Organ Function in *HS-65$_{BV}$+VP*.

	Baseline	Post-Resuscitation		
	−10 min	24 h	48 h	72 h
Creatinine (mg/dl)				
CTR	1.3±0.2	1.5±1.4	1.4±1.7 [11]	0.9±0.1[b] [10]
EPO	1.0±0.2	0.9±0.2 [10]	0.9±0.2	0.9±0.1
Blood Urea Nitrogen (mg/dl)				
CTR	10±5	22±22	15±24	7±2
EPO	9±3	9±3	7±3	7±2
AST (U/l)				
CTR	35±7	400±501[c]	188±204	113±118
EPO	32±6	168±101[†]	85±43	67±26
ALT (U/l)				
CTR	51±10	126±109[c]	107±51[c]	100±36
EPO	53±8	82±24*	86±26	58±23[c]
Troponin I (ng/ml)				
CTR	0.22±0.18	1.47±2.40[c]	0.48±1.06	0.22±0.24
EPO	0.15±0.09	0.41±0.29	0.20±0.47	0.12±0.09*
Neurologic Deficit Score				
CTR	0±0	26±45[a]	15±45	2±6
EPO	0±0	13±35	0±0	0±0

Numbers in brackets indicate when the sample size decreased from the initial twelve animals. Values are mean ± SD. CTR, control; EPO, erythropoietin. The data was analyzed using two-way repeated measures ANOVA. There was no overall significant treatment effect and no overall statistically significant interactions between treatment and time. *$p \leq 0.05$ and †$p \leq 0.01$ denote statistically significant differences between groups at the specified time points. [a]$p \leq 0.05$, [b]$p \leq 0.01$, and [c]$p \leq 0.001$ denote statistically significant differences *vs* baseline using the Holm-Sidak test for multiple comparisons showing the differences only when they occurred in one of the two groups.

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Table 5. Effect of EPO on Plasma Cytokines in *HS-65$_{BV}$+VP*.

	Baseline	End BR	Post-Resuscitation	
	−10 min	210 min	24 h	72 h
IL-6 (pg/ml)				
CTR	42±28 [34;25]	79±55 [63; 34]	794±2515 [59, 53]	115±152 [66; 43]
EPO	30±28 [16;43]	106±73 [94; 57]	58±72 [25;30]	34±24 [26;28]
IL-8 (pg/ml)				
CTR	18±13 [14;22]	34±4 [24;27]	17±22 [6;15]	10±5 [12;8]
EPO	18±14 [15;15]	18±12 [15;17]	7±4 [5;3]	8±5 [7;2]
IL-10 (pg/ml)				
CTR	23±50; [8;8]	70±81 [33;28]	16±14 [11;9]	11±9 [8;4]
EPO	12±7 [12;9]	207±281 [70; 174][b†]	15±16 [7;8]	10±6 [8;3]
TNF-α (pg/ml)				
CTR	31±12 [30;9]	31±9 [33;10]	24±13 [24;11]	29±11 [28;12]
EPO	24±7 [21;6]	37±26 [27;15][a]	20±8 [18;4]	24±5 [22;5]

Samples were available for each of the 12 control (CTR) pigs including the 10 that survived at 72 hours; but, for only 10 of the erythropoietin (EPO) treated pigs, which included all the survivors. Values are mean ± SD showing also the median with interquartile range in brackets as values for several time events were not normally distributed. BR, blood reinfusion; IL, interleukin; TNF-α, tumor necrosis factor-α. The data was analyzed using two-way repeated measures ANOVA. There was no overall statistically significant treatment effect. There was a statistically significant time effect for IL-8 ($p = 0.011$), IL-10 ($p \leq 0.001$) and TNF-α ($p = 0.006$) and there was a borderline statistically significant interactions between treatment and time for IL-10 ($p = 0.062$). †$p \leq 0.003$ denotes a statistically significant difference between groups at the specified time point. [a]$p \leq 0.05$, [b]$p \leq 0.001$ denote statistically significant differences from baseline within each group using the Holm-Sidak test for multiple comparisons.

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Table 6. Effect of Fluid Resuscitation on Plasma Cytokines in *HS-65$_{BV}$+VP*.

	Baseline	End BR	Post-Resuscitation	
	−10 min	210 min	24 h	72 h
IL-6 (pg/ml)				
NS	30±22 [23;23]	62±27 [57; 24]	40±23 [38;20]	42±25 [38;33]
No NS	43±33 [33;45]	120±77 [101; 68]	880±2620 [83; 132]	115±164 [69; 61]
IL-8 (pg/ml)				
NS	16±14 [10;17]	16±11 [17;17]	9±9 [5;4]	8±4 [6;5]
No NS	20±11 [19;13]	37±42 [32;27] *	16±23 [5;9]	11±5 [11;9]
IL-10 (pg/ml)				
NS	25±53 [9;11]	84±120 [38;35]	8±4 [7;4]	10±6 [9;3]
No NS	11±5 [9;6]	181±262 [67; 206][a]*	23±17 [14;28]	12±9 [8;3]
TNF-α (pg/ml)				
NS	28±15 [21;16]	30±10 [29;10]	26±13 [25;17]	30±11 [27;13]
No NS	27±5 [27;5]	38±24 [31;16]	18±7 [19;5]	23±4 [22;3]

Samples were available for all 11 pigs in each group that survived the initial 24 hours and for all the 9 that survived at 72 hours from the group without fluid resuscitation. Values are mean ± SD showing also the median with interquartile range in brackets as values for several time events were not normally distributed. BR, blood reinfusion; NS, normal saline; IL, interleukin; TNF-α, tumor necrosis factor-α. The data was analyzed using two-way repeated measures ANOVA. There was no overall statistically significant treatment effect. There was a statistically significant time effect for IL-8 ($p = 0.008$), IL-10 ($p \leq 0.001$), and TNF-α ($p = 0.008$). *$p \leq 0.05$ denotes a statistically significant difference between groups at the specified time points. [a]$p \leq 0.001$ denotes a statistically significant difference from baseline using the Holm-Sidak test for multiple comparisons.

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animals that did not receive fluids, 11 were also resuscitated but only 9 were alive at 72 hours; a difference however that was not statically significant.

Organ injury and function

The post-resuscitation effect on various organs was assessed daily in *HS-65$_{BV}$+VP*. As shown in Table 4, EPO treated animals had an attenuated increase in AST, ALT, troponin I, and less neurological deficit at some point during the post-resuscitation phase. There were statistically insignificant differences suggesting less kidney injury in the EPO group. There was no difference in the percentage of lung water at 72 hours between EPO and control pigs (81.0±0.6% *vs* 81.8±1.0%).

Plasma cytokines

Plasma IL-6, IL-8, IL-10, and TNF-α were measured in the *HS-65$_{BV}$+VP* series, analyzing the effects of EPO (Table 5) and the effects of fluid resuscitation (Table 6). Overall there was a time effect with increases in IL-8 and IL-10 by the end of blood reinfusion reversing to baseline by 72 hours. Most prominently, EPO was associated with an increase in IL-10 by the end of blood reinfusion (Table 5). Administration of normal saline blunted increases in IL-8 and in IL-10 (Table 6).

Hematological effects

Pooled data from animals that survived in the *HS-50$_{BV}$* and *HS-65$_{BV}$+VP* series (18 animals in control group and 17 in EPO group) showed no differences between treatment groups at baseline with cell counts within normal for swine [29]. Red blood cell count and hematocrit increased relative to baseline in both

Table 7. Hematological Effects of EPO in *HS-50$_{BV}$* and *HS-65$_{BV}$+VP*.

		Baseline	72 h Post-Resuscitation
Red Blood Cells (10^6/μl)	CTR	5.5±0.4[18]	6.0±0.8[a]
	EPO	5.6±0.6[17]	6.8±0.7*[c]
Hematocrit (%)	CTR	31.5±1.7	34.4±4.8[a]
	EPO	31.6±2.1	39.3±4.5*[c]
White Blood Cells (10^3/μl)	CTR	18.0±4.0	20.9±4.9
	EPO	20.0±3.5	21.0±5.5
Platelets (10^3/μl)	CTR	312±68	332±106
	EPO	304±85	397±164[a]

Values are mean ± SD. CTR, control; EPO, erythropoietin. Data was pooled from *HS-50$_{BV}$* and *HS-65$_{BV}$+VP series*. Numbers in bracket indicate pooled sample size. Unpaired *t*-test was used to compare differences in the pooled hematological data between treatment groups at given time points. *$p \leq 0.05$. Paired *t*-test was used to compare pooled hematological data from *HS-50$_{BV}$* and *HS-65$_{BV}$+VP series* at baseline and post-resuscitation within each treatment group. [a]$p \leq 0.05$ and [c]$p \leq 0.001$. There were no statistically significant differences between groups at baseline.

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groups at 72 hours post-resuscitation but to a greater extent in the EPO group (Table 7). Over the same time interval, platelet count increased but only in the EPO group whereas the white blood cell count remained unchanged.

Discussion

The present study failed to demonstrate a beneficial (or detrimental) effect of EPO on initial resuscitability or subsequent survival in a swine model of HS regardless of its severity. The work was conducted in three consecutive HS series modeling mild severity, high severity with high fatality despite aggressive fluid resuscitation, and high severity with low fatality associated with vasopressin infusion and low-fluid or no-fluid resuscitation. EPO in the last series featuring high severity with low fatality increased plasma levels of the anti-inflammatory cytokine IL-10, attenuated lactatemia, and lessened transient injury to the liver, heart, and brain based on enzyme release and clinical neurological deficit. In addition, the study addressed several current aspects of HS management showing the efficacy of vasopressin infusion and restrictive fluid resuscitation.

EPO Effect

The dose of EPO chosen for the present experiments (1,200 U/kg) was extrapolated from a dose previously used in a study targeting sudden cardiac arrest victims (90,000 U) [21]. A lower dose (12,000 U) was deemed effective in a pilot human assessing potential protection after acute myocardial infarction [30]. In the present study, we confirmed that the chosen EPO dose – delivered through the intraosseous route – reached the bloodstream (Figure 3) attaining plasma levels that exceeded 30 U/ml for the initial 60 minutes decreasing to approximately 20 U/ml after 120 minutes. Only a brief exposure to EPO is required to trigger a sustained protective effect during HS [31] and levels above 5 U/ml are sufficient to elicit cytoprotection [32]. Accordingly, the lack of effects of EPO on resuscitability and survival occurred despite a sustained presence of EPO in the bloodstream and evidence of biological activity given the significant increase in hematocrit and platelets after 72 hours (Table 5).

The lack of impact on initial resuscitability from HS likely reflected the mechanism of death. Animals died preceded by progressive reductions in blood pressure that at some point precipitously compromised coronary perfusion and cardiac function. However, there was no evidence of protracted myocardial ischemia before demise upon which EPO could have exerted an acute "protective" effect, as previously reported during resuscitation from cardiac arrest [20]. The lack of myocardial ischemia was shown in series *HS-65$_{BV}$* (the series with the highest HS severity and mortality), in which the lactate gradient across the coronary circuit was negative (i.e., lactate utilization) and the PCO_2 gradient was not increased [25], both indicating absence of myocardial ischemia (Table 2). At the same time, substantial systemic lactic acidosis developed in series *HS-65$_{BV}$* and series *HS-65$_{BV}$+VP*, indicating critical reductions in oxygen delivery prompting anaerobic metabolism in other tissues. In *HS-65$_{BV}$+VP*, EPO attenuated increases in lactic acid, consistent with a beneficial effect at the mitochondrial level as we have reported in a rat model of cardiac arrest [20]. Likewise, there was attenuation of markers of organ injury in EPO treated pigs in the *HS-65$_{BV}$+VP* series. The protective effects at the cell level reported by us [20] and others [32] in rats involve at the very least Akt activation (phosphorylation); a kinase that plays a key role in cell survival and other adaptive responses including mitochondrial bioenergetic function and enhanced myocardial contractility [33,34]. In addition, plasma IL-10 was higher in pigs that received EPO at the end of blood reinfusion. IL-10 mediates anti-inflammatory effects and EPO can increase production of IL-10 [35–37]; pointing to the pleiotropic effect of EPO and mediation of additional effects that could be potentially beneficial for HS. The levels of IL-6 and IL-8 (pro-inflammatory cytokines) were lower at 24 and 72 hours in pigs treated with EPO but the differences were not statistically significant.

Studying the potential clinical relevance of these effects at the organ level was beyond the scope of the present study. In clinical settings, patients are exposed to repetitive injuries after stabilization (i.e., biological, infectious, sterile, etc.) and information on whether organ susceptibility to subsequent injuries could be ameliorated by EPO would be of substantial clinical interest.

Hemodynamic Response to Hemorrhagic Shock

A prominent (adaptive) chronotropic response maintained the cardiac index in *HS-50$_{BV}$* at ~80% of baseline, enabling increases in oxygen extraction to preserve aerobic metabolism and thereby yielding only minor increases in lactic acid. In series *HS-65$_{BV}$* and *HS-65$_{BV}$+VP*, however, the cardiac index was maintained at ~62% of baseline and the oxygen extraction reached its maximum level prompting systemic lactic acidosis; most likely from tissues excluded from the circulation following redistribution of blood flow towards vital organs. Accordingly, in this swine model, the critical transition from "compensated" to "uncompensated" HS occurred after removing 50% of the blood volume.

Vasopressin

Hemodynamic crises, including HS, trigger the release of vasopressin as part of a prominent neuroendocrine stress response. However, the endogenous vasopressin response is time-limited and exogenous administration has been suggested for hemodynamic stabilization in sepsis [38,39] and HS [40,41]. In models of HS, vasopressin was comparably superior to fluid administration or epinephrine [42,43] and has been proposed as the "preferred" vasopressor agent for hemodynamic stabilization during HS [41]. In a small clinical study in civilians suffering traumatic injury with hypotension, vasopressin infusion reduced the need of resuscitation fluids [44]. Clinicaltrials.gov lists two studies examining the effects of vasopressin in civilians with HS; the AVERT Shock study by Sims, C and the VITRIS study by Wenzel, V. [45].

These reasons prompted us to select vasopressin in our last series (*HS-65$_{BV}$+VP*), with the dose chosen according to a previous study by Voelckel *et al.*, also in swine [42]. The timing for initiation of vasopressin infusion was based on series *HS-65$_{BV}$*, noticing that demise began to occur after removing greater than 50% of the blood volume (Figure 2). The effect of vasopressin infusion on resuscitability was impressive, increasing from 25% (in *HS-65$_{BV}$*) to 92% (in *HS-65$_{BV}$+VP*) for the same degree of blood volume removal (Figure 2). Moreover, 83% of the animals in *HS-65$_{BV}$+VP* were alive at 72 hours and with essentially no organ dysfunction (Table 4).

Fluid Resuscitation

There is growing consensus that aggressive fluid resuscitation during HS can be detrimental [46]. Coagulation is compromised consequent to dilution of clotting factors and reduced activity by hypothermia and acidosis. Concomitantly, the endogenous fibrinolytic system is activated, tilting the hemostatic balance toward bleeding [47]. Excessive fluid also drives edema formation, which may affect the lungs and other tissues [48]. Large amounts of normal saline can precipitate hyperchloremic acidosis which has been associated with increased risk of renal injury [49] and

activation of inflammatory cascades [50]. For this and other (logistic) reasons, resuscitation with minimal or no fluid is gaining acceptance. The Tactical Combat Casualty Care Guidelines recommend fluid administration during HS only when there is altered mental status (in the absence of head injury) and weak or absent peripheral pulses.

In our initial two series (i.e., *HS-50$_{BV}$* and *HS-65$_{BV}$*), we administered normal saline 3 times the amount of blood removed according to the old paradigm resulting in a substantial increase in cardiac index (*via* preload increase) exceeding baseline levels. However, despite high initial resuscitability, the 72 hour survival was less than in the more severe *HS-65$_{BV}$+VP* series suggesting that excess fluid could have been detrimental. In the *HS-65$_{BV}$+VP* series, we examined the current paradigm and administered normal saline half the amount of blood removed in half of the animals and no fluids in the other half observing no differences in resuscitability or survival. Normal saline, however, had as positive effect on cardiac index, aortic pressure, and left ventricular dP/dt$_{max}$ while reducing systemic oxygen extraction and the generation of lactic acid (Figure 8, Table 3). Thus, there was hemodynamic and metabolic benefit elicited by fluid administration which could be critical under conditions of more severe HS and when vasopressin alone is not sufficient.

Limitations

Extrapolation of the current findings to real-life battlefield resuscitation from hemorrhagic shock is limited by several factors. Upmost is modeling HS without additional tissue injury. Although HS can occur in military and (more so) in civilian settings with low-grade or unassociated tissue injury, HS in the battlefield occurs typically accompanied by substantial tissue injury. Tissue injury compounds the severity of HS partly by the specific organ dysfunction, wound contamination, and amplification of the inflammatory response. In addition, the rate of blood withdrawal was controlled by a predetermined algorithm and did not follow the natural profile of bleeding after injury. The present studies, however, allowed assessing the effects of the studied interventions on severe HS without the confounding effects of tissue injury and uncontrolled bleeding. In subsequent series we incorporated the elements of tissue injury and uncontrolled bleeding by using a liver laceration model. Other limitations include the use of anesthesia, masking some of the physiologic responses to hypovolemic shock. The naturally occurring neuropeptide in swine is lysine vasopressin whereas in humans it is arginine vasopressin (the one used in the present studies). Thus, potency may vary contingent on specific receptors and vascular beds and translation of these findings to humans will need to consider human dose-responses. Despite these limitations, our model reproduced key characteristics of resuscitation from hemorrhagic shock and provided mechanistic information under highly controlled conditions that would be difficult to obtain in a more realistic model.

Conclusions

EPO given during HS in a swine model of HS failed to alter resuscitability and 72 hour survival regardless of HS severity and concomitant treatment with fluids and vasopressin. EPO, however, attenuated lactate increases and acute injury to the liver, heart, and brain based on enzyme release and neurological deficit scores. The studies also showed that vasopressin infusion with restrictive fluid administration is highly effective for hemodynamic stabilization and subsequent survival.

Supporting Information

Datafile S1 Hemodynamic and metabolic data from the three hemorrhagic shock series.
(XLSX)

Checklist S1 ARRIVE guidelines checklist.
(PDF)

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Author Contributions

Conceived and designed the experiments: RJG. Performed the experiments: VBL KW BMC. Analyzed the data: VBL KW BMC RJG. Contributed reagents/materials/analysis tools: AB YM SV JR. Wrote the paper: VBL RJG.

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Appendix 2

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RESEARCH ARTICLE

Vasopressin Infusion with Small-Volume Fluid Resuscitation during Hemorrhagic Shock Promotes Hemodynamic Stability and Survival in Swine

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Abstract

Introduction

Current management of hemorrhagic shock (HS) in the battlefield and civilian settings favors small-volume fluid resuscitation before controlling the source of bleeding. We investigated in a swine model of HS the effects of vasopressin infusion along with small-volume fluid resuscitation; with erythropoietin (EPO) and HS severity as additional factors.

Methods

HS was induced in 24 male domestic pigs (36 to 41 kg) by blood withdrawal (BW) through a right atrial cannula modeling spontaneous bleeding by a mono-exponential decay function. The initial 12 pigs received no fluids; the last 12 pigs received normal saline (NS) half the BW volume. Pigs were randomized 2:1 to receive intraosseously vasopressin (0.04 U/kg·min^{-1}) or vehicle control from minute 7 to minute 210. Pigs assigned to vasopressin were further randomized 1:1 to receive EPO (1,200 U/kg) or vehicle control and 1:1 to have 65% or 75% BW of their blood volume. Shed blood was reinfused at 210 minutes and the pigs recovered from anesthesia.

Results

Survival at 72 hours was influenced by vasopressin and NS but not by EPO or % BW. Vasopressin with NS promoted the highest survival (8/8) followed by vasopressin without NS (3/8), NS without vasopressin (1/4), and neither treatment (0/4) with overall statistical significance (log-rank test, $p = 0.009$) and each subset different from vasopressin with NS by Holm-Sidak test. Vasopressin increased systemic vascular resistance whereas NS increased cardiac output.

Conclusion

Vasopressin infusion with small-volume fluid resuscitation during severe HS was highly effective enabling critical hemodynamic stabilization and improved 72 hour survival.

Introduction

Hemorrhagic shock after penetrating trauma in the battlefield accounts for a high percentage of potentially survivable injuries [1]. A report from operations Iraqi Freedom and Enduring Freedom between October 2001 and June 2011 showed that 87% of battlefield fatalities occurred before arrival to a medical treatment facility with 24% deemed potentially survivable [2]. Of these potentially survivable injuries, 91% were associated with hemorrhagic shock.

Fluid resuscitation is hemodynamically effective but it is logistically constrained in the battlefield and not free of complications. In large quantities, fluids can worsen acute traumatic coagulopathy by dilution and hypothermia and dislodge freshly formed clots [3–5]. Accordingly, hemodynamic stabilization with limited fluid resuscitation in victims suffering severe hemorrhagic shock until arrival to a medical treatment facility is considered advantageous and expected to save lives [4,5]. Similar considerations apply to hemorrhage associated with trauma in civilian populations [6,7].

In previous work, we had hypothesized that administration of erythropoietin (EPO) early during hemorrhagic shock could minimize tissue injury through activation of mitochondrial protective mechanisms and help improve resuscitability and survival [8,9]. Although EPO attenuated transient tissue injury it failed to elicit survival benefits [9]. While conducting these studies we observed that vasopressin infusion was highly effective for hemodynamic stabilization and promoted survival under conditions of severe hemorrhagic shock in which aggressive fluid resuscitation had failed. Similar observations have been made by other investigators suggesting that vasopressin could be a highly effective hemodynamic intervention for resuscitation from hemorrhagic shock [10,11]. Our studies further suggested that vasopressin infusion could act in conjunction with small-volume fluid resuscitation to enable sustained hemodynamic stabilization obviating the need to administer large amounts of fluids. The focus of our previous studies, however, was on EPO and the aforementioned observations on the effects of vasopressin were uncontrolled and made in separate series.

We therefore designed a study to specifically investigate the role of vasopressin infusion and small-volume fluid resuscitation for hemodynamic stabilization under conditions of severe hemorrhagic shock maintaining the focus on battlefield relevance and constraints for deployment in far-forward scenarios. The experiments were conducted in a swine model of hemorrhagic shock induced by blood withdrawal through a 14-F cannula advanced into the right atrium. A closed-loop system was developed to model spontaneous bleeding according to a mono-exponential decay function. Shed blood was reinfused at 210 minutes. Resuscitated animals were recovered from anesthesia and observed for up to 72 hours.

We used a factorial design to separately assess the effects of vasopressin infusion, small-volume fluid resuscitation, EPO, and severity of hemorrhagic shock in 24 pigs.

Materials and Methods

The studies were approved by the Institutional Animal Care and Use Committee (IACUC) at Rosalind Franklin University of Medicine and Science (approval number 12–23) and by the

United States Army Medical Research and Materiel Command Animal Care and Use Review Office (ACURO) and were conducted according to institutional guidelines.

Animal housing and husbandry

Animals were group-housed in the Biological Resource Facility (AAALAC accredited facility) at Rosalind Franklin University of Medicine and Science in which lights are set at the recommended illumination levels with a 12/12 hour cycle controlled via automatic timers. Temperature was maintained between 61 and 81°F. Resting mats and Aspen Sani-Chip bedding from a certified vendor (Harlan Laboratories, Indiana) were used. Assessment for general health and well-being, possible injuries, or death was performed daily by animal care technicians and the day before, during, and after the experiment by the investigators.

Animal preparation

Basic preparation. Twenty-four domestic pigs (36 to 41 kg) were sedated with ketamine hydrochloride (30 $mg \cdot kg^{-1}$ intramuscularly). Anesthesia was induced with propofol (2 $mg \cdot kg^{-1}$ through an ear vein) and the animal intubated with a size 7.5 tracheal tube initiating positive pressure ventilation with a volume controlled ventilator (840 Ventilator System, Nellcor Puritan Bennett, Boulder, CO) set to deliver a tidal volume of 10 $ml \cdot kg^{-1}$ and peak flow of 60 $l \cdot min^{-1}$. Respiratory rate was adjusted to maintain an end-expired PCO_2 ($P_{ET}CO_2$) between 35 and 45 mmHg (Capnogard, Novometrix Medical Systems, Wallingford, CT). Anesthesia was continued using isoflurane (1.75% to 2.5%) and a 1:1 mixture of nitrous oxide and oxygen yielding an FiO_2 of 0.50 adjusted to maintain a surgical plane of anesthesia throughout the experiment. The electrocardiogram was recorded through limb leads using a defibrillator/monitor (Agilent Heartstream XL, Agilent Technologies, Santa Clara, CA). All invasive procedures were performed with sterile technique. A 7-Fr high-fidelity micro-tip catheter transducer (Millar Instruments, Houston, TX) was advanced through the right femoral artery into the descending thoracic aorta for pressure measurement. A balloon-tipped pulmonary artery catheter (Edwards Lifescience Corp, Irvine, CA) was advanced through the left cephalic vein into the pulmonary artery for measuring core temperature and thermodilution cardiac output along with pressures in the right atrium and pulmonary artery. A 6-F high-fidelity micro-tip pressure transducer pigtail catheter (Millar Instruments, Houston, TX) was advanced through the surgically exposed left carotid artery into the left ventricle for pressure measurement. A 14-Fr cannula (Bio-Medicus, Medtronic, Minneapolis, MN) was advanced through the left external jugular vein into the right atrium and used for blood withdrawal. Core temperature was maintained between 37.5°C and 38.5°C with a water-circulated blanket (Blanketrol II, Cincinnati SubZero, Cincinnati, OH). Vasopressin and EPO were administered through the left proximal tibia accessed using an EZ-IO intraosseous infusion system (VidaCare Corp, Santo Antonio, TX).

Hemorrhagic shock protocol. Blood was withdrawn into a 2,000 ml blood transfer bag (Charter Medical, Salen, NC) containing heparin (~10 $U \cdot ml^{-1}$ of the anticipated blood volume withdrawn) using a roller pump (model 313S, Watson Marlow, Inc., Wilmington, MA). The heparinized transfer bag was placed on an electronic scale (model 2200, Doran Scales, Inc., Batavia, IL) enabling continuous gravimetric measurement of the rate of blood withdrawal (blood density = 1.06 g/ml). A close-loop system with input from the electronic scale and output to the roller pump was developed by us (RJG and AB) in LabVIEW 6.0. For this application, we used a mono-exponential decay function to mimic spontaneous bleeding after traumatic injury (i.e., high initial bleeding with progressively slower rates as blood pressure declines and hemostatic mechanisms are activated and/or externally applied). The system was

highly precise maintaining the target withdrawal volume within 1 ml at any given time during the withdrawal interval. The blood withdrawn was kept in a water bath at 37.5°C and returned over 30 minutes using a blood transfusion filter (PALL Biomedical, Port Washington, NY) at 210 minutes from the start of blood withdrawal to model a situation in which evacuation and arrival to a medical treatment facility from the initial injury is delayed.

Recovery from anesthesia and survival. Upon completion of the acute resuscitative phase, all catheters were removed, vessels ligated, and the skin wounds stapled, all under sterile conditions. The animal was allowed to recover from anesthesia and the endotracheal tube removed after resumption of spontaneous breathing. The animal was then returned to its pen and monitored every 60 minutes until it was able to right itself to sternal recumbency. Thereafter the animal was monitored every 4 hours for the initial 24 hours and at a minimum interval of 8 hours until completion of the 72 hours. For analgesia, a fentanyl dermal patch was applied and maintained throughout the 72 hour post-resuscitation period. If additional analgesia was needed, 2.2 mg/kg of flunixin meglumine was administered intramuscularly. The neurological status was evaluated at 24, 48, and 72 hours post-resuscitation using a neurological deficit score (0 = best; 420 = worst) and a cerebral performance category score (1 = normal; 2 = mild disability; 3 = severe disability; and 4 = coma) [12]. The pig was euthanized at 72 hours by intravenous injection of euthanasia solution (pentobarbital sodium and phenytoin sodium; 5 ml, Vedco Inc., St Joseph, MO) or earlier for humanitarian reason in the event of any of the following; moderate to severe pain and distress unalleviated by analgesic agents, inability to eat or drink unassisted after 24 hours post-surgery, non-weight bearing or paralysis after 24 hours, depression or lethargy after 48 hours, profuse diarrhea, infection not resolved with antimicrobial therapy, lack of righting reflex, and cyanosis with difficulty breathing. None of the animals required earlier euthanasia for humane reason.

Study design

A factorial design was used to separately investigate the effects of vasopressin infusion, small-volume fluid resuscitation, EPO, and the hemorrhagic shock severity. The experiments were randomized by blocks with the investigators blind to the assignments (except for fluid resuscitation), unblinding the assignments only after completion of data collection and verification of data integrity.

Each of the four factors analyzed and the corresponding distributions of the remaining factors for each of the analyses are shown in Fig 1. The rationale and approach for investigating the aforementioned factors is detailed below.

Vasopressin. As discussed above, the addition of vasopressin infusion in an series from a previous study markedly improved initial resuscitation and subsequent 72 hour survival relative to a preceding series in which only fluid resuscitation with normal saline was used before blood reinfusion for the same hemorrhagic shock severity [9]. For the present study, pigs were randomized 2:1 to vasopressin or no vasopressin. Vasopressin (Pitressin, JHP Pharmaceuticals, Rochester, MI) or vehicle control was infused intraosseously using a syringe pump (PHD 2000 Syringe Pump Series, Harvard Apparatus, Holliston, MA) at a constant rate of 0.04 U/kg·min^{-1} from minute 7 of blood withdrawal (i.e., 16% of the blood volume removed) until minute 210 coincident with the start of blood reinfusion.

Fluid resuscitation. The preceding study [9] also showed that in the presence of vasopressin initial resuscitability and 72 hour survival could be achieved successfully administering a reduced amount of normal saline (corresponding to half the amount of blood withdrawn) or no fluids at all. Thus, for the present series we also examined whether normal saline could be required. The approach involved starting the series without fluid resuscitation and performing

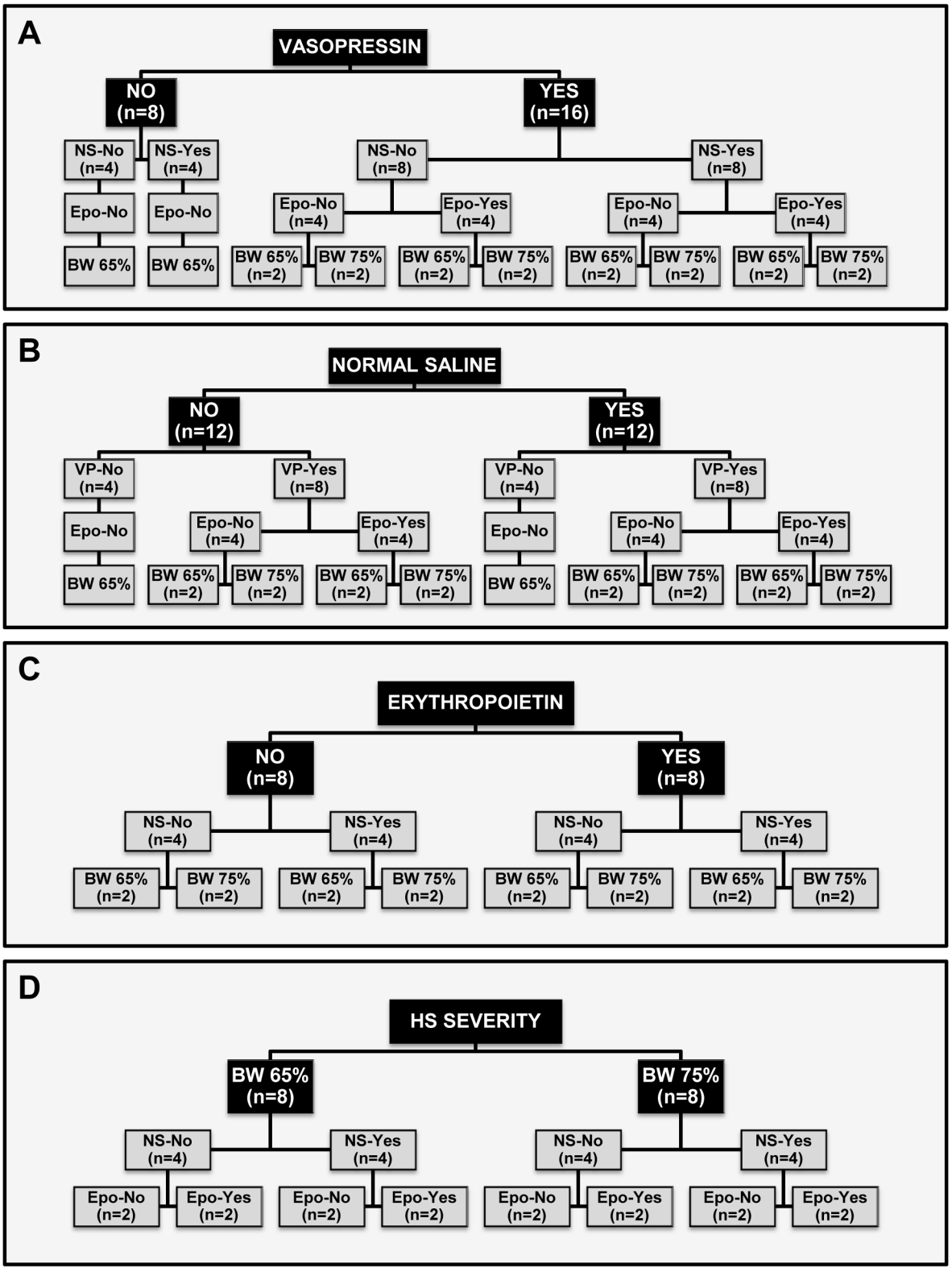

Fig 1. Distributions of the remaining factors for each of the four factor analyses. (**A**) Vasopressin effect, showing the 2:1 randomization with 16 pigs allocated to vasopressin also randomized to normal saline, erythropoietin, and level of hemorrhagic shock severity and 8 pigs allocated to no-vasopressin randomized only to normal saline but not to erythropoietin and exposed to the lowest hemorrhagic shock severity; (**B**) Normal saline effect, showing the 1:1 randomization with 12 pigs allocated to each level and a balanced distribution of the remaining factors; (**C**) Erythropoietin effect for the 16 pigs that received

vasopressin, showing the 1:1 randomization with 8 pigs allocated to each level and a balanced distribution of the remaining factors; and (**D**) Hemorrhagic shock severity effect for the 16 pigs that received vasopressin, showing the 1:1 randomization with 8 pigs allocated to each level and a balanced distribution of the remaining factors. NS, normal saline; Epo, erythropoietin; BW, blood withdrawal.

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an interim analysis after 12 experiments. The interim analysis showed a 72 hour survival of 25% (3/12) for the initial 12 experiments prompting for the last 12 experiments administration of normal saline from minute 90 to minute 120 through an ear vein. The volume of normal saline administered corresponded to half the volume of blood withdrawn; thus, 19.5 ml/kg was administered when 65% of the blood volume was removed–estimated as 60 ml/kg–and 22.5 ml/kg when 75% of the blood volume was removed.

Erythropoietin (EPO). In keeping with the original objective of our funded project but incorporating knowledge gained from the previous series, in which EPO had no effect on resuscitation and 72 hour survival but attenuated organ dysfunction under conditions of severe but survivable hemorrhagic shock [9], we randomized in the subset of animals receiving vasopressin to also receive EPO or vehicle control. Similar to preceding series, EPO was administered as a single intraosseous bolus dose of 1,200 U/kg after 14% of the blood volume had been removed (i.e., 6 minutes from the start of blood withdrawal).

Hemorrhagic shock severity. The severity of hemorrhagic shock was varied in the subset of animals receiving vasopressin by randomizing the blood withdrawal to 65% or to 75% of the blood volume. It is pertinent to highlight that in our previous study the highest amount of blood withdrawn was 65% of the estimated blood volume [9]. A mono-exponential decay function as described above was used for both blood withdrawal targets, stopping after 60 minutes for the 65% and after 80 minutes for the 75% blood withdrawal subsets.

Measurements

Blood analysis. Blood samples were collected from the aorta and pulmonary artery and were processed on site for pH, PO_2, PCO_2, hemoglobin, base excess, and lactate using a cartridge based device (OPTI CCA-TS Blood Gas and Electrolyte Analyzer, OPTI Medical Systems, Roswell, GA) and for common hemoglobin types (oxy-, met-, carboxy-, and reduced-) using a co-oximeter (AVOXimeter 4000, AVOX systems Inc., San Antonio, TX). O_2 content in the aorta (CaO_2) and pulmonary artery (CvO_2) was calculated according to the following equation:

$$O_2\ Content\left(\frac{ml}{dl}\right) = Hemoglobin\left(\frac{g}{dl}\right) \times 1.39\left(\frac{ml}{g}\right) \times S_FO_2 + 0.003\left(\frac{ml}{dl}\cdot mmHg^{-1}\right) \times PO_2\ (mmHg)$$

where 1.39 denotes ml of O_2 bound to 1 g of hemoglobin (Hufner's number), S_FO_2 the fraction of oxyhemoglobin relative to the four hemoglobin types, and 0.003 the O_2 solubility coefficient. Oxygen delivery and consumption index were calculated from CaO_2 multiplied by cardiac index and from the difference between CaO_2 and CvO_2 multiplied by cardiac index respectively, both estimated in ml/min·m^{-2}. Additional blood from the aortic samples had their plasma separated and stored at -80°C for subsequent batch analysis of glucose, urea nitrogen, creatinine, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, creatine kinase, and cardiac troponin I at the Captain James A. Lovell Federal Health Care Center, North Chicago, IL.

Hemodynamic measurements. Thermodilution cardiac output was measured in duplicate after bolus injection of 0.9% NaCl (5 ml) into the right atrium (HP-Philips M012AT cardiac output module, Amsterdam, The Netherlands). Cardiac output was normalized to body surface area using the Kelley equation (body surface area [m^2] = 0.073·body-weight$^{2/3}$ [kg]) [13].

Aortic and left ventricular pressure signals were calibrated and zeroed using a Millar PCU 2000 Box. Pressure signals measured using fluid-filled systems were calibrated using a digital pressure gauge (DPG1000, Omega Engineering) and zeroed to mid-cavity level. All signals were sampled and digitized at 250 Hz using a 16-bit data acquisition board (AT-MIO-16XE-50; National Instruments, Austin, TX) and analyzed using custom-developed software (Labview 6.0, National Instruments).

Cardiac function. Indices of cardiac function were derived from left ventricular and pulmonary artery pressures, reporting the left and right ventricular stroke work index (LVSWI and RVSWI, respectively) corresponding to the stroke volume index times the difference between systolic and end-diastolic left ventricular pressures and between the mean pulmonary and right atrial pressures, respectively, expressed in centijoules (cJ) by multiplying by 0.013332 [14]. The systemic vascular resistance index (SVRI) was calculated from the difference between mean aortic and mean right atrial pressure divided by cardiac index and reported in dynes·s/cm^{-5}·m^{-2}.

Statistical analysis

The statistical analysis was performed using Sigmaplot 11.0 (Systat Software, Inc., San Jose, CA). The survival effect of each of the four studied factors was analyzed using the Kaplan-Meier method and the log-rank test using the Holm-Sidak pairwise multiple comparisons test when applicable. The risk contribution of each of the studied factors on survival time was analyzed using the Cox proportional hazard analysis. Because only vasopressin and normal saline had an independent survival effect, the analysis of explanatory (hemodynamic and metabolic) variables was limited to these two factors using two-way repeated ANOVA analyzing the separate effects of vasopressin and normal saline identifying main effects, interactions, and differences at specific time points. Samples sizes for the separate factor analyses of 8 or higher per group were deemed adequate to identify clinically relevant effects based in previous work with similar experimental protocols. Data are shown as means ± SEM in figures and ± SD in tables and text. A two-tail $p \leq 0.05$ was considered statistically significant.

Results

No unexpected adverse events occurred. Demise was the consequence of hemorrhagic shock severity during the acute phase. Animals that survived the acute phase and were recovered from anesthesia survived the 72 hour observation interval recovering baseline neurological function and overall performance.

Baseline

No significant differences among groups were observed at baseline (Tables 1 and 2) except for cardiac index and indices of left and right ventricular work that were higher in the group assigned to receive normal saline.

Survival

Fig 2 shows the survival effect of vasopressin and normal saline, demonstrating a significantly higher survival rate associated with the combination of vasopressin and normal saline compared to each of the individual interventions or to none of them (Fig 2A). In addition, separate survival analyses performed for each of the main factors while maintaining a balanced distribution of the remaining factors showed a survival benefit associated with vasopressin infusion

Table 1. Effect of vasopressin on indices of organ function/injury.

	Baseline	BW	HS	End BR	Survival
	-10 min	60 min	150 min	240 min	72 h
Glucose (mg/dl)					
No-Vasopressin	95±21[8]	119±70[8]	185±34[3]	143±7[3]	118[1]
Vasopressin	103±54[16]	228±140[16]†b	137±102[12]	92±59[11]	107±7[11]
Blood Urea Nitrogen (mg/dl)					
No-Vasopressin	11±3	12±3	16±3[a]	16±3[a]	9
Vasopressin	8±3	11±3[b]	15±4[b]	14±4[b]	8±2
Creatinine (mg/dl)					
No-Vasopressin	0.9±0.1	1.3±0.1[b]	1.9±0.4[b]	1.9±0.4[b]	0.8
Vasopressin	1.0±0.1	1.4±0.2[b]	1.8±0.5[b]	1.7±0.4[b]	0.9±0.1
Aspartate Aminotransferase (U/l)					
No-Vasopressin	43±11	43±11	48±3	62±4	54
Vasopressin	38±9	32±12	77±144	93±112	140±117
Alanine Aminotransferase (U/l)					
No-Vasopressin	69±14	65±14	62±5	72±8	104
Vasopressin	64±8	58±12	50±6	60±8	178±74*[b]
Alkaline Phosphatase (U/l)					
No-Vasopressin	196±38	193±36	236±47	245±47	200
Vasopressin	199±60	193±53	236±70[a]	254±85[b]	200±66
Creatine Kinase (U/l)					
No-Vasopressin	1325±923	1384±817	1458±438	1277±192	1479
Vasopressin	1352±489	1337±432	1456±608	1574±675	8443±7679
Cardiac Troponin I (ng/dl)					
No-Vasopressin	0.04±0.05	0.10±0.11	1.32±0.59[b]	1.43±0.80[b]	0.04
Vasopressin	0.03±0.05	0.03±0.05	0.11±0.18‡	0.10±0.16‡	0.22±0.33

Numbers in brackets indicate the sample size reflecting animals alive at the specific time point except for HS 150 minute in the no-vasopressin group in which two samples were not available. Values are mean SD. BW, blood withdrawal; HS, hemorrhagic shock; BR, blood reinfusion. The data was analyzed using two-way repeated measures ANOVA reporting statistically significant differences between groups at specified time points

*$p \leq 0.05$

†$p \leq 0.01$, and

‡$p \leq 0.001$, and statistically significant differences from baseline within each group using the Holm-Sidak test for multiple comparisons

[a]$p \leq 0.01$, and

[b]$p \leq 0.001$.

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(Fig 2B) and normal saline (Fig 2C). Use of EPO (Fig 2D) and increasing hemorrhagic shock severity (Fig 2E) had no statistically significant impact on survival.

Proportional hazard risk

Table 3 shows hazard ratios for each factor demonstrating a significantly reduced risk on survival time associated with administration of vasopressin and administration of normal saline but not EPO or hemorrhagic shock severity.

Effects of vasopressin on hemodynamic and metabolic parameters

Fig 3 depicts the hemodynamic and metabolic effects of vasopressin. Vasopressin increased systemic vascular resistance resulting in an initial increase in mean aortic pressure attaining

statistical significance at minute 15 from the start of blood withdrawal. However, vasopressin also blunted the chronotropic response to hemorrhagic shock attaining statistical significance from minute 60 to minute 120, further reducing cardiac index despite no adverse effect on left ventricular work index. This effect precluded a sustained increase in mean aortic pressure (Fig 3A). Vasopressin intensified lactic acidosis and the base excess deficit, likely related to a flow dependent reduction in systemic oxygen consumption (VO_2), but this effect was transient attaining values similar to pigs treated without vasopressin before blood reinfusion (Fig 3B). The $P_{ET}CO_2$ largely followed changes in cardiac index consistent with its flow dependency under low-flow states (Fig 3B). Blood reinfusion restored cardiac index to baseline halting the progression of lactic acidosis initiating the reversal of hemodynamic and metabolic abnormalities.

Table 2. Effect of normal saline on indices of organ function/injury.

	Baseline	BW	HS	End BR	Survival
	-10 min	60 min	150 min	240 min	72 h
Glucose (mg/dl)					
No-Normal Saline	104±60[12]	202±141[12]b	172±97[5]	112±49[4]	104±8[3]
Normal Saline	99±25[12]	181±126[12]a	134±94[10]	100±62[10]	109±8[9]
Blood Urea Nitrogen (mg/dl)					
No-Normal Saline	10±2	12±3b	18±3c	17±3c	9±3
Normal Saline	8±3	10±4b	13±4c	14±4c	8±2
Creatinine (mg/dl)					
No-Normal Saline	1.0±0.1	1.3±0.1c	2.0±0.5c	1.7±0.3c	0.9±0.2
Normal Saline	1.0±0.1	1.4±0.2c	1.7±0.4c	1.7±0.4c	0.9±0.1
Aspartate Aminotransferase (U/l)					
No-Normal Saline	36±10	31±14	36±19	53±35	106±67
Normal Saline	43±8	40±10	89±157	100±115	141±128b
Alanine Aminotransferase (U/l)					
No-Normal Saline	64±12	59±16	50±10	58±15	153±56c
Normal Saline	68±8	61±9	54±6	64±6	178±81c
Alkaline Phosphatase (U/l)					
No-Normal Saline	185±46	180±43	194±58	206±61	228±45a
Normal Saline	211±58	206±50	257±60b	271±77c	191±68a
Creatine Kinase (U/l)					
No-Normal Saline	1186±444	1193±335	1439±468	1384±439	9541±7322c
Normal Saline	1501±791	1512±716	1465±629	1593±711	7304±8028c
Cardiac Troponin I (ng/dl)					
No-Normal Saline	0.04±0.05	0.05±0.08	0.32±0.34a	0.29±0.35a	0.05±0.03
Normal Saline	0.02±0.05	0.05±0.09	0.37±0.67	0.43±0.76a	0.24±0.35

Numbers in brackets indicate the sample size reflecting animals alive at the specific time point except for HS 150 minute in the no-normal saline group in which two samples were not available. Values are mean SD. BW, blood withdrawal; HS, hemorrhagic shock; BR, blood reinfusion. The data was analyzed using two-way repeated measures ANOVA reporting statistically significant differences from baseline within each group using the Holm-Sidak test for multiple comparisons

[a] $p \leq 0.05$

[b] $p \leq 0.01$, and

[c] $p \leq 0.001$.

There were no statistically significant differences between groups at any specified time point.

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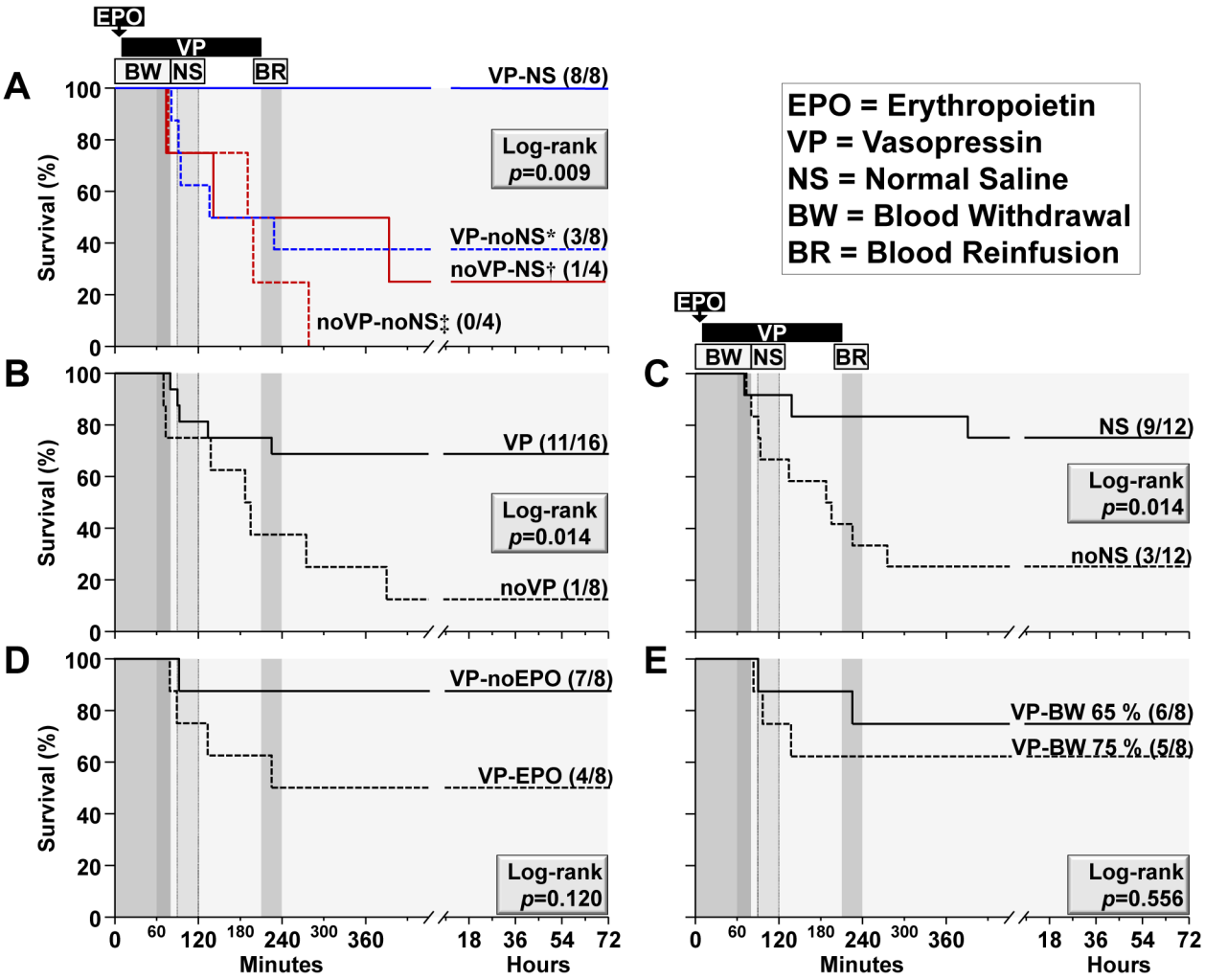

Fig 2. Survival analysis. Kaplan-Meier survival curves analyzed using the log-rank test and the Holm-Sidak test for multiple comparisons when applicable. The *p*-value for each log-rank test results is shown inside each graph. (**A**) Kaplan-Maier survival curves comparing the effects of vasopressin with normal saline (VP-NS), VP without NS (VP-noVP), no VP with NS (noVP-NS), and neither VP nor NS (noVP-noNS). Pairwise comparisons by the Holm-Sidak test demonstrated significantly lower survival for VP-noNS (*$p = 0.035$), noVP-NS (†$p = 0.0198$), and noVP-noNS (‡$p = 0.001$) compared with VP-NS. B through E show Kaplan-Maier survival curves for the independent effects of vasopressin (**B**), normal saline (**C**), erythropoietin (**D**), and hemorrhagic shock severity (**E**) with the remaining factors allocated as shown in Fig 1. In parentheses, number of animals alive at 72 hour survival relative to the initial subset.

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Effects of vasopressin on organ function/injury

These changes are shown on Table 1. Blood glucose increased during hemorrhagic shock attaining statistical significance only in vasopressin-treated pigs normalizing by 72 hours. Blood urea nitrogen and creatinine increased mildly in both groups during hemorrhagic shock

Table 3. Hazard ratios for main factors.

Covariate	Coefficient	SE	*p*-value	Hazard Ratio	95%CI
Vasopressin	-3.02	1.24	0.015	0.049	0.004–0.559
Normal Saline	-1.88	0.77	0.014	0.152	0.034–0.688
Erythropoietin	2.20	1.17	0.060	8.989	0.910–88.766
Blood Withdrawal 75%	0.84	0.93	0.365	2.324	0.374–14.435

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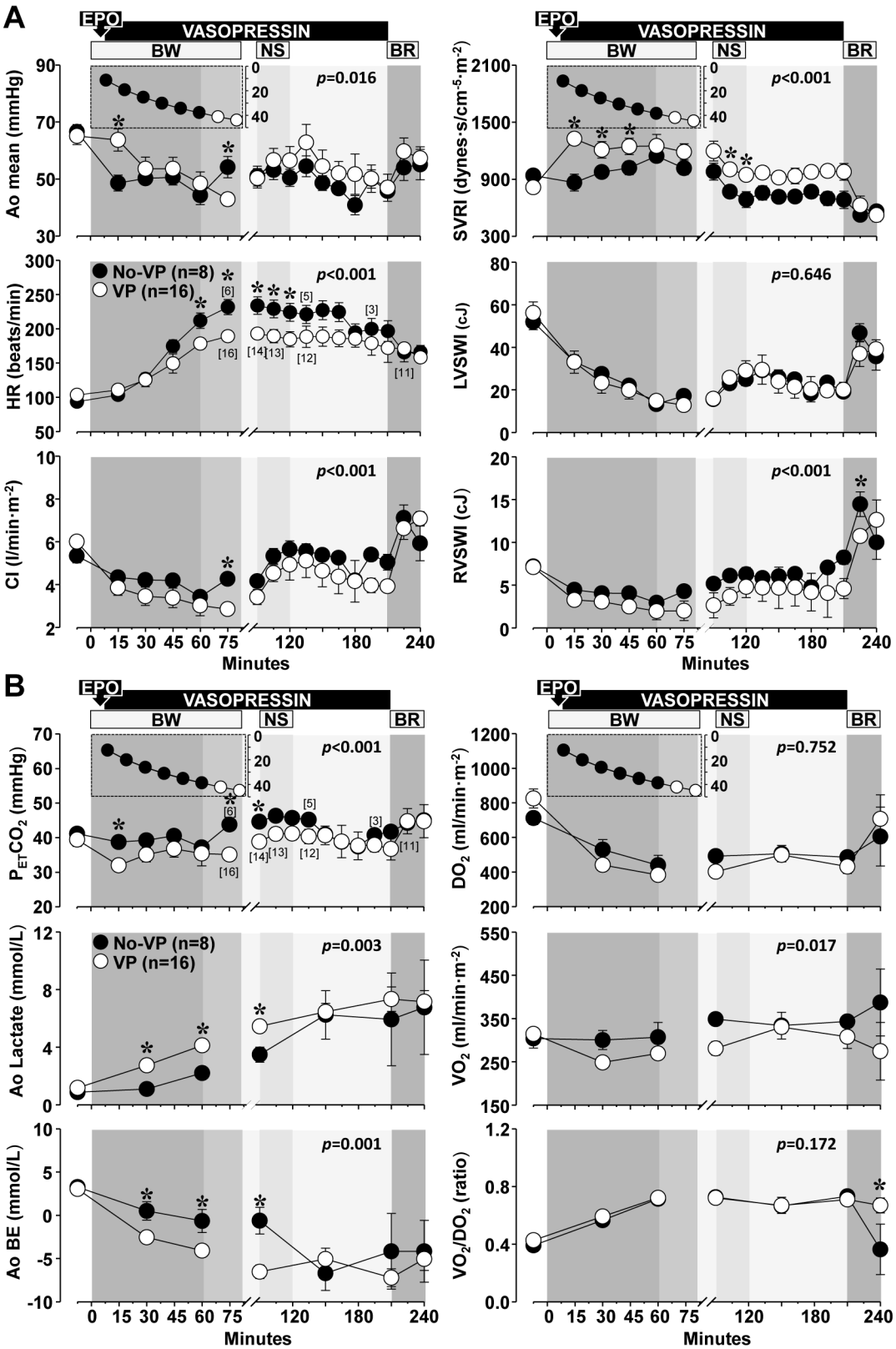

Fig 3. Effects of vasopressin on hemodynamic and metabolic function during hemorrhagic shock. EPO, erythropoietin; BW, blood withdrawal; NS, normal saline; BR, blood reinfusion. The inset depicts the time course of the blood withdrawal (ml/kg) ending at 60 minutes in the 65% BW subset and at 80

minutes in the 75% BW group. Numbers in brackets indicate when the number of animals decreased from the preceding time point. Values are shown as mean ± SEM. Differences between groups were analyzed by two-way repeated measures ANOVA. Overall statistical significances for the treatment effect are shown inside each graph. *$p \leq 0.05$ denotes statistically significant differences at the specified time point. Open circles denote vasopressin (VP) and closed circles denote vehicle control (No-VP). (**A**) Effects on hemodynamic and myocardial function. Ao, aortic pressure; HR, heart rate; CI, cardiac index; SVRI, systemic vascular resistance index; LVSWI, left ventricular stroke work index; RVWI, right ventricular stroke work index. (**B**) Effects on metabolic variables. $P_{ET}CO_2$, end-tidal carbon dioxide; Ao, aortic; BE, base excess; DO_2, oxygen delivery index; VO_2, oxygen consumption index; VO_2/DO_2, oxygen consumption and delivery ratio. There was an overall statistically significant interaction between treatment and time for Ao BE ($p = 0.040$).

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normalizing by 72 hours. A statistically insignificant increase in aspartate aminotransferase was observed associated with increases in alkaline phosphatase during hemorrhagic shock followed by an increase in alanine aminotransferase by 72 hours in vasopressin treated pigs. There was also a statistically insignificant increase in creatine kinase at 72 hours in vasopressin treated pigs. There was a mild increase in cardiac troponin I during hemorrhagic shock in control pigs but not in vasopressin-treated pigs.

Hemodynamic and metabolic effects of normal saline

Fig 4 depicts the hemodynamic and metabolic effects of normal saline. There were group differences before administration of normal saline likely reflecting the sequential allocation to treatment with normal saline in the last half of the series. Coincident with administration of normal saline from minute 90 to 120, left ventricular work index increased–attributed to preload augmentation–leading to a higher cardiac index and lowering the systemic vascular resistance index (Fig 4A). This effect was associated with attenuation of lactic acidosis (Fig 4B). Upon return of shed blood, animals treated with normal saline had hemodynamic and metabolic parameters closer to baseline.

Effects of normal saline on organ function/injury

These changes are summarized on Table 2 and were similar to those described above in relation to the vasopressin effects; namely, increases in glucose, blood urea nitrogen, creatinine, and cardiac troponin I during hemorrhagic shock that reversed by 72 hours but without differences between groups. Aspartate aminotransferases increased at 72 hours in normal saline treated pigs. Alanine aminotransferase increased in both groups also by 72 hours preceded by increases in alkaline phosphatase. Elevation in creatine kinase occurred in both groups by 72 hours.

Neurological deficit and overall performance

Pigs that survived 72 hours exhibited minimal or no clinical neurological deficits and had adequate overall performance.

Effect of EPO

Within the subset of pigs that received vasopressin, EPO failed to confirm a previously reported beneficial effect attenuating acute organ injury. In fact, pigs that received EPO had a lower mean aortic pressure, a blunted inotropic response, a higher systemic oxygen extraction ratio, and higher levels of aspartate aminotransferase and alkaline phosphatase during hemorrhagic shock. Their neurological deficit score was higher and overall performance category worse at 24 hours returning to baseline by 72 hours. Although not statistically significant, survival was lower in pigs that received EPO.

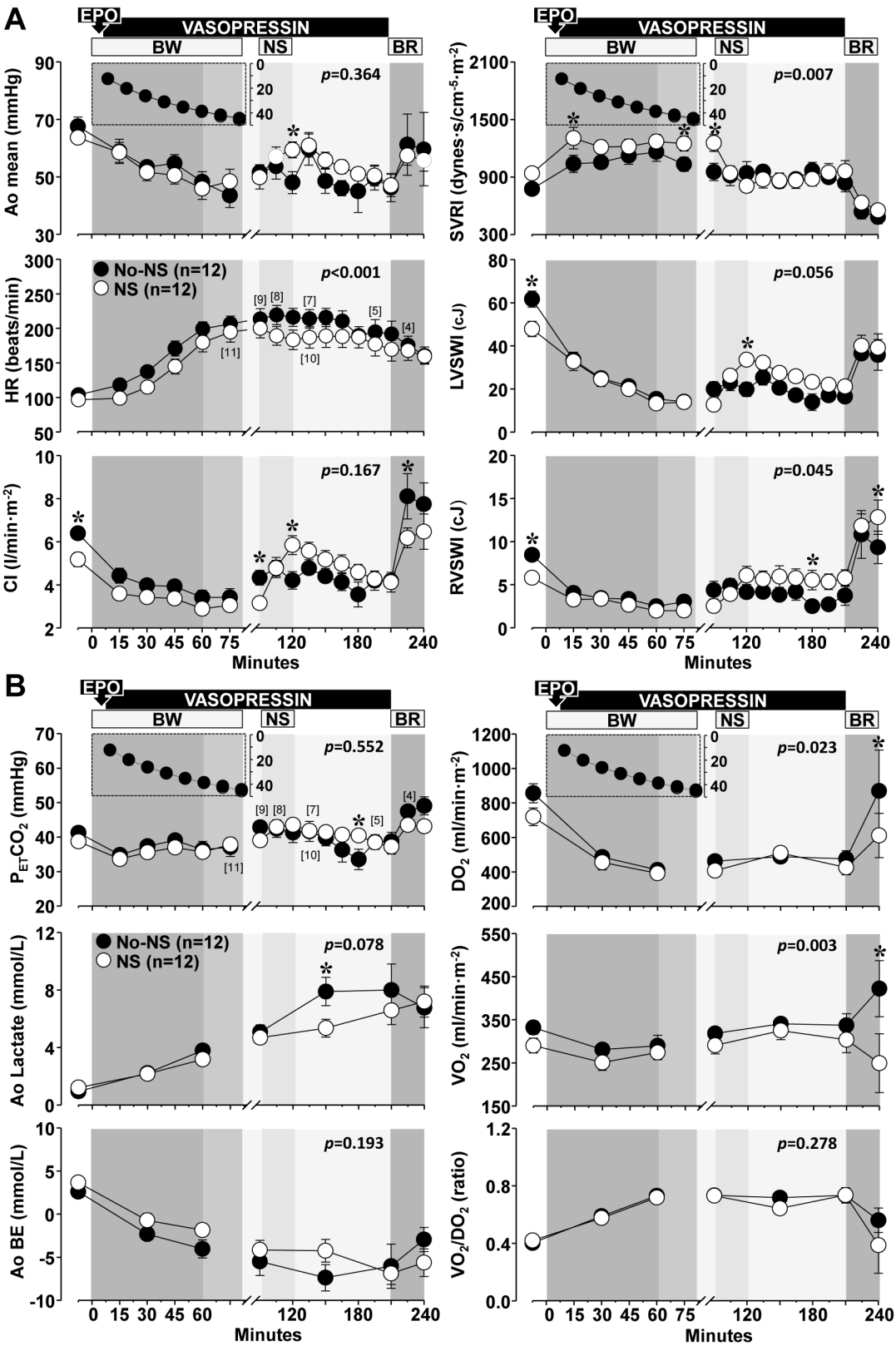

Fig 4. Effects of normal saline on hemodynamic and metabolic function during hemorrhagic shock. EPO, erythropoietin; BW, blood withdrawal; NS, normal saline; BR, blood reinfusion. The inset depicts the time course of the blood withdrawal (ml/kg) ending at 60 minutes in the 65% BW subset and at 80

minutes in the 75% BW group. Numbers in brackets indicate when the number of animals decreased from the preceding time point. Values are shown as mean ± SEM. Differences between groups were analyzed by two-way repeated measures ANOVA. Overall statistical significances for the treatment effect are shown inside each graph. *$p \leq 0.05$ denotes statistically significant differences at the specified time point. Open circles denote normal saline (NS) and closed circles denote no fluid administration (No-NS). (**A**) Effects on hemodynamic and myocardial function. Ao, aortic pressure; HR, heart rate; CI, cardiac index; SVRI, systemic vascular resistance index; LVSWI, left ventricular stroke work index; RVWI, right ventricular stroke work index. There was an overall statistically significant interaction between treatment and time for CI ($p < 0.001$), LVSWI ($p = 0.007$), and RVWI ($p = 0.009$). (**B**) Effects on metabolic variables. $P_{ET}CO_2$, end-tidal carbon dioxide; Ao, aortic; BE, base excess; DO_2, oxygen delivery index; VO_2, oxygen consumption index; VO_2/DO_2, oxygen consumption and delivery ratio.

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Discussion

The present study demonstrates a marked survival benefit associated with early and sustained administration of vasopressin in conjunction with small-volume fluid resuscitation in a swine model of severe hemorrhagic shock. Vasopressin acted by increasing systemic vascular resistance whereas normal saline acted by increasing cardiac index through a preload effect. Although vasopressin intensified lactic acidosis, this effect was counterbalanced by normal saline. The combination of vasopressin and normal saline averted early demise and secured sufficient hemodynamic stability for the 210 minutes of hemorrhagic shock before blood reinfusion, modeled to simulate delayed arrival to a medical treatment facility where control of the bleeding source and resuscitation with blood products could be performed. Administration of EPO conferred no additional survival benefit.

Vasopressin

Vasopressin is a nonapeptide synthesized by the paraventricular and supraoptic nuclei in the hypothalamus and stored in the posterior pituitary. It is released in response to osmotic and baroreceptor signaling. In the circulation, vasopressin acting *via* V_1 receptors on smooth muscle cells increases vascular tone and promotes vasoconstriction. The effect is mediated in part through closure of ATP sensitive K^+ channels preventing cell membrane hyperpolarization and thus maintaining calcium channels operational, making cytosolic calcium available for contraction. Vasopressin also blunts increases in cyclic guanosine monophosphate and reduces NO synthase production [11,15]. The vascular effects are predominantly arteriolar–increasing vascular resistance with relative minor effects on venous tone [16]. Vasopressin is released during hemorrhagic shock and other hemodynamic crises as part of the neuroendocrine stress response; however, the reserve of vasopressin is limited and exogenous administration has been suggested for hemodynamic stabilization in sepsis [17–19] and hemorrhagic shock [11,20,21]. In previous studies also using swine models of hemorrhagic shock, vasopressin was comparably superior to fluid administration and to epinephrine [10,22]. In a dog model of hemorrhagic shock, vasopressin given before administering fluids was more effective that when used after fluid administration [23]. In our own previous studies using a similar swine model of controlled hemorrhagic shock, we observed a marked survival effect produced by vasopressin infusion under conditions in which aggressive fluid resuscitation failed [9]. Vasopressin has indeed been proposed as a "preferred" vasopressor agent for hemodynamic stabilization during hemorrhagic shock [11].

The dose chosen for the present studies followed studies by Voelckel *et al*., also in swine [10]. We began the vasopressin infusion at 7 minutes into hemorrhagic shock based on previous experience with the model in which demise started after removing more than 50% of the blood volume using a linear function. The mono-exponential decay function chosen to model spontaneous bleeding resulted in faster earlier blood loss. Thus, we elected to start the vasopressin infusion before critical reduction in blood volume occurred. We assumed that such approach would be deployable in the battlefield through the intraosseous route using a small

pre-loaded and pre-programmed infusion pump upon recognition of severe bleeding with the option of administering fluids as a secondary intervention guided in the battlefield by combat casualty care criteria.

Consistent with previous observations [24], vasopressin blunted the baroreceptor response to hypovolemia such that not only the heart rate but also the cardiac index was lower than in vehicle control animals (Fig 3A). This effect was associated with a higher oxygen extraction ratio and higher lactate levels early during hemorrhagic shock but without compromising the ability of vasopressin to promote sustained hemodynamic stability and improve survival without organ injury or dysfunction. Moreover, vasopressin infusion appeared to have protected the myocardium as minor increases in cardiac troponin I were observed only in control animals (Table 1).

Fluid resuscitation

The aggressiveness of fluid administration during hemorrhagic shock before control of the bleeding source is been tempered in both military [4,5] and civilian settings [6,7]. Aggressive fluid administration can compromise coagulation by dilution of clotting factors and hypothermia. Concomitantly, there is activation of the fibrinolytic system, tipping the hemostatic balance toward bleeding and prompting greater use of blood products [25]. Excessive fluid administration also risks pulmonary edema and edema in other territories contributing to the development of compartment syndromes and intestinal dysfunction [26]. There is also concern that when normal saline is the fluid administered in large amounts it can precipitate renal injury through development of hyperchloremic acidosis [27] and also prompt activation of inflammatory cascades [28]. In addition, it is logistically disadvantageous to carry large amounts of fluids for deployment in the battlefield. For these reasons, small-volume fluid resuscitation is gaining acceptance in military and civilian settings with clinical trials showing better outcomes compared to aggressive fluid resuscitation protocols [6,7].

The present study showed that small-volume fluid resuscitation could be highly effective when used in conjunction with vasopressin infusion during severe hemorrhagic shock. In the present study each of the 8 pigs treated with vasopressin infusion and small-volume fluid resuscitation survived 72 hours without evidence of organ dysfunction.

Limitations

The main limitation of the present study is the absence of tissue injury that typically accompanies hemorrhagic shock in the battlefield and the ensuing inflammatory response. In addition, the necessity of anesthesia could mask some of the physiologic responses to hypovolemic shock. Finally, the naturally occurring neuropeptide in swine is lysine vasopressin whereas in humans it is arginine vasopressin. Thus, potency of the effect may vary contingent on specific receptors and vascular beds and translation of these findings to humans will need to consider human dose-responses.

Conclusions

The present findings support the concept that early and sustained administration of vasopressin could be highly effective for hemodynamic stabilization in the setting of severe hemorrhagic shock and work in conjunction with small-volume fluid resuscitation to initiate and maintain critical hemodynamic stability until arrival to a medical treatment facility.

Supporting Information

S1 Dataset. The primary dataset pertaining to this study.
(XLSX)

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Non-endorsement disclaimer

The views, opinions, and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

Author Contributions

Conceived and designed the experiments: RJG. Performed the experiments: K. Whitehouse K. Whittinghill AB. Analyzed the data: K. Whitehouse K. Whittinghill RJG. Contributed reagents/materials/analysis tools: AB JR. Wrote the paper: RJG.

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Appendix 3

Gazmuri RJ, Whitehouse K, Shah K, Whittinghill K, Baetiong A, Radhakrishnan J. Early and sustained vasopressin infusion augments the hemodynamic efficacy of restrictive fluid resuscitation and improves survival in a liver laceration model of hemorrhagic shock (undergoing peer review by the *Journal of Trauma and Acute Care Surgery*)

Early and Sustained Vasopressin Infusion Augments the Hemodynamic Efficacy of Restrictive Fluid Resuscitation and Improves Survival in a Liver Laceration Model of Hemorrhagic Shock

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ABSTRACT

Background: Current management of hemorrhagic shock favors restrictive fluid resuscitation before control of the bleeding source. We investigated the additional effects of early and sustained vasopressin infusion in a swine model of hemorrhagic shock produced by liver laceration.

Methods: Forty male domestic pigs (32 to 40 kg) had a liver laceration inflicted with an X-shaped blade clamp, 32 received a second laceration at minute 7.5, and 24 received two additional lacerations at minute 15. Using a two-by-two factorial design, animals were randomized 1:1 to receive vasopressin infusion (0.04 U/kg·min^{-1}) or vehicle control given intraosseously from minute 7 until minute 240 and 1:1 to receive normal saline (NS, 12 ml/kg) intravenously at minute 30 or no fluids.

Results: Kaplan-Meier curves showed overall survival differences (log-rank test, p=0.095) favoring vasopressin with NS (8/10) over vasopressin without NS (4/10), NS without vasopressin (3/10), and no intervention (3/10). Logistic regression showed that vasopressin improved survival at 240 minutes (p=0.042). Vasopressin augmented mean aortic pressure between 10 and 20 mm Hg without intensifying the rate of bleeding from liver laceration, which was virtually identical to that of control animals (33.9±5.1 and 33.8±4.8 ml/kg). Vasopressin increased systemic vascular resistance and reduced transcapillary fluid extravasation augmenting the volume of NS retained (6.5±2.7 *vs* 2.4±2.0 ml/kg by minute 60). The cardiac output and blood flow to the myocardium, liver, spleen, kidney, small bowel, and skeletal muscle at minute 120 and minute 180 were comparable or higher in the vasopressin group.

Conclusions: Early and sustained vasopressin infusion provided critical hemodynamic stability during hemorrhagic shock induced by liver laceration and increased the hemodynamic efficacy of restrictive fluid resuscitation without intensifying bleeding or compromising organ blood flow resulting in improved 240 minute survival.

Level of Evidence: V (animal studies).

BACKGROUND

Current recommendations for initial management of hemorrhagic shock in combat operations and civilian trauma favor hypotensive resuscitation through restrictive fluid administration, deferring full hemodynamic stabilization until control of the bleeding source (1-8). Aggressive fluid resuscitation may worsen acute traumatic coagulopathy by dilution and hypothermia and dislodge freshly formed clots (2,4,7). Aggressive fluid resuscitation also poses logistical constraints for far-forward military deployment (3,8). Thus, restrictive fluid resuscitation is becoming standard of care in military (3,8) and civilian trauma (1,4) with concomitant efforts to optimizing the quality of fluids based on their hemodynamic, oxygen carrying capacity, metabolic, and hemostatic effects (8). In addition, strategies to provide sustained hemodynamic stability are needed to favorably impact the management of hemorrhagic shock in the battlefield, especially with anticipated longer evacuation times (8).

Recent studies in our laboratory, using swine models of controlled hemorrhage (9,10), and previous studies by other investigators in smaller animal models (11,12), support the use of vasopressin infusion to promote rapid hemodynamic stabilization and enhance the efficacy of subsequent restrictive fluid resuscitation. Vasopressin may be superior to other vasopressor agents and fluids alone (11,13-16) and has been proposed for the clinical management of hemorrhagic shock associated with trauma (13,17). Clinicaltrials.gov lists three clinical studies; two have been completed (18,19) and one is active (AVERT Shock). In these trials, vasopressin is being studied for the management of hemorrhagic shock only after initial stabilization efforts, which include administration of intravenous fluids.

We proposed the use of sustained vasopressin infusion started early after the onset of hemorrhagic shock to promote rapid hemodynamic stability and enhance the efficacy of restrictive fluid re-

suscitation; thus minimizing the risk of death before control of the bleeding source. Nearly 90% of all injury related deaths in combat operations occur before arrival to a medical treatment facility. Of these, ~25% are deemed potentially survivable and are associated with bleeding from internal organs or large surface areas not amenable to compression (20). Accordingly, early use of vasopressin infusion may be especially effective in this patient population and apply to similar civilian scenarios. Yet, there is valid concern that vasopressin administration in the presence of non-compressible injuries could accentuate the rate of bleeding. Thus, information on the effects of vasopressin infusion during hemorrhagic shock in the presence of non-compressible injuries in clinically relevant large animal model is of paramount importance for clinical translation.

We therefore investigated in a swine model of hemorrhagic shock produced by liver laceration (21) the effects of early initiation of vasopressin infusion followed by restrictive fluid resuscitation without attempting hemostasis. We hypothesized that vasopressin infusion would promote hemodynamic stability without intensifying bleeding securing survival for at least 4 hours, modeling a scenario of severe injury and delayed access to definitive care (8,22). We assessed the effects on bleeding rate, systemic hemodynamic function, organ blood flow distribution, and 4 hour survival.

METHODS

The studies were approved by the Institutional Animal Care and Use Committee at Rosalind Franklin University of Medicine and Science (approval number 12-23) and by the United States Army Medical Research and Materiel Command Animal Care and Use Review Office and were conducted according to institutional guidelines.

Animal Housing and Husbandry

Animals were group housed in pens in the Biological Resource Facility (AAALAC accredited facility) at Rosalind Franklin University of Medicine and Science. Lights were set at the recommended illumination levels of a 12/12-hour cycle controlled via automatic timers. Temperature was maintained between 61 °F and 81 °F. Resting mats were provided using Aspen Sani-Chip bedding from a certified vendor (Harlan Laboratories; Indianapolis, IN). Health assessment for general health and well-being, possible injuries, or death was performed daily by animal care technicians and veterinarians and by investigators the day before each experiment.

Animal Preparation

Forty male domestic pigs (32.3 to 40.3 kg) were sedated with ketamine hydrochloride (30 $mg \cdot kg^{-1}$ intramuscularly). Anesthesia was induced with propofol (2 $mg \cdot kg^{-1}$ through an ear vein) and the animal intubated with a size 7.5 tracheal tube initiating positive pressure ventilation with a volume controlled ventilator (840 Ventilator System, Nellcor Puritan Bennett; Boulder, CO) set to deliver a tidal volume of 10 $mL \cdot kg^{-1}$, peak flow of 60 $l \cdot min^{-1}$, and FiO_2 of 0.5. Respiratory rate was adjusted to maintain an end-expired PCO_2 ($P_{ET}CO$) between 35 and 45 mmHg (Capnogard, Novometrix Medical Systems; Wallingford, CT). Anesthesia was continued using isoflurane (1.5% to 2.0%) and a 1:1 mixture of nitrous oxide and oxygen. The electrocardiogram was rec-

orded through a defibrillator/monitor (Heartstream XL, Agilent Technologies; Santa Clara, CA). A 6 F dual lumen pressure transducer catheter (Langston, Vascular Solutions; Minneapolis, MN) was advanced through the right femoral artery into the descending thoracic aorta for pressure measurement. A balloon-tipped pulmonary artery catheter (Edwards Lifescience Corp; Irvine, CA) was advanced through the left cephalic vein into the pulmonary artery for measuring core temperature and cardiac output by bolus thermodilution along with pressures in the right atrium and pulmonary artery. A 7 F dual lumen pressure transducer pigtail catheter (Langston, Vascular Solutions; Minneapolis, MN) was advanced through the surgically exposed left carotid artery into the left ventricle for pressure measurement. An intraosseous needle was placed in the left proximal tibia using an electrical drill (EZ-IO Intraosseous Infusion System, Teleflex Medical; Research Triangle Park, NC) and used for vasopressin infusion (Figure 1A). Core temperature was maintained between 37.5°C and 38.5°C with a water-circulated blanket (Blanketrol II, Cincinnati SubZero; Cincinnati, OH).

Experimental Protocol

After confirming hemodynamic and metabolic stability, a 20-cm midline laparotomy was performed starting from the xiphoid process. At time zero, the left lateral lobe of the liver was exteriorized and a laceration inflicted at 8 cm from the apex and 6 cm from the edge with a custom built, sharpened steel, X-shaped, 5-cm blade clamp to simulate a shotgun injury with collateral damage. The laceration entailed closing the clamp and repeating the maneuver after opening and rotating 90 degrees. As described under *Study Design*, additional liver lacerations were inflicted in subsets of animals at minute 7.5 (in the same lobe, 12 cm from the apex and 4 cm from the edge) and at minute 15 (two lacerations, one on the left medial lobe 10 cm from the apex and 4 cm from the edge and another 14 cm from the apex and 6 cm from the edge). Blood flowing

from the liver was continuously collected into a canister placed on an electronic scale (Doran 2200; Batavia IL) using a vacuum suction device (Easy Go, Precision Medical; Northampton, PA). An additional suction catheter was placed in the left paracolic gutter and connected to a roller pump (Watson Marlow; Wilmington, MA) also directing the blood into the canister. The setup enabled continuous gravimetric monitoring of the bleeding rate. In addition, pre-weighed laparotomy pads were placed in dependent intra-abdominal areas including both paracolic gutters to collect blood that might have escaped suctioning, and measured blood volume by differential weight to determine – with the suctioned blood – the total volume of blood loss (blood density = 1.06 g/dl). No efforts were made to control the bleeding.

During the experiment, mechanical ventilation was maintained at the preset ventilatory settings adjusting the isoflurane level to secure a surgical plane. Pigs were monitored for a maximum of 240 minutes and those that survived were euthanized by intravenous injection (5 ml) of pentobarbital sodium and phenytoin sodium mixture (Euthasol, Virbac, Ottawa, Canada). Necropsy was performed sampling organs of interest for determining blood flow by a microsphere technique described below.

Measurements

Organ blood flow: Fluorescent microspheres (15 μm in diameter) of four distinct colors (Interactive Medical Technologies – Stason Laboratories, Irvine, CA) were injected into the left ventricle at baseline and at minutes 60, 120, and 180 of hemorrhagic shock. The microspheres were supplied in 10 ml vials (50 million/ml suspension) and were kept during the experiment in a water bath at room temperature. Five minutes before injection, the vial was placed in an ultrasonicator and 2 ml – i.e., 10 million microspheres – were transferred to a 3 ml syringe, which was also kept in the ultrasonicator until injection. For reference organ, blood was withdrawn from the aor-

ta at 1 ml·min^{-1} for 5 minutes starting 2 minutes before microsphere injection and collected into a heparinized syringe using a syringe pump (PHD 2000 Syringe Pump Series, Harvard Apparatus, Holliston, MA). At the end of the experiment, ~3 g tissue samples were obtained from the left ventricular epicardium and endocardium at mid-cavity level, left and right renal cortex, left and right intercostal skeletal muscles, small bowel 10 cm from the duodenal junction, right liver lobe, and spleen. The left and right adrenal glands were collected in their entirety.

The tissue and reference organ blood samples were stored at 4 °C and shipped to Interactive Medical Technologies for processing, which involved overnight digestion in alkaline reagents at 60 °C, mixing with a microsphere counting reagent, centrifugation, and filtering to retrieve the microspheres for flow cytometry analysis. Organ blood flow (Q_i, ml/min/g) was calculated according to Q_i,= (C_i)·(Q_r)/C_{ref}; where (C_i) denotes microspheres per gram of tissue, (Q_r) reference organ blood withdrawal rate (ml/min), and C_{ref} microspheres in the reference blood sample. Total blood flow (Q_T, ml/min) was calculate by substituting the total number of microspheres injected (C_T) for C_i according to Q_T,= (C_T)·(Q_r)/C_{ref}. Blood flow was expressed as percentage of baseline with blood flow to the left ventricular layers and to paired organs reported correspondingly combined.

Blood analysis: Blood analysis for pH, PO_2, PCO_2, hemoglobin, base excess, and lactate along with metabolic and hemodynamic calculations was performed as previously described (10).

Hemodynamic and metabolic measurements: Indices of cardiac and hemodynamic function along with systemic oxygen delivery and consumption were calculated as previously described (9).

Study Design

We used a two-by-two factorial design, randomizing 40 pigs – through five blocks of eight animals each – to vasopressin infusion or vehicle control and to normal saline or no fluids; each applying a 1:1 randomization ratio. The severity of injury was adjusted as the study progressed intended to achieve an overall mortality of ~50% (i.e., to optimize statistical power for assessing survival). To this end and before randomization, we conducted 6 pilot experiments inflicting one liver laceration, which yielded an average blood loss of 28 ml/kg (~45% of the blood volume). We considered the model sufficiently severe given the additional tissue injury and commenced the 40 randomized experiments while scheduling interim analyses after each block of eight, examining overall survival without unblinding. The first analysis showed 25% mortality prompting the addition of a second liver laceration at minute 7.5. The second analysis showed 37.5% mortality prompting the addition of a third and a fourth liver laceration at minute 15 and the completion of the series without additional analyses. The randomization blocks are shown in the supplemental digital content (SDC) (Figure 1-SDC). The strategy provided adequate sample size to examine the various hemodynamic and metabolic effects of the interventions and their effect with an overall mortality of 55%.

Vasopressin and Normal Saline

Vasopressin (Pitressin [20 U/ml], JHP Pharmaceuticals; Rochester, MI) – or equal volume of normal saline (vehicle) – was infused intraosseously at a constant rate of 0.04 U/kg·min^{-1} using an infusion pump (Alaris Pump, Model 8100; San Diego, CA) from minute 7 (i.e., immediately before the second liver laceration) until the end of the experiment at minute 240 with the investigators blind to the treatment assignment. The dose chosen for the present studies followed studies by Voelckel *et al.*, also in swine (13), and studies by us using controlled hemorrhagic shock protocols (9,10). For restrictive fluid resuscitation, normal saline, 12 ml/kg, (Baxter; Deerfield,

IL) was infused through an ear vein from minute 30 to minute 60 using a peristaltic pump (B. Braun Medical Inc.; Bethlehem, PA).

Statistical Analysis

Survival was analyzed using Kaplan-Meier curves and log-rank test and by multiple logistic regression assessing the contribution of liver lacerations, vasopressin infusion, and normal saline (SigmaPlot 11.0, Systat Software, Inc.; San Jose, CA). Continuous repeatedly measured variables were analyzed using a linear mixed effect model treating time as a continuous variable to assess main effects and interactions and as a categorical variable to assess differences at specific times (SPSS 23.0, IBM Corp.; Armonk, NY). Samples sizes of 20 per group for the main effects were deemed adequate based on similar previous studies. Data are shown as means ± SEM in Figures and as means ± SD in Tables and text.

RESULTS

Baseline

Hemodynamic and metabolic parameters were comparable among groups before initiation of the experiment as shown in the various Figures and Tables.

Survival

Kaplan-Meier curves (Figure 2, A-D) depict the independent effects of liver lacerations, vasopressin infusion, normal saline, and the combination of the latter two on survival. Analysis of the four subsets resulting from the combination of vasopressin and normal saline (Figure 2D) showed survival differences (p=0.095) favoring vasopressin with normal saline. Multivariate logistic regression showed that mortality was increased by the number of liver lacerations (p=0.015) but was reduced by vasopressin infusion (p=0.042).

Bleeding profile

Liver lacerations prompted rapid bleeding that spontaneously ceased within ~15 to 30 minutes (Figure 1B, 1C, and 1D). The total blood loss was 26.8±3.5 ml/kg after one laceration, 34.2±2.8 ml/kg after two lacerations (p<0.001 *vs* one), and 36.1±3.4 ml/kg after four lacerations (p<0.001 *vs* one laceration). Vasopressin did not alter the bleeding profile, which completely overlapped with the profile of control pigs (Figure 1C), and the total blood loss was the same with and without vasopressin (33.9 ± 5.1 and 33.8 ± 4.8 ml/kg). Normal saline elicited similar bleeding profiles (Figure 1D) with slightly higher but statistically insignificant total blood loss (34.4 ± 4.9 *vs* 33.2 ± 4.9 ml/kg).

Hemodynamic and Metabolic Effects

Vasopressin effect: Vasopressin increased systemic vascular resistance yielding a mean aortic pressure ~10 to 20 mmHg higher than controls (Figure 3) and blunted the chronotropic response to hemorrhagic shock mostly during the initial 40 to 80 minutes as expected (23) while increasing the left ventricular stroke work index. Cardiac index transiently decreased in both groups at the onset of hemorrhagic shock but increased with vasopressin infusion toward minute 240. Systemic oxygen delivery and oxygen consumption were initially lower with vasopressin but subsequently stabilized toward minute 240. Arterial lactate was initially higher with vasopressin, coincident with the lower oxygen consumption, but subsequently stabilized and exhibited a downward trend. Also noted was a transient decrease in hematocrit after normal saline administration in the vasopressin group.

Normal saline effect: Normal saline transiently increased mean aortic pressure, cardiac index, and left ventricular stroke work index and lowered systemic vascular resistance (Figure 4). Normal saline also attenuated arterial lactate increased without substantial effects on systemic oxygen delivery or consumption. The hematocrit transiently decreased with normal saline.

A complete set of hemodynamic and metabolic measurements is available as SDC (Figures 2-5 SDC). With vasopressin, there was a higher rate of maximal left ventricular pressure decrease suggesting a lusitropic effect and a lower pulmonary vascular resistance (Figure 2-SDC).

Total and Regional Blood Flow Effects

Vasopressin effect: The total blood flow paralleled changes in cardiac index. With vasopressin, blood flow to the left ventricle, skeletal muscle, and small bowel was transiently reduced coincident with the blunted chronotropic response to hemorrhagic shock but increased thereafter attaining statistical significant higher levels than control for small bowel and the kidneys (Table 1A).

Normal saline effect: The total blood flow also paralleled changes in cardiac index attaining higher levels with normal saline after the bolus, accompanied by increases in blood flow to the left ventricle, kidneys, liver, small bowel, spleen, and skeletal muscle (Table 1B).

Fluid Retentive effect of Vasopressin

The transient reduction in hematocrit observed in the vasopressin group after normal saline (Figure 5) suggested that vasopressin elicited an intravascular volume retentive effect. Because bleeding from liver lacerations had largely ceased by minute 30 before administering the normal saline bolus, we calculated the fluid retentive effect by estimating the animal's blood volume at minute 30 and used the serially changes in hematocrit to calculate changes in plasma volume. As shown in Figure 5, the expansion of the intravascular volume after the normal saline bolus was the largest in the presence of vasopressin (i.e., 6.5±2.7 ml/kg corresponding to 54 % of the volume infused) but limited to ~60 to 90 minutes. A detailed description of calculations and findings is available as SDC (Methods-SDC).

Additional Interactions

The addition of normal saline to vasopressin infusion increased cardiac index, mean aortic pressure, and left ventricular stroke work index while further blunting the chronotropic response to hemorrhagic shock and lessening the increase in systemic vascular resistance. Normal saline also attenuated lactate increases and promoted higher organ blood flows without reductions in left ventricular blood flow at minute 60 as shown in SDC (Figures 6-7 SDC and Table 1-SDC).

DISCUSSION

The liver laceration model allowed assessing the effects of vasopressin infusion in a setting of non-compressible internal bleeding and tissue injury. Bleeding spontaneously ceased after 15 to 30 minutes from the first liver laceration despite maintaining the abdomen open and regardless of systemic blood pressure. The model mimicked clinical scenarios in which a conservative approach is adopted (24,25), reserving interventions (e.g., angioembolization or damage control surgery) for uncontrolled bleeding (26). The adjustment of hemorrhagic shock severity by inflicting additional lacerations yielded an overall mortality of 55% with variability in severity that was evenly distributed through the various interventions adding an element of potential translational relevance.

Vasopressin Effect

Vasopressin is a nonapeptide synthesized in the hypothalamus and stored in the neurohypophysis. It is released during hemorrhagic shock as part of a generalized neuroendocrine response. Yet, the vasopressin response is short-lived and exogenous vasopressin administration has been advocated (27). We selected vasopressin for its effect on V1A receptors, to increase systemic vascular resistance and thereby prevent critical reductions in coronary perfusion pressure that could precipitate cardiac arrest. Vasopressin increased systemic vascular resistance by ~50% and mean aortic pressure by 10 to 20 mmHg. The vasoconstrictive effect was limited to the systemic circulation without affecting (or even decreasing) the pulmonary vascular resistance. This effect could be advantageous during hemorrhagic shock. Use of norepinephrine or phenylephrine in a similar animal model of hemorrhagic shock increased pulmonary vascular resistance and compromised oxygenation (28); an adverse effect not observed in the present study.

The rationale for investigating vasopressin infusion early after onset of hemorrhagic shock stemmed from our previous findings in a swine model of controlled bleeding. In this model, demise begun to occur after withdrawing > 50% of the blood volume yielding 75% acute mortality despite aggressive fluid resuscitation (9). Yet, vasopressin infusion started upon removing 50% of the blood volume markedly reduced mortality to only 8%. In additional studies in which we withdraw blood according to a mono-exponential decay function (to model spontaneous bleeding with faster initial blood loss), vasopressin infusion started at minute 7 also markedly improved survival (10). As noted in Figure 2 (B and D), vasopressin infusion prevented demise within the initial 60 minutes with later demise mitigated by normal saline (Figure 2D). Yet, our intention was not to define a precise time for the initiation of vasopressin infusion but to propose its use as the initial intervention for severe hemorrhagic shock. A similar favorable effect was reported recently in a rat model of uncontrolled hemorrhagic shock (29).

We expected that vasopressin infusion would secure myocardial perfusion at the expense of blood flow to other organs. Yet, there was no preferential blood flow redistribution. Instead, there was early and transient reduction in total blood flow (i.e., by minute 60) that also affected the myocardium and probably accounted for the high early lactate levels. This effect coincided with the blunted chronotropic response to hemorrhagic shock (23). Accordingly, it was transient and after the initial 60 minutes, the total and organ blood flows in the vasopressin group were comparable or even higher than in the control group.

Unexpectedly, we observed that vasopressin infusion enabled more normal saline to be retained intravascularly. We attributed the effect to systemic vasoconstriction elicited by V1A receptors leading to reduction in the capillary hydrostatic pressure driving transcapillary fluid efflux. A similar effect was recently reported using selective V1A receptor agonists in a sheep model of

septic shock (30,31), but attributed to an anti-inflammatory effect helping preserve capillary permeability. These observations may have clinical relevance whereby greater intravascular volume retention would support hemodynamic function while minimizing post-resuscitation edema formation (32-34).

Importantly, vasopressin did not intensify bleeding from liver lacerations, which spontaneously ceased early into hemorrhagic shock (Figure 1C) regardless of the effect on blood pressure (Figure 3) and despite a prominent increase in blood flow (arterial) to the liver (Table 1A). Yet, we cannot exclude that reductions in portal blood flow (35) and other effects of vasopressin – i.e., enhanced platelet aggregation (36) and coagulation (37-39) – could have contributed to local hemostasis. Accordingly, bleeding from liver lacerations did not preclude the hemodynamic benefits elicited by vasopressin infusion. Yet, further work – preferable in large animal models – would be important to extend these observations to other organs. To this end, vasopressin was hemodynamically more effective than fluids in swine after incision across the mesenterial shaft (40). Thus, vasopressin infusion through vasoconstriction may reduce or at least not intensify bleeding (except from untreated large arterial lacerations) while achieving or exceeding blood pressure target currently set for fluid resuscitation (29,41,42). This effect of vasopressin could resolve the current therapeutic conflict when hemorrhagic shock coexists with traumatic brain injury (8) enabling increasing cerebral perfusion pressure without resorting to fluids.

The choice of intraosseous route for vasopressin infusion reflected our intent to prioritize the use of vasopressin over fluids upon recognition of severe hemorrhagic shock given its easier access and stability, reserving the intravenous route for subsequent fluid administration.

Normal Saline Effect

Use of normal saline for fluid resuscitation is debated. The Tactical Combat Casualty Care Guidelines recommend among crystalloids the use of balanced salt solutions arguing against use of normal saline (8). Normal saline – especially in large amounts – may be detrimental to the kidney through hyperchloremic acidosis and activation of inflammatory cascades (43,44). Yet, a recent study in critically ill patients showed no differences between normal saline and Plasma-Lyte 148 (45). The amount of fluids given in this study was modest and therefore strategies that restrict fluid resuscitation may ease concerns about adverse effects of normal saline. Despite the relatively small volume of normal saline given in the present study, it served – while on vasopressin – to improve total and organ blood flow and favor survival. Logistically, a restrictive fluids strategy would markedly facilitate the cost-effective development and use of fluids being developed with oxygen carrying capacity (46,47) and other useful hemostatic, metabolic, and hemodynamic features (8).

Limitations

The use of anesthesia could have masked physiologic adaptations to hypovolemic shock while protecting tissues from ischemic injury. The native neuropeptide in swine is lysine vasopressin whereas in humans it is arginine vasopressin. Thus, translation to humans might require consideration on the specific activity and corresponding pharmacological effect. In addition, the present experiments examined acute effects. It would be important to examine the effects on organ function and survival after closure of the abdomen and recovery from anesthesia.

Conclusions

The present study demonstrates a survival benefit elicited by early and sustained vasopressin infusion during severe hemorrhagic shock in swine. Vasopressin helped preserve or enhance perfusion to key organs despite increases in peripheral vascular resistance. Vasopressin did not inten-

sify bleeding from liver lacerations despite its pressor effect and helped retain intravascularly the bolus of normal saline administered. Accordingly, the present study supports vasopressin infusion for severe hemorrhagic shock given to rapidly achieve critical hemodynamic stability while enhancing the hemodynamic efficacy of restrictive fluid resuscitation until control of the source of bleeding is achieved.

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Non-Endorsement Disclaimer

The views, opinions and/or findings contained in this report are those of the authors and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

AUTHOR CONTRIBUTION

Raúl J. Gazmuri designed the study; analyzed and interpreted the data; prepared the figures and tables; and wrote the manuscript. Kasen Whitehouse trained in the liver laceration technique and contributed to the design of the study, analysis and interpreted the data, and to the draft of fig-

ures, tables, and the manuscript. Kruti Shah participated in the all the experiments and performed the data analysis related to the rate of blood loss. Karla Whittinghill assisted in the experiments and contributed to the data analysis. Alvin Baetiong participated in the experiments and conceptualized the approach to electronically monitor continuously the rate of blood loss. Jeejabai Radhakrishnan contributed with the analysis of the systemic metabolic effects and literature review. All authors read and approved the final manuscript.

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LEGENDS TO FIGURES

Figure 1: (**A**) Swine model of hemorrhagic shock induced by liver laceration depicting the steel clamp built to inflict the lacerations, the intraosseous (IO) access for vasopressin infusion, the ear vein access for fluid resuscitation, and the gravimetric approach to monitor the rate of bleeding; $P_{ET}CO_2$, end-tidal PCO_2; CO, cardiac output. (**B**-**D**) Temporal bleeding profiles showing the effect of liver lacerations, vasopressin infusion, and normal saline.

Figure 2: Kaplan-Meir curves showing the effects of (**A**) liver lacerations, (**B**) vasopressin infusion, (**C**) normal saline, and (**D**) combination of vasopressin infusion and normal saline on 240-minute survival. The *p*-values for survival – calculated using the log-rank test – are shown in each graph.

Figure 3: Graphs comparing the effects of vasopressin infusion and vehicle control on selected hemodynamic and metabolic variables. Differences were analyzed by a linear mixed effect model treating time as continuous variable to assess the main effects of time (min) and vasopressin infusion (VP) and their interaction (VP x min) and treating time as categorical variable to assess differences at specific fixed times (*$p \leq 0.05$, †$p \leq 0.01$; ‡$p \leq 0.001$). The data are shown as mean ± SEM. LL, liver lacerations; NS, normal saline; MAP; mean aortic pressure; SVRI, systemic vascular resistance index; LVSWI, left ventricular stroke work index; Ao, aortic; DO_2, systemic oxygen delivery index; VO_2, systemic oxygen consumption index; Hct, hematocrit.

Figure 4: Graphs comparing the effects of normal saline on selected hemodynamic and metabolic variables. Differences were analyzed by a linear mixed effect model treating time as continuous variable to assess the main effects of time (min) and normal saline (NS) and their interaction (NS x min) and treating time as categorical variable to assess differences at specific fixed times

(*$p \leq 0.05$, †$p \leq 0.01$; ‡$p \leq 0.001$). The data are shown as mean ± SEM. LL, liver lacerations; NS, normal saline; MAP; mean aortic pressure; SVRI, systemic vascular resistance index; LVSWI, left ventricular stroke work index; Ao, aortic; DO_2, systemic oxygen delivery index; VO_2, systemic oxygen consumption index; Hct, hematocrit.

Figure 5: Changes in plasma volume relative to the estimated plasma volume at minute 30; time at which normal saline (12 ml/kg) was started and given over 30 minutes in half of the pigs. Values are mean ± SEM. VP, vasopressin; NS, normal saline.

Table 1: Effects of vasopressin infusion and normal saline on blood flow distribution (percentage of baseline)

	Baseline	Hemorrhagic Shock			Main effect (*p*-value)		Interaction (*p*-value)
	-10 min	60 min	120 min	180 min	min	VP or NS	VP or NS x min
A. Effects of Vasopressin Infusion							
Total Flow Index							
No-Vasopressin	100 ± 0[20]	80 ± 33[16]	70 ± 28[13]	64 ± 26[8]	<0.001	0.248	0.073
Vasopressin	100 ± 0[20]	66 ± 29[20]	81 ± 31[15]	77 ± 25[13]			
Left Ventricle							
No-Vasopressin	100 ± 0	172 ± 85	168 ± 70	172 ± 49	<0.001	0.328	0.606
Vasopressin	100 ± 0	122 ± 71*	163 ± 68	177 ± 102			
Kidneys							
No-Vasopressin	100 ± 0	52 ± 27	37 ± 25	40 ± 26	<0.001	0.425	0.003
Vasopressin	100 ± 0	51 ± 31	64 ± 29†	69 ± 40†			
Adrenal Glands							
No-Vasopressin	100 ± 0	85 ± 31	109 ± 32	107 ± 44	0.717	0.683	0.845
Vasopressin	100 ± 0	57 ± 103	99 ± 178	114 ± 206			
Liver							
No-Vasopressin	100 ± 0	365 ± 286	271 ± 262	193 ± 107	0.024	0.935	0.618
Vasopressin	100 ± 0	353 ± 402	347 ± 320	283 ± 174			
Small Bowel							
No-Vasopressin	100 ± 0	95 ± 22	74 ± 31	76 ± 28	<0.001	0.185	0.017
Vasopressin	100 ± 0	77 ± 31*	85 ± 34	98 ± 40*			
Spleen							
No-Vasopressin	100 ± 0	69 ± 69	37 ± 30	36 ± 32	<0.001	0.317	0.080
Vasopressin	100 ± 0	44 ± 41	61 ± 68	60 ± 59			
Skeletal Muscle							
No-Vasopressin	100 ± 0	92 ± 47	86 ± 30	80 ± 21	0.002	0.277	0.588
Vasopressin	100 ± 0	72 ± 30*	84 ± 29	83 ± 30			
B. Effects of Normal Saline							
Total Flow Index							
No-Normal Saline	100 ± 0[20]	55 ± 22[18]	65 ± 24[11]	68 ± 17[7]	<0.001	0.106	0.260
Normal Saline	100 ± 0[20]	90 ± 30[18]‡	83 ± 32[17]*	74 ± 30[14]			
Left Ventricle							
No-Normal Saline	100 ± 0	116 ± 62	141 ± 62	175 ± 109	<0.001	0.220	0.743
Normal Saline	100 ± 0	173 ± 88†	181 ± 69	176 ± 74			
Kidneys							
No-Normal Saline	100 ± 0	40 ± 31	44 ± 29	61 ± 22	<0.001	0.399	0.417
Normal Saline	100 ± 0	63 ± 21†	57 ± 31	56 ± 44			
Adrenal Glands							
No-Normal Saline	100 ± 0	45 ± 31	73 ± 45	89 ± 55	0.943	0.663	0.127
Normal Saline	100 ± 0	94 ± 104	123 ± 162	123 ± 196			
Liver							
No-Normal Saline	100 ± 0	226 ± 241	230 ± 150	222 ± 76	0.032	0.155	0.920
Normal Saline	100 ± 0	490 ± 397‡	365 ± 349	261 ± 184			
Small Bowel							
No-Normal Saline	100 ± 0	75 ± 25	74 ± 23	88 ± 36	0.002	0.433	0.514
Normal Saline	100 ± 0	95 ± 28*	83 ± 38	90 ± 39			
Spleen							
No-Normal Saline	100 ± 0	30 ± 32	27 ± 17	29 ± 7	<0.001	0.283	0.045
Normal Saline	100 ± 0	82 ± 65‡	65 ± 64*	62 ± 60			
Skeletal Muscle							
No-Normal Saline	100 ± 0	63 ± 21	76 ± 25	78 ± 27	0.001	0.135	0.367
Normal Saline	100 ± 0	98 ± 45‡	91 ± 30	84 ± 27			

Numbers in brackets indicate animals alive at the specific time point. Values are mean ± SD. Differences were ana-

lyzed by a linear mixed effect model treating time as continuous variable to assess the main effects of time (min) and vasopressin infusion (VP) or normal saline (NS) and their interaction (VP x min or NS x min) and treating time as categorical variable to assess differences at specific fixed times (*$p \leq 0.05$, †$p \leq 0.01$; ‡$p \leq 0.001$).

Figure 1

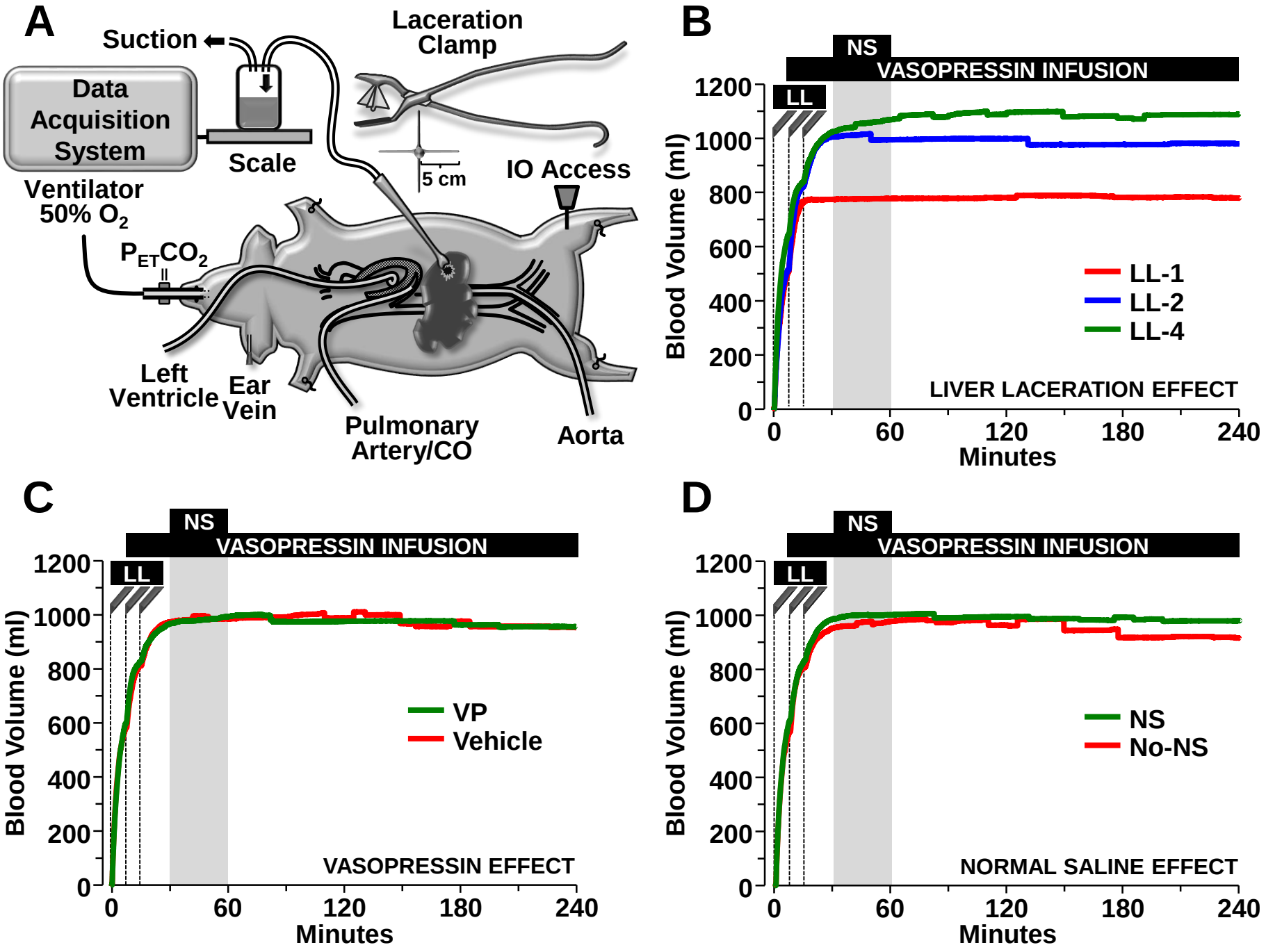

A
Suction
Laceration Clamp
Data Acquisition System
Scale
5 cm
IO Access
Ventilator 50% O_2
$P_{ET}CO_2$
Left Ventricle
Ear Vein
Pulmonary Artery/CO
Aorta
B
NS
VASOPRESSIN INFUSION
LL
Blood Volume (ml)
1200
1000
800
600
400
200
0
LL-1
LL-2
LL-4
LIVER LACERATION EFFECT
0
60
120
180
240
Minutes
C
NS
VASOPRESSIN INFUSION
LL
Blood Volume (ml)
VP
Vehicle
VASOPRESSIN EFFECT
Minutes
D
NS
VASOPRESSIN INFUSION
LL
Blood Volume (ml)
NS
No-NS
NORMAL SALINE EFFECT
Minutes

Figure 2

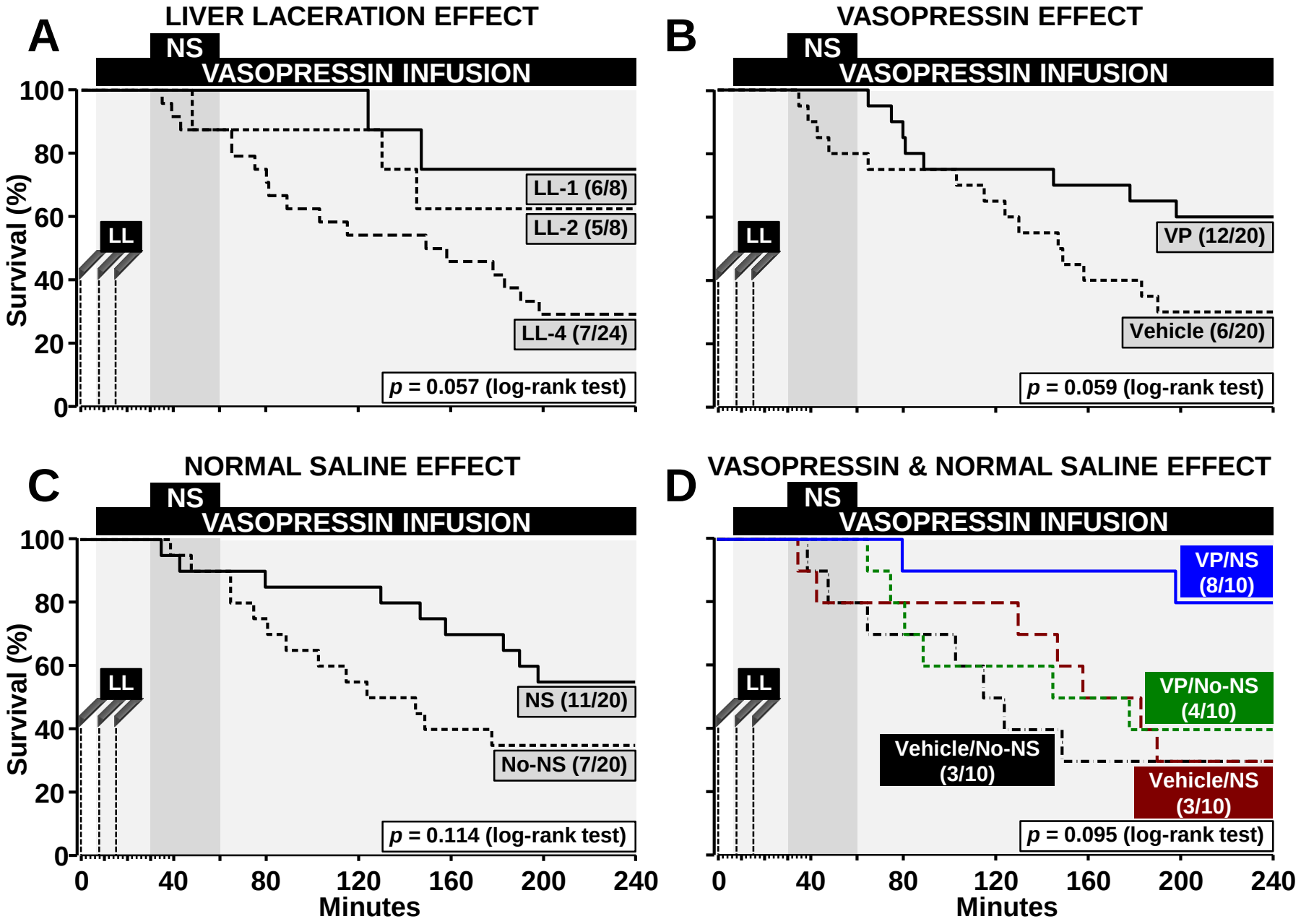

A
LIVER LACERATION EFFECT
NS
VASOPRESSIN INFUSION
LL
LL-1 (6/8)
LL-2 (5/8)
LL-4 (7/24)
p = 0.057 (log-rank test)
B
VASOPRESSIN EFFECT
NS
VASOPRESSIN INFUSION
LL
VP (12/20)
Vehicle (6/20)
p = 0.059 (log-rank test)
C
NORMAL SALINE EFFECT
NS
VASOPRESSIN INFUSION
LL
NS (11/20)
No-NS (7/20)
p = 0.114 (log-rank test)
D
VASOPRESSIN & NORMAL SALINE EFFECT
NS
VASOPRESSIN INFUSION
LL
VP/NS (8/10)
VP/No-NS (4/10)
Vehicle/No-NS (3/10)
Vehicle/NS (3/10)
p = 0.095 (log-rank test)
Survival (%)
0
20
40
60
80
100
0
40
80
120
160
200
240
Minutes

Figure 3

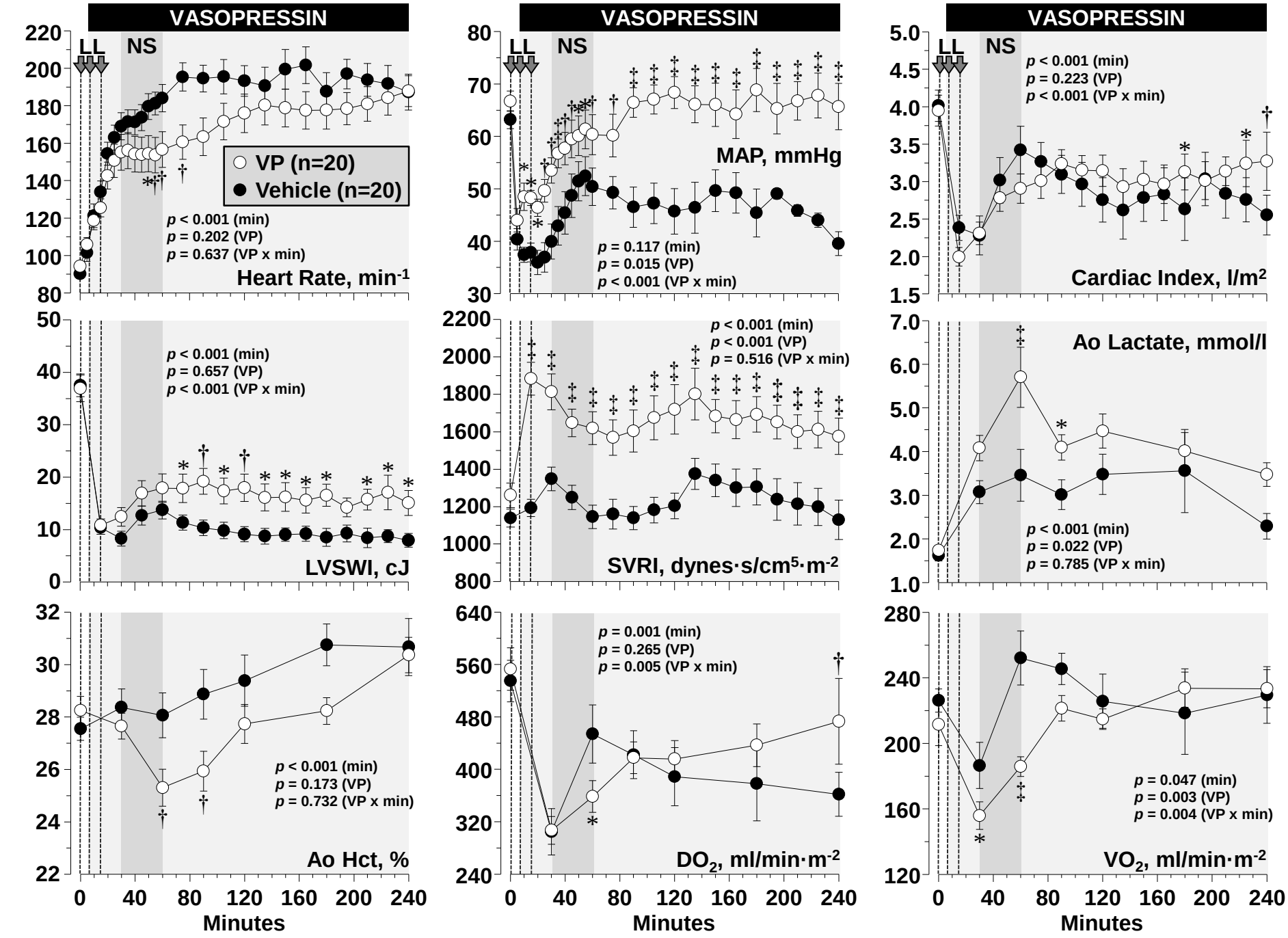

VASOPRESSIN
LL
NS
VP (n=20)
Vehicle (n=20)
p < 0.001 (min)
p = 0.202 (VP)
p = 0.637 (VP x min)
Heart Rate, min-1
p = 0.117 (min)
p = 0.015 (VP)
p < 0.001 (VP x min)
MAP, mmHg
p < 0.001 (min)
p = 0.223 (VP)
p < 0.001 (VP x min)
Cardiac Index, l/m2
p < 0.001 (min)
p = 0.657 (VP)
p < 0.001 (VP x min)
LVSWI, cJ
p < 0.001 (min)
p < 0.001 (VP)
p = 0.516 (VP x min)
SVRI, dynes·s/cm5·m-2
Ao Lactate, mmol/l
p < 0.001 (min)
p = 0.022 (VP)
p = 0.785 (VP x min)
p < 0.001 (min)
p = 0.173 (VP)
p = 0.732 (VP x min)
Ao Hct, %
p = 0.001 (min)
p = 0.265 (VP)
p = 0.005 (VP x min)
DO2, ml/min·m-2
p = 0.047 (min)
p = 0.003 (VP)
p = 0.004 (VP x min)
VO2, ml/min·m-2
Minutes
Minutes
Minutes

Figure 4

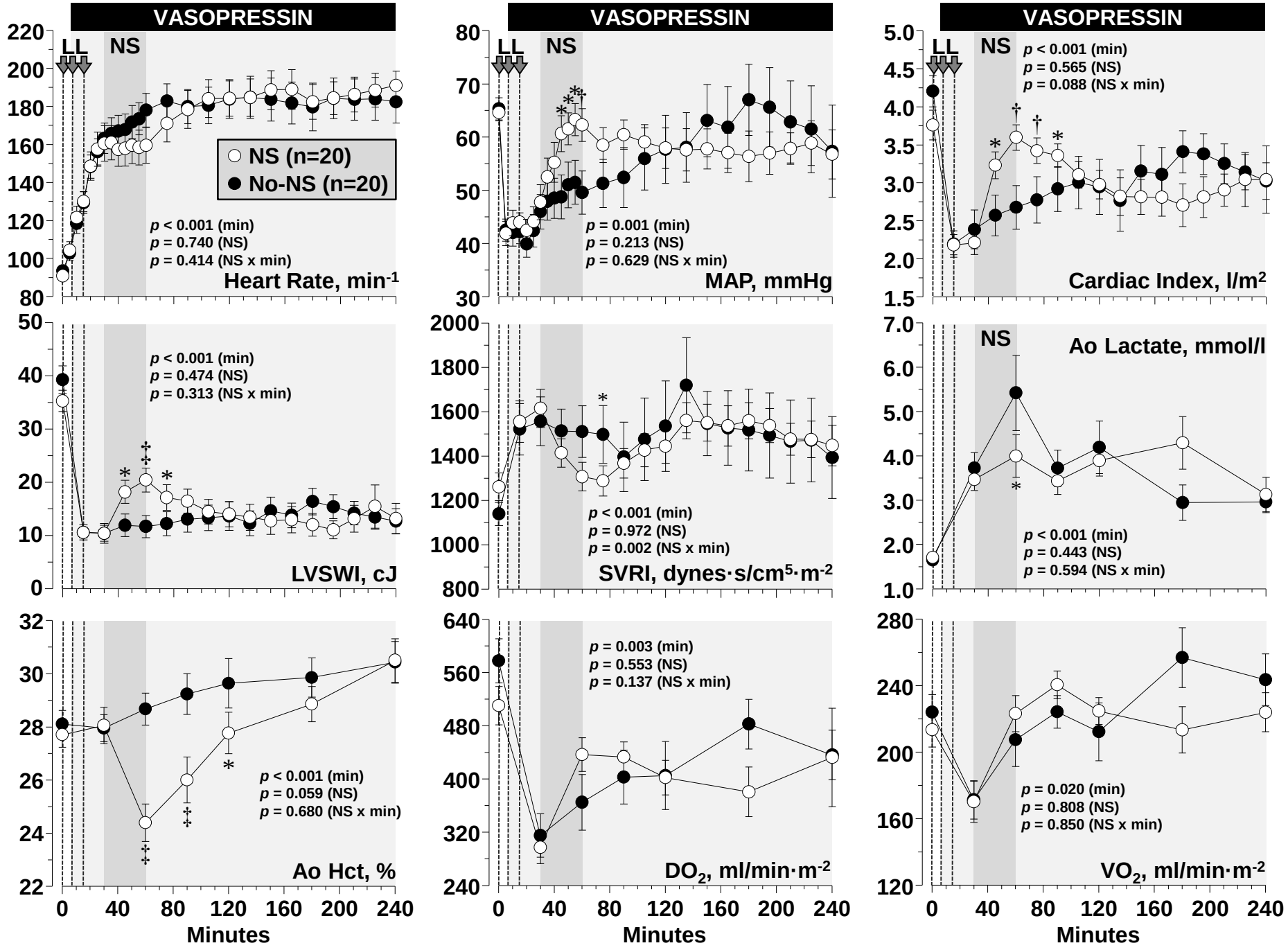
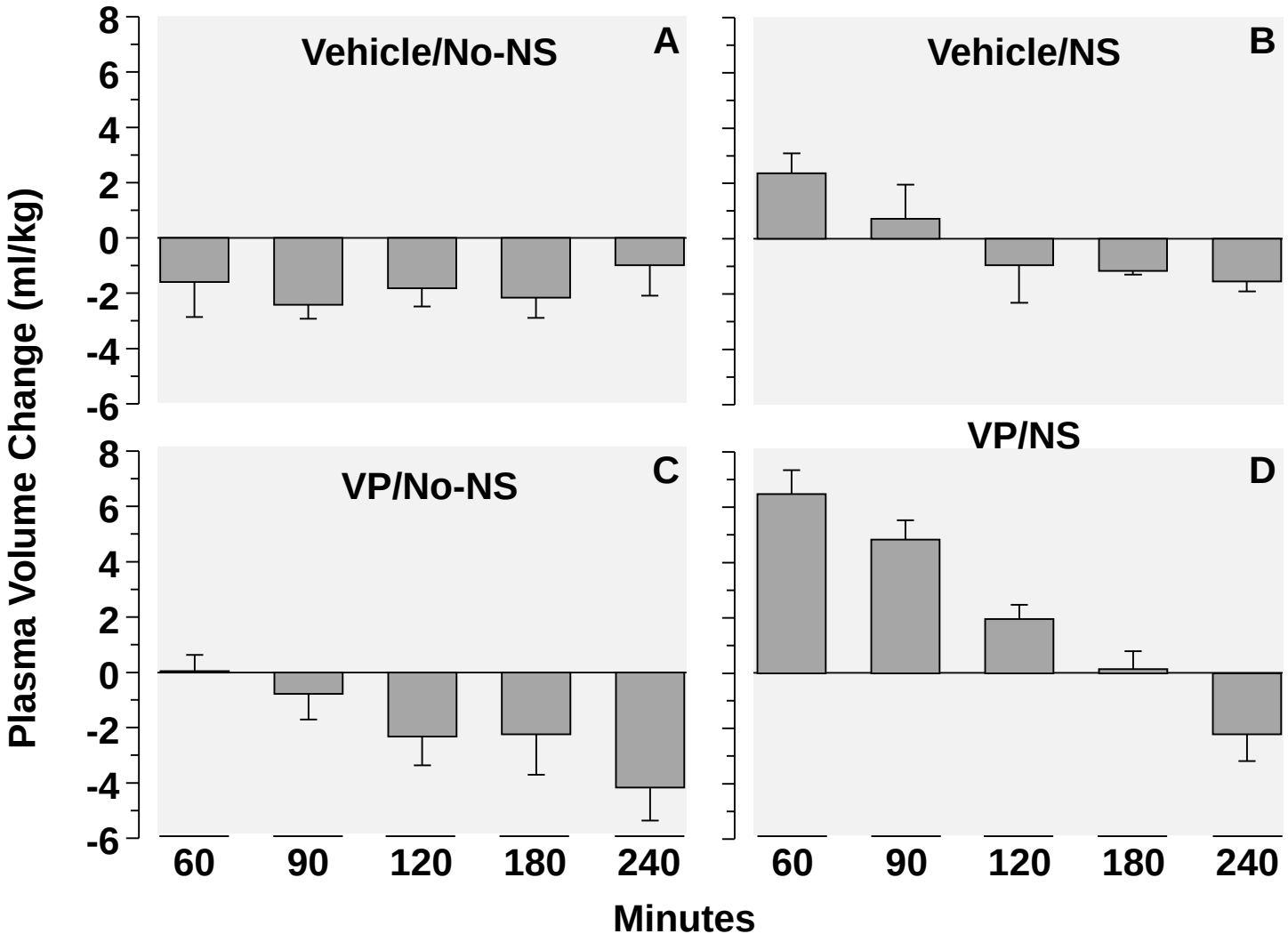

VASOPRESSIN
LL
NS
NS (n=20)
No-NS (n=20)
p < 0.001 (min)
p = 0.740 (NS)
p = 0.414 (NS x min)
Heart Rate, min-1
p = 0.001 (min)
p = 0.213 (NS)
p = 0.629 (NS x min)
MAP, mmHg
p < 0.001 (min)
p = 0.565 (NS)
p = 0.088 (NS x min)
Cardiac Index, l/m2
p < 0.001 (min)
p = 0.474 (NS)
p = 0.313 (NS x min)
LVSWI, cJ
p < 0.001 (min)
p = 0.972 (NS)
p = 0.002 (NS x min)
SVRI, dynes·s/cm5·m-2
Ao Lactate, mmol/l
p < 0.001 (min)
p = 0.443 (NS)
p = 0.594 (NS x min)
p < 0.001 (min)
p = 0.059 (NS)
p = 0.680 (NS x min)
Ao Hct, %
p = 0.003 (min)
p = 0.553 (NS)
p = 0.137 (NS x min)
DO2, ml/min·m-2
p = 0.020 (min)
p = 0.808 (NS)
p = 0.850 (NS x min)
VO2, ml/min·m-2
Minutes

Figure 5


Vehicle/No-NS
A
Vehicle/NS
B
VP/No-NS
C
VP/NS
D
Plasma Volume Change (ml/kg)
8
6
4
2
0
-2
-4
-6
60
90
120
180
240
Minutes

Supplemental Digital Content

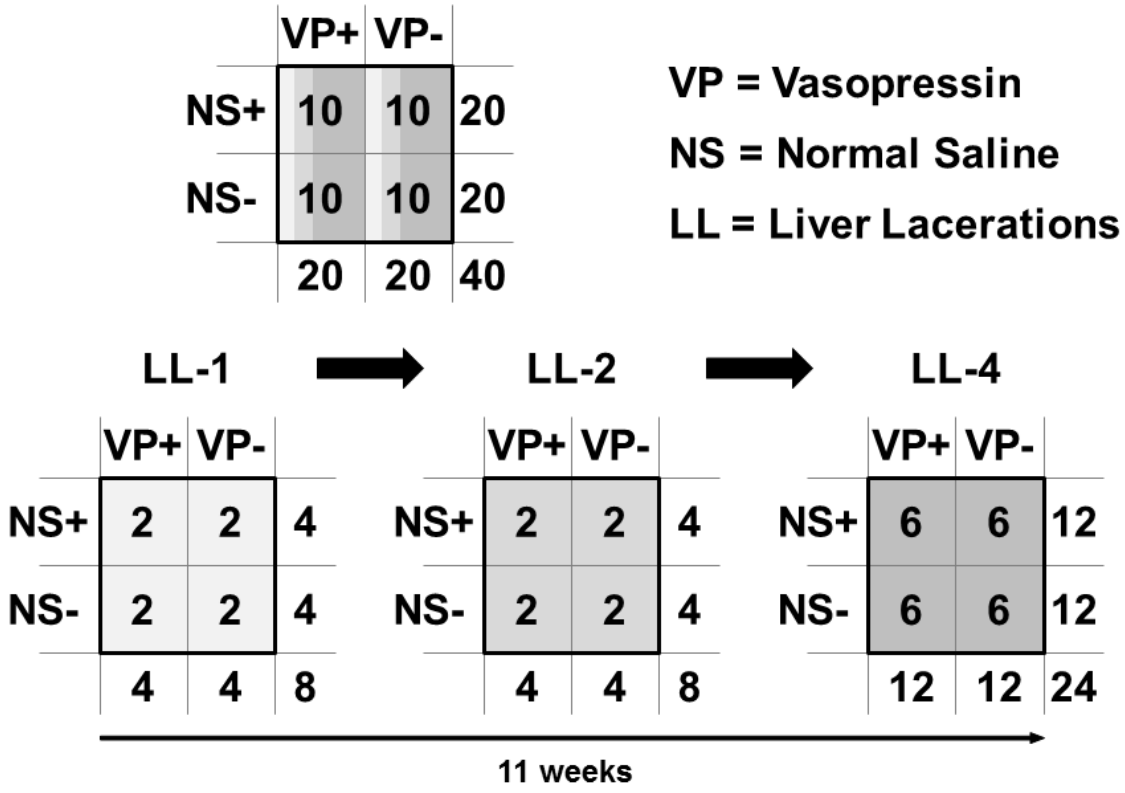


Figure S1: Study design showing the factorial distribution and block randomization in groups of 8, sequentially increasing the severity of hemorrhagic shock by additional liver lacerations yielding a balanced distribution to assess the effects of vasopressin infusion and small-volume fluid resuscitation (normal saline). The experiments were conducted over an interval of 11 weeks.

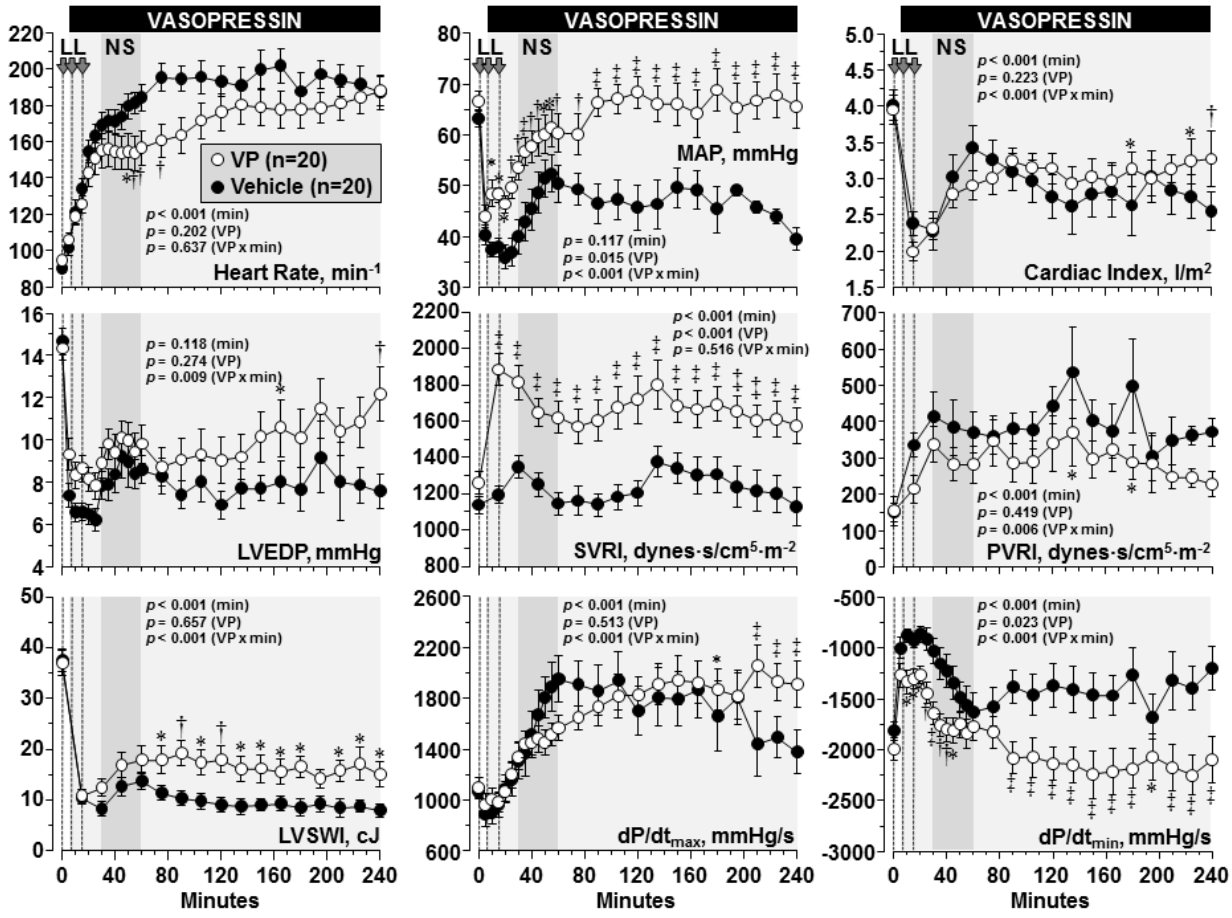


Figure S2: Graphs comparing the effects of vasopressin infusion and vehicle control on selected parameters of hemodynamic and cardiac function. Differences were analyzed by a linear mixed effect model treating time as continuous variable to assess the main effects of time (min) and vasopressin (VP) and their interaction (VP x min) and treating time as categorical variable to assess differences at specific fixed times (*$p \leq 0.05$, †$p \leq 0.01$; ‡$p \leq 0.001$). The data are shown as mean ± SEM. LL, liver lacerations; NS, normal saline; MAP; mean aortic pressure; LVEDP, left ventricular end-diastolic pressure; SVRI, systemic vascular resistance index; PVRI, pulmonary vascular resistance index; LVSWI, left ventricular stroke work index; dP/dt, rate of left ventricular pressure change.

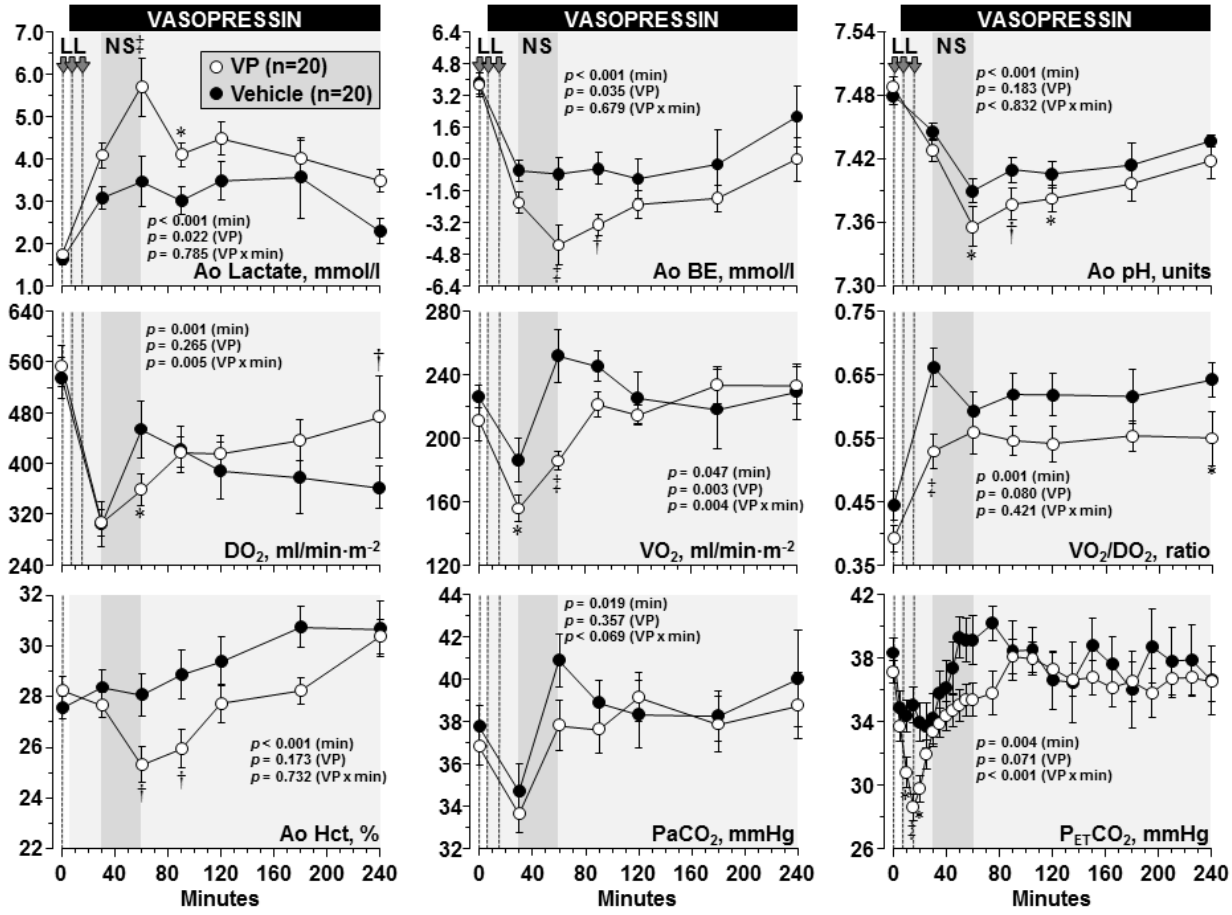


Figure S3: Graphs comparing the effects of vasopressin infusion and vehicle control on selected parameters of metabolic function. Differences were analyzed by a linear mixed effect model treating time as continuous variable to assess the main effects of time (min) and vasopressin (VP) and their interaction (VP x min) and treating time as categorical variable to assess differences at specific fixed times (*$p \leq 0.05$, †$p \leq 0.01$; ‡$p \leq 0.001$). The data are shown as mean ± SEM. LL, liver lacerations; NS, normal saline; Ao, aortic; BE; base excess, DO_2, systemic oxygen delivery index; VO_2, systemic oxygen consumption index; Hct, hematocrit, $PaCO_2$, arterial PCO_2; $P_{ET}CO_2$, end-tidal PCO_2.

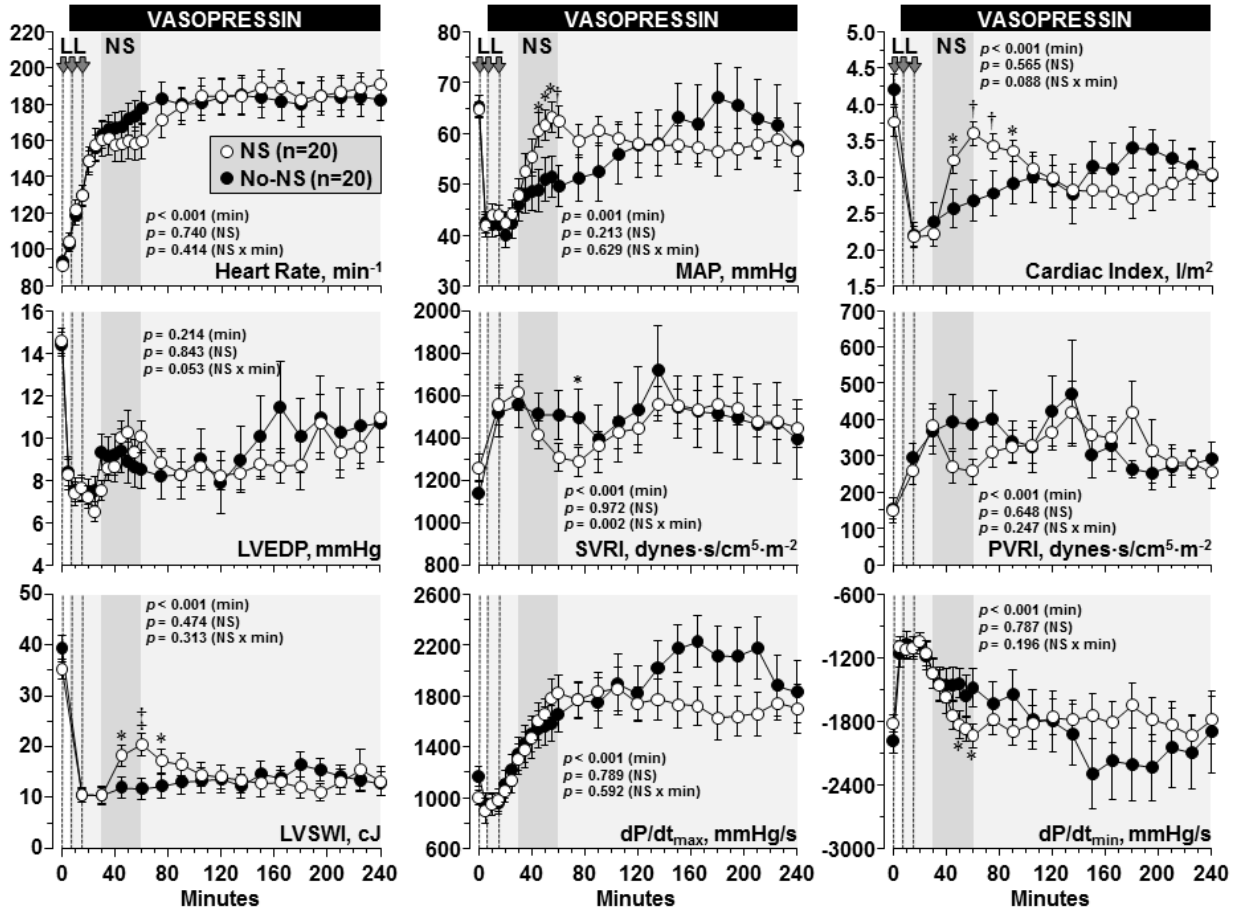


Figure S4: Graphs comparing the effects of normal saline or no fluids on selected parameters of hemodynamic and cardiac function. Differences were analyzed by a linear mixed effect model treating time as continuous variable to assess the main effects of time (min) and normal saline (NS) and their interaction (NS x min) and treating time as categorical variable to assess differences at specific fixed times (*$p \leq 0.05$, †$p \leq 0.01$; ‡$p \leq 0.001$). The data are shown as mean ± SEM. LL, liver lacerations; NS, normal saline; MAP; mean aortic pressure; LVEDP, left ventricular end-diastolic pressure; SVRI, systemic vascular resistance index; PVRI, pulmonary vascular resistance index; LVSWI, left ventricular stroke work index; dP/dt, rate of left ventricular pressure change.

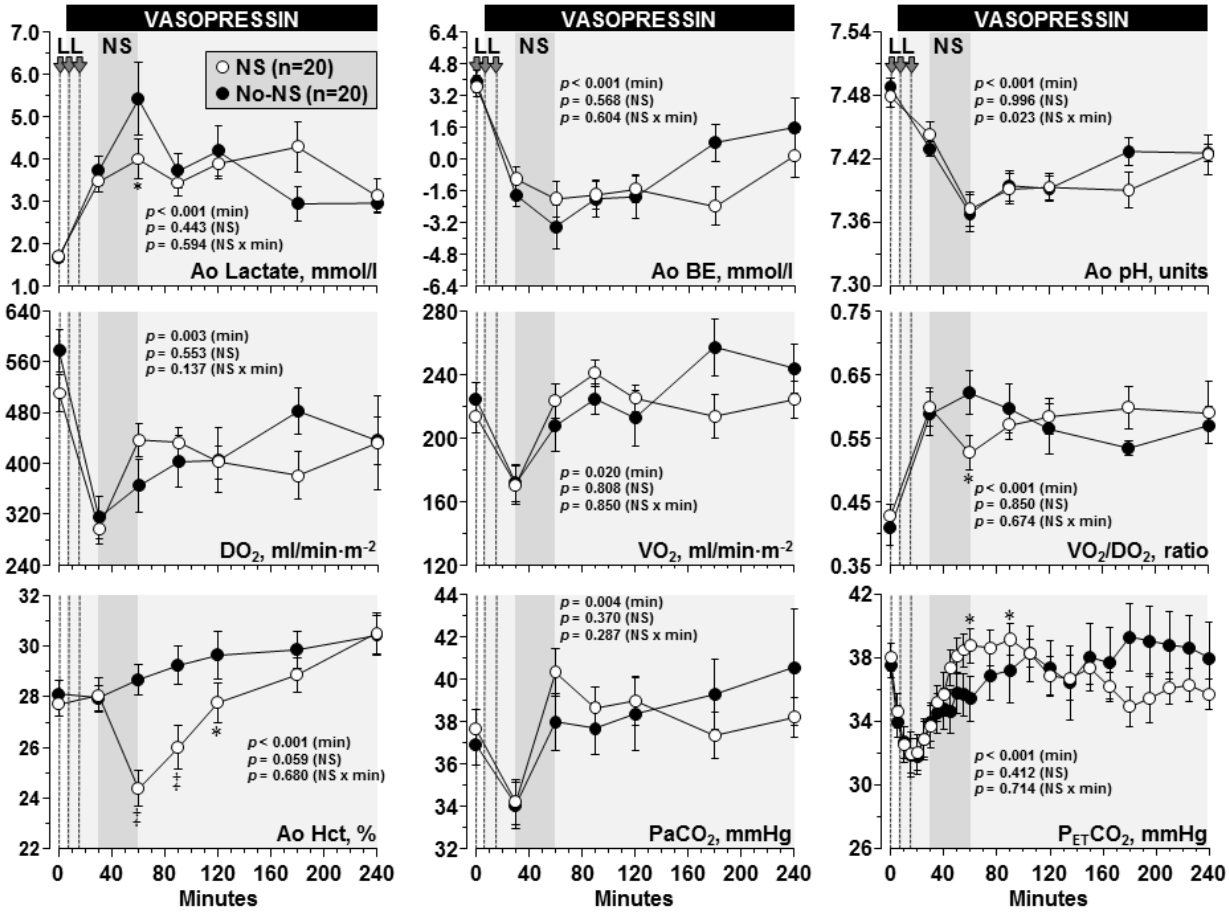


Figure S5: Graphs comparing the effects of normal saline or no fluids on selected metabolic parameters. Differences were analyzed by a linear mixed effect model treating time as continuous variable to assess the main effects of time (min) and normal saline (NS) and their interaction (NS x min) and treating time as categorical variable to assess differences at specific fixed times (*$p \leq 0.05$, †$p \leq 0.01$; ‡$p \leq 0.001$). The data are shown as mean ± SEM. LL, liver lacerations; NS, normal saline; Ao, aortic; BE; base excess, DO_2, systemic oxygen delivery index; VO_2, systemic oxygen consumption index; Hct, hematocrit, $PaCO_2$, arterial PCO_2; $P_{ET}CO_2$, end-tidal PCO_2.

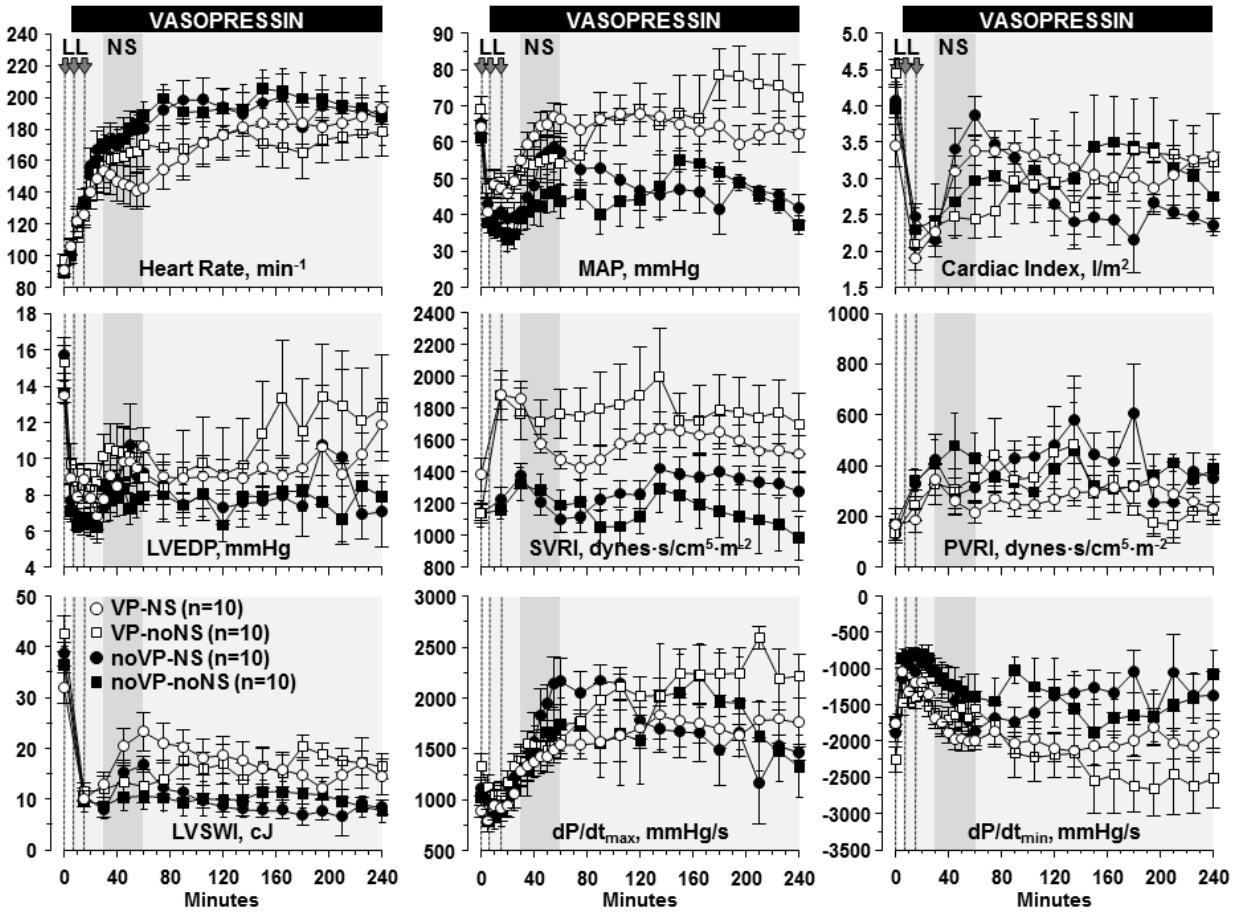


Figure S6: Graphs comparing hemodynamic and cardiac function among the four groups resulting from treatment with vasopressin infusion, normal saline, and their respective controls. The data are shown as mean ± SEM. LL, liver lacerations; NS, normal saline; MAP; mean aortic pressure; LVEDP, left ventricular end-diastolic pressure; SVRI, systemic vascular resistance index; PVRI, pulmonary vascular resistance index; LVSWI, left ventricular stroke work index; dP/dt, rate of left ventricular pressure change.

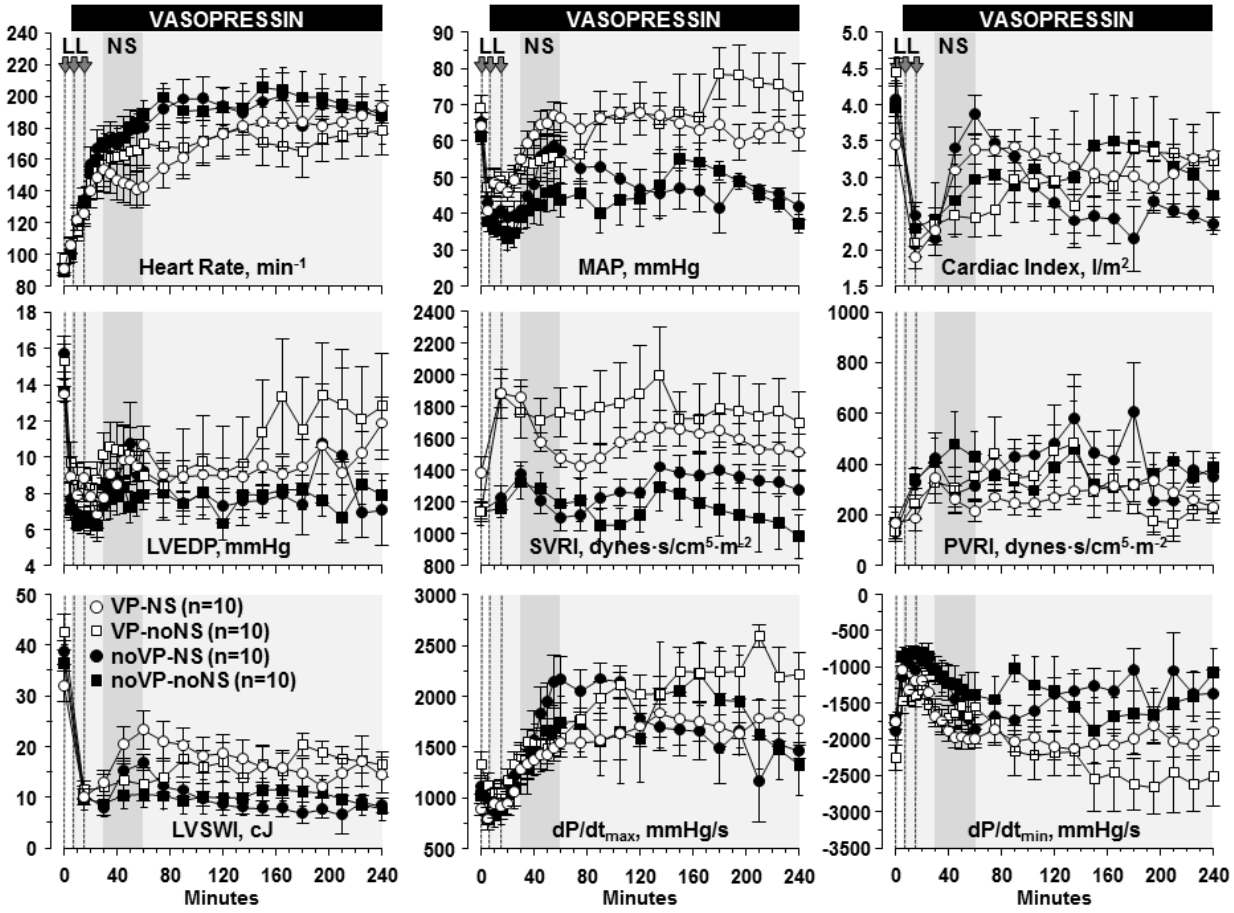


Figure S7: Graphs comparing selected metabolic parameters among the four groups resulting from treatment with vasopressin infusion, normal saline, and their respective controls. The data are shown as mean ± SEM. LL, liver lacerations; NS, normal saline; Ao, aortic; BE; base excess, DO_2, systemic oxygen delivery index; VO_2, systemic oxygen consumption index; Hct, hematocrit, $PaCO_2$, arterial PCO_2; $P_{ET}CO_2$, end-tidal PCO_2.

Table S1: Effect of vasopressin and normal saline on distribution of blood flow (percentage baseline)

	Baseline	Hemorrhagic Shock		
	-10 min	60 min	120 min	180 min
Total Flow Index				
NoVP–NoNS	100 ± 0[10]	65 ± 23[8]	65 ± 25[5]	73 ± 22[3]
NoVP-NS	100 ± 0[10]	96 ± 35[8]	74 ± 31[8]	58 ± 29[5]
VP-NoNS	100 ± 0[10]	47 ± 18[10]	66 ± 24[6]	65 ± 16[4]
VP-NS	100 ± 0[10]	85 ± 26[10]	91 ± 31[9]	82 ± 28[9]
Left Ventricle				
NoVP–NoNS	100 ± 0	137 ± 33	140 ± 54	196 ± 51
NoVP-NS	100 ± 0	208 ± 108	185 ± 76	158 ± 48
VP-NoNS	100 ± 0	99 ± 76	142 ± 72	159 ± 146
VP-NS	100 ± 0	145 ± 61	178 ± 66	185 ± 86
Kidneys				
NoVP–NoNS	100 ± 0	40 ± 25	33 ± 27	51 ± 18
NoVP-NS	100 ± 0	64 ± 24	40 ± 25	34 ± 30
VP-NoNS	100 ± 0	39 ± 37	53 ± 29	69 ± 24
VP-NS	100 ± 0	63 ± 21	72 ± 29	69 ± 47
Adrenal Glands				
NoVP–NoNS	100 ± 0	67 ± 28	97 ± 39	128 ± 55
NoVP-NS	100 ± 0	104 ± 22	116 ± 27	94 ± 37
VP-NoNS	100 ± 0	28 ± 21	53 ± 42	59 ± 36
VP-NS	100 ± 0	87 ± 142	129 ± 228	139 ± 247
Liver				
NoVP–NoNS	100 ± 0	343 ± 317	287 ± 195	254 ± 115
NoVP-NS	100 ± 0	386 ± 272	262 ± 310	156 ± 95
VP-NoNS	100 ± 0	133 ± 98	182 ± 91	199 ± 29
VP-NS	100 ± 0	573 ± 473	457 ± 374	320 ± 200
Small Bowel				
NoVP–NoNS	100 ± 0	93 ± 22	77 ± 22	93 ± 27
NoVP-NS	100 ± 0	96 ± 23	72 ± 37	65 ± 26
VP-NoNS	100 ± 0	60 ± 17	72 ± 26	84 ± 45
VP-NS	100 ± 0	94 ± 32	94 ± 38	104 ± 39
Skeletal Muscle				
NoVP–NoNS	100 ± 0	72 ± 20	81 ± 35	84 ± 10
NoVP-NS	100 ± 0	112 ± 58	89 ± 28	78 ± 26
VP-NoNS	100 ± 0	56 ± 21	72 ± 16	74 ± 36
VP-NS	100 ± 0	87 ± 30	92 ± 33	87 ± 29

Numbers in brackets indicate animals alive at the specific time point. Values are mean ± SD. VP, vasopressin; NS, normal saline.

SUPPLEMENT-METHODS

Fluid Retentive effect of Vasopressin

Simultaneous analysis of the four groups resulting from the factorial design revealed that the prominent reduction in hematocrit during vasopressin infusion following normal saline administration at minute 30 was limited to the 10 pigs that received normal saline while on vasopressin infusion. To further explore this effect we first calculated the percentage change in plasma volume relative to the estimated plasma volume at minute 30 according to PV_{change} (%) = ($Hct_{30min}/Hct_{>30min}$ – 1)/(1– Hct_{30min}/100) x 100 (1); where Hct_{30min} denotes the arterial hematocrit measured at minute 30 and $Hct_{>30min}$ denotes any of the arterial hematocrits measured at minutes 60, 120, 180, or 240 minutes. Next, the blood volume at minute 30 was estimated as the baseline blood volume (i.e., 65 ml/kg in swine) minus the total blood bled from the liver lacerations (given that by minute 30 the bleeding had largely ceased) and multiplied by the percentage change in plasma volume. The effect is shown in Figure 1D. In the absence of vasopressin or normal saline the plasma volume decreased by ~ 2 ml/kg and remained unchanged throughout hemorrhagic shock. Normal saline in the absence of vasopressin expanded the plasma volume by 2.4 ± 2.0 ml/kg at minute-60 corresponding to 20 % of the normal saline infused but the gain was rapidly lost ending with a negative plasma volume of 1.6 ± 1.0 ml/kg by minute-240. However, normal saline in the presence of vasopressin infusion expanded the plasma volume by 6.5 ± 2.7 ml/kg at minute-60 corresponding to 54 % of the volume infused and the effect lasted longer; yet, not preventing plasma volume loss of -2.2 ± 3.0 ml/kg towards the end of hemorrhagic shock. Vasopressin infusion in the absence of normal saline had no initial effect on plasma volume and did not prevent late reduction in plasma volume of -4.2 ± 3.8 ml/kg.

REFERENCE

1. Nygren A, Redfors B, Thoren A, Ricksten SE. Norepinephrine causes a pressure-dependent plasma volume decrease in clinical vasodilatory shock. *Acta Anaesthesiol Scand* 2010;54:814-820.