Splenectomy Prevents Death from Decompensation in Hemorrhagic Shock in Swine

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Abstract

Controversy exists on whether to perform a splenectomy in large animal models of hemorrhagic shock, as hemorrhage-induced splenic contraction returns erythrocytes into circulation. However, existing studies have not examined splenectomy in otherwise lethal models. We hypothesized that a failure to remain constricted may cause the spleen to act as a volume sink, increasing early mortality. We performed splenectomy in 9 swine (NoSpl) and subjected them to polytrauma and hemorrhage (1.5h shock, 12.5h resuscitation/observation) similar to a historical group of 14 swine (Spl). Survival was significantly improved to 89% (versus 43% in the Spl group, p=0.02) and the need for fluids was significantly slowed in the NoSpl group. Rapid crystalloid resuscitation in Spl animals caused erythrocyte, platelet, and plasma protein dilution that reverted in under 2h, suggesting the added fluid extravasated. Total oxygen delivery always exceeded consumption in both groups, suggesting oxygen was not a limiting factor. However, the hind-limb oxygen extraction ratio was higher in the Spl group and plasma glucose dropped significantly in the Spl but not the NoSpl group, suggesting a potential hyper-metabolic state in some tissues in the Spl group. Plasma sodium and potassium were also more disrupted in the Spl group. Inflammatory cytokines and leukocytes increased earlier in the NoSpl animals but were more resolved by 14h. Thromboelastography was similar between groups. Our findings identify the spleen as a weak point in compensation and support no splenectomy when studying early mortality from decompensation and splenectomy when studying late mortality from organ injury.

Introduction

In recent conflicts, 90% of deaths from combat-related potentially survivable injuries were the result of hemorrhage or its sequelae (1). Preventing death from hemorrhage requires overcoming three hurdles, you must: 1) stop the bleeding, 2) compensate for the hypovolemia, and 3) keep the organ systems from failing. Generally, they need to be addressed in that order, i.e., if hemorrhage is not stopped, no amount of cardiovascular compensation is sufficient. Likewise, decompensation may be fatal long before organ dysfunction develops or progresses to organ failure (2, 3). These challenges involve different mechanisms, which means a treatment designed to prevent organ damage may not affect decompensation. If care is not taken, decompensatory deaths may be misinterpreted as failure of treatment, rather than a failure to properly test the treatment.

We recently encountered this difficulty in a 15-hour swine hemorrhage + polytrauma protocol (not yet published). Despite hemorrhage sufficient to cause greater than 50% mortality, both survivors and non-survivors in the untreated group had low levels of histological organ damage, making it difficult to determine if the study drug could reduce organ injury. Increasing shock severity to increase organ damage would likely only further increase mortality from decompensation, making the study impossible. We needed a method to reduce or prevent decompensation that would not invalidate the model for the purposes of pre-hospital combat casualty care.

We looked to the spleen for a solution. It is well known that the spleen filters out older, less flexible erythrocytes for controlled destruction and that swine and canine spleens are contractile. During a stressed state, sympathetic stimulation triggers constriction, forcing sequestered red blood cells back into circulation to support blood volume and oxygen transport

(4-6). The human spleen was not originally believed to have this capability (7, 8), but recent studies have shown that human spleens constrict, though their smaller volume (4, 9) and typically shorter duration of contraction make it difficult to monitor (10-12). The question of whether or not to include splenectomy in large animal models of shock has been controversial for years. Most studies provide no clear rationale for it and there is "little evidence in the literature to support or refute the need to remove the spleen in acute porcine hemorrhagic shock models" (4). Some include splenectomy to remove variability caused by inconsistent degrees of splenic contraction in response to hemorrhage (13, 14), while others argue leaving the spleen in brings the hematocrit closer to human, improving applicability. One group compromised by spraying the exterior of the spleen with adrenaline to induce maximal contraction prior to excision (oral communication). This focus on oxygen carrying capacity, however, means the effect of splenic contraction on vascular volume has been overlooked.

Vascular volume is a key determinant of hemodynamics, because, regardless of cardiac performance, cardiac output is *bound* by venous return. The pressure driving venous return comes from the elastic recoil of stretched blood vessels, i.e., from the *stressed volume* (15). Stressed volume is defined as the volume of blood in excess of *unstressed volume* (the volume needed to fill the vasculature without stretching it). Without stressed volume, there is no cardiac output. The threshold between unstressed and stressed volume is dependent on the degree to which the vasculature is constricted. In response to hemorrhage, vessels constrict, transforming more of the total remaining volume into stressed volume, maintaining venous return (15). Constriction of the spleen has the same effect, compensating for hypovolemia (not just hypoxia). However, after severe hemorrhage when the body has reached its compensatory limits, it must maintain that splenic contraction or risk transforming the remaining stressed volume into

unstressed volume, causing venous return/cardiac output to plummet, potentially faster than can be corrected with exogenous resuscitation fluid.

Likewise, resuscitation only improves hemodynamics if it increases stressed volume. Added fluid expands the part of the vasculature with the least resistance, i.e., arteries constrict with more force than veins, so generally venous volume will increase before arterial volume in response to fluid (one reason resuscitation does not always improve functional capillary density). The spleen may represent another "weak" spot, such that some of the early resuscitation goes to refilling it. If the spleen decreases its constriction in response to resuscitation instead of resisting it, the increased volume in the spleen will be unstressed and will not increase blood pressure, even with an ideal fluid like fresh whole blood. If the fluid used is a crystalloid, as is often the case in resource-poor conditions like combat casualty care, it may even contribute to later complications. Crystalloid dilution of plasma lowers oncotic pressure, resulting in the fluid leaking out as edema. It is not known if the vasculature or spleen can re-constrict as easily after this fluid escapes. While crystalloids buy time, barring additional resuscitation, they may leave the individual in a worse condition than before: hypovolemic *and* edematous.

Our hypothesis is that the spleen represents both an initial volume source and a subsequent volume sink, the latter increasing decompensation and reducing the effectiveness of resuscitation. The objective of this study was to determine if splenectomy improves the ability of swine to maintain a compensatory state after polytrauma, hemorrhage, and resuscitation, increasing short-term (<14 h) survival and reducing need for resuscitation volume.

Methods

Swine model

Research was conducted in compliance with the Animal Welfare Act, the implementing Animal Welfare Regulations, and the principles of the Guide for the Care and Use of Laboratory Animals, National Research Council. The facility's Institutional Animal Care and Use Committee approved all research conducted in this study. The facility where this research was conducted is fully accredited by AAALAC. The model is a modification of an existing polytrauma model incorporating a blunt chest trauma, a penetrating liver injury (uncontrolled hemorrhage), and controlled hemorrhage (16). Because this study was undertaken to improve the animal model for future studies there are some additional procedural differences, detailed below and summarized in **Figure 1**, between the splenectomy group (NoSpl, N=9) and historical group with the spleen left in (Spl, N=14).

Preparation

Briefly, 40±5 kg male Yorkshire crossbred swine were anesthetized using atropine (0.04-0.4 mg/kg) and Telazol (6-8 mg/kg) before transport to the procedural room. An ear vein catheter was placed upon arrival, followed by endotracheal intubation. Swine were maintained on isoflurane (1.0-3.0%) during instrumentation via anesthesia machine and automatic ventilator (Draeger Medical Apollo Gas system and Infinity Explorer Monitoring System, Telford, PA). Ventilation was initially set to a tidal volume of 10 ml/kg body weight, 20 breaths per minute, and peak pressure at 20 cm H_2O. Settings were adjusted, as needed, to maintain end tidal pCO_2 around 40 mmHg. In the NoSpl group, fraction of inspired oxygen (FiO_2) was kept between 0.21 and 0.3 to simulate an austere environment where oxygen may not be available. FiO_2 was not as tightly controlled in the Spl group and may have been as high as 0.4. Cut downs with blunt dissections were performed to isolate the carotid artery and jugular vein. A pressure transducer-

tipped catheter (3.5Fr. Mikro-Tip, Millar Instruments, Inc., Houston, TX) was placed non-occlusively into the carotid artery for blood pressure monitoring. A Swan Ganz catheter (8Fr., 110cm, Q-Tip, heparin Coated; ICU Medical, San Clemente, CA) was advanced into the pulmonary artery via the jugular vein for measurement of continuous cardiac output via thermodilution (Q2 Plus SO_2Continuous Cardiac Output Computer, ICU Medical, San Clemente, CA). An additional cut down with blunt dissection was performed for isolation and cannulation of the femoral artery and vein (8 Fr side port/percutaneous catheter inducers; Argon Medical Devices, Athens, TX) for arterial hemorrhage, venous and arterial blood sampling, and intravenous infusion of resuscitation fluid. A second pressure transducer-tipped catheter was inserted through the hind port of the arterial catheter for blood pressure monitoring. Hemodynamic and cardiac data were collected utilizing the data acquisition instrumentation rack/Biomedical Data Recorder (DAQ) and Physiological Data Recorder program (Dynamic Research Evaluation Workstation- DREW, US Army Institute of Surgical Research, San Antonio, TX). Once lines were placed, a midline laparotomy was conducted for cystotomy and urine collection via catheter. In NoSpl animals, the spleen was then isolated and vessels ligated using two umbilical cord tapes per blood vessel along with two sutures before cutting and removal of the spleen.

Prior to obtaining baseline readings, swine were transitioned from isoflurane to intravenous anesthesia through the ear vein catheter. The Spl group was given an initial bolus of sufentanil (2.5 µg/kg) and midazolam (0.1 mg/kg) followed by a continuous rate infusion (CRI) of sufentanil (1.0-20.0 µg/kg/h) and midazolam (0.07-0.15 mg/kg/h). However, due to a shortage in sufentanil supply, the NoSpl group was kept at a similar anesthetic depth with propofol (3-12 mg/kg/h) and buprenorphine (2-8 ug/kg/h) in place of sufentanil and midazolam. Isoflurane was

then reduced to reach a minimum alveolar concentration (MAC) ratio between 0.2 - 0.8. After a stabilization period of at least 10 minutes to adjust to the change in anesthetic, the baseline (BL) blood sample was drawn.

Poly-trauma Injury

To simulate a penetrating abdominal trauma, the left medial hepatic lobe of the liver was isolated, two lap sponges were placed (one below and one above) to capture any un-suctioned blood from the hemorrhage, and, using a custom scalpel, two cross-shaped puncture wounds (each of the 4 arms was 1" long) were made on the midline, 12 cm and 8 cm from the tip of the lobe. These wounds were allowed to bleed freely for 60 seconds. The uncontrolled hemorrhage volume was continuously quantified by suctioning shed blood into a canister on a balance. The two lap sponges were removed for weighing (to determine total uncontrolled hemorrhage), followed by immediate packing of the wound with five new lap sponges.

To create the lung contusion injury, we fired a captive bolt against a steel plate (3-inch by 5-inch, 1 cm thick, with 1 cm thick foam padding) to distribute the force of the injury. The plate was held against the ribs below the right front leg using a strap around the torso at consistent tension (15 lbs) to improve reproducibility. The animals were removed from the ventilator for a few seconds at the time of the lung injury to avoid tension pneumothorax injuries.

Immediately following completion of the liver and lung injuries, we began controlled hemorrhage (100 ml/min) via the femoral artery. Blood was drawn until the mean arterial pressure (MAP) reached 30 mmHg. This signaled the start of shock (T = 0 h) and the drawing of the B0 blood sample. We then maintained a MAP of 30 ± 5 mmHg, turning the pump on and off as needed, until the full controlled hemorrhage was drawn. For the Spl animals, the controlled

hemorrhage was 24 ml/kg body weight. However, since the uncontrolled hemorrhage was variable (3.1 ± 1.6 ml/kg), this led to inter-animal variations in the total hemorrhage. To improve the model going forward, the volume removed in controlled hemorrhage for the NoSpl animals was calculated so that the total hemorrhage (controlled + uncontrolled) would equal 24 mL/kg. With regards to total loss of volume, the two groups remained essentially equivalent, with the known 3.1-ml/kg greater hemorrhage in the Spl group offset by ~4.5 ml/kg of blood volume (estimate of splenic blood volume (17)) removed with the excision of the spleen in the NoSpl group.

After completion of hemorrhage, no additional blood was removed or fluid given until resuscitation at T = 1.5 h with up to 3x the shed blood volume of Lactated Ringer's (LR) Fluid. Resuscitation started with a 60 ml bolus of LR given over one minute, after which swine were resuscitated at 1.5 ml/kg/min as needed to maintain MAP at 65 ± 5 mmHg. While NoSpl animals were allowed resuscitation fluids until the end at T = 14 h, in the original protocol (Spl group), resuscitation was halted at T = 2.5 h. However, this restriction only caused a slight reduction in the volume given to two animals (see Results), so overall resuscitation volume remained consistent between groups. After resuscitation, only maintenance fluids (LR) were given (0.5 ml/kg/h) for the remainder of the protocol. After collection of the final blood sample (see below), animals were euthanized with sodium pentobarbital (Euthasol; 120 mg/kg).

Differences between Spl and NoSpl group

In addition to those mentioned above, there were differences between the NoSpl and Spl procedures with respect to the order of injuries and the time of blood draws (**Figure 1**). In the Spl group the lung injury occurred prior to laparotomy. To reduce the amount of time between

the start and completion of injuries going forward, the lung injury was moved to after the liver injury in the NoSpl group. For the Spl group, 13 ml blood samples were taken at baseline, at T = 0, 1.5, and at the end of resuscitation (~2.5 h), then hourly from that through T = ~13.5 h. The final blood draw, however, was taken 15 h after the lung injury (placing it at T = 14.4 h on average). To reduce temporal variability, for this study (NoSpl), the blood samples were taken at baseline, at T = 0, 1.5, and 2 h, then hourly through the final sample at T = 14 h. Due to the slight offset for the blood samples taken after the start of resuscitation, NoSpl samples are compared to the following Spl sample (e.g., the B10 sample was at 10 h in NoSpl vs ~10.5 h in Spl).

Blood sample analysis

Complete blood cell count (CBC), blood gases, and electrolytes were determined immediately after blood draw by HemaTrue hematology analyzer (Heska Corp. Loveland, CO) and ABL 800 Flex blood analyzer (Radiometer, Brea, CA). Thromboelastography was performed within an hour of blood draw with a Haemoscope 5000 (Haemonetics Corp., Braintree, MA). For each time point a 5 mL blood aliquot was centrifuged (15 minutes, 15,000 rcf) and the plasma aliquoted and frozen (-80 ºC) for later analysis. For cytokine and chemokine expression analysis, we used the MAGPIX System (Luminex, Austin, TX) with a Milliplex MAP Kit Porcine Cytokine/Chemokine Magnetic Bead Panel (PCYTMAG-23K, Millipore Sigma, Burlington, MA) per manufacturer instructions.

Oxygen delivery (DO_2 in L of O_2 per min) and oxygen extraction (VO_2 in L of O_2 per min) were calculated using the arterial and venous oxygen concentrations (c_AO_2 and c_VO_2 in mL O_2 per dL blood) and cardiac output (CO in L/min) using the equations $DO_2 = CO \times c_AO_2 \times 0.01$ and $VO_2 = CO \times (c_AO_2 - c_VO_2) \times 0.01$. Oxygen concentrations were calculated from the

measured total hemoglobin (tHb in g/dL), oxygen saturation (sO_2 in %), and partial pressure of oxygen (PO_2 in mmHg) using the equation $cO_2 = (tHb \times 1.34 \times sO_2) + (PO_2 \times 0.003)$. For the whole-body VO_2 calculation, we used the sO_2 of the mixed venous blood in the pulmonary artery measured by the Swan-Ganz catheter. Oxygen extraction ratio (OER) for the hind-limb was calculated using the equation $OER = (c_AO_2 - c_VO_2)/ c_AO_2$ where c_AO_2 and c_VO_2 were from the femoral artery and vein, respectively.

Data Analysis

Data is expressed as Mean ± Standard Deviation. Continuous measures were analyzed by Student's t-test. Kaplan-Meier curves were analyzed by log-rank test. Significance was set to $p < 0.05$. Microsoft Excel was used for statistical analysis.

Results

Survival, shock severity, and hemodynamics

Though the total blood loss between groups was similar, the amount of controlled hemorrhage required to initially reduce MAP to 30 mmHg was 9.9±2.7 ml/kg in the Spl group as compared to 14.0±5.2 ml/kg in the NoSpl group ($p = 0.05$). Survival was significantly greater in the NoSpl group than in the Spl group (89% vs. 43%, $p = 0.02$) (**Figure 2**). Lactate and base excess were similar between groups at all time points in the experiment (**Figure 3A and B**). Similarly, there were no differences in venous pH, except where it dropped more in the Spl group at B0, which may reflect the greater time since lung injury in the Spl group (**Figure 3C**). Hemodynamically, mean arterial pressure (MAP) was significantly elevated in the NoSpl vs the

Spl group for the first 6 h post-injury, particularly at the end of shock (T = 1.5 h) (**Figure 4A**). Breaking MAP into its components of diastolic arterial pressure (DAP) and pulse pressure (PP), we observed DAP in the NoSpl group remained significantly elevated through T = 10 h (**Figure 4B**). While PP in the Spl group was similar to NoSpl early on (except at the end of shock), it increased to the point it was significantly greater than NoSpl PP (**Figure 4C**). By T = 14 h, Spl PP was approximately double its baseline value. There was an early trend for elevated heart rate (HR) in the NoSpl group (significant at the end of hemorrhage), but HR was equivalent between the two groups from the end of shock through the rest of the study (**Figure 4D**).

Fluids and volume effects

The effect of splenectomy on fluid resuscitation was profound. Though Spl animals were only resuscitated for 1 h, all but two animals reached the maximum 72 ml/kg of LR (and those two reached 67 and 68 ml/kg), indicating pressure stopped increasing with fluids (**Figure 5A**). In contrast, only the sole non-survivor in the NoSpl group reached the maximum resuscitation in under an hour of resuscitation. Most of the NoSpl animals needed little fluid to maintain their MAP above 60 mmHg. As a result, even by T = 5 h, only a third of NoSpl animals had reached maximum LR.

Splenic ejection of red blood cells (RBCs) in response to hemorrhage in the Spl group manifested as a significant increase in the circulating RBC concentration at B1 (**Figure 5B**). LR resuscitation caused a massive dilution of blood, such that between B1 and B2, RBC concentration went from being significantly elevated in the Spl group to being significantly lowered relative to the NoSpl group. This change was short-lived, however, and the RBC concentration began to rise again until RBC concentration was again significantly greater than

that of the NoSpl group. After B4, there was a slow downward trend in RBC concentration in both groups, but it remained higher by a consistent and significant amount in the Spl group. Since the resuscitation fluid lacked protein, a similar dilution response can be seen with total protein (except there was no corresponding influx of protein from the spleen) (**Figure 5C**). The differential response to fluid resuscitation was also apparent when we examined cardiac output (CO). During shock, CO was significantly higher in the NoSpl group (**Figure 5D**). Within 30 min of the start of resuscitation, NoSpl CO returned to baseline, whereas the Spl CO overshot baseline to become briefly significantly elevated compared to NoSpl.

Oxygen dynamics

Our direct measurement of CO, hemoglobin, and arterial and venous blood gases enabled us to calculate oxygen transport. There was a small but significant difference between groups in arterial oxygen saturation at the femoral artery that started with baseline and was present throughout (excepting the large variation at B8 from an animal 5 minutes from death) (**Figure 6A**). In contrast to arterial saturation, venous oxygen saturation at the femoral vein varied considerably over the course of the experiment. It started the same at baseline in both groups and plummeted during shock, though to a lesser extent in the NoSpl group, perhaps reflecting the greater CO in that group (longer RBC transit times allow more off-loading of oxygen). However, despite equivalent CO from B3 onward, femoral vein sO_2 remained elevated in the NoSpl group through B6, suggesting additional factors. The resulting oxygen extraction ratio for the hind limb was significantly higher or trended higher in the Spl group from B0 through B8 (**Figure 6B**). Oxygen delivery, DO_2, for the body as a whole was similar between groups through the end of shock at B1, when delivery fell to approximately half of baseline (**Figure 6C**). The Spl group

DO_2 returned to baseline levels at B2 with resuscitation, but levels rapidly rejoined those of the NoSpl group, which never recovered to baseline despite better survival. VO_2 never dropped below baseline in either group (**Figure 6D**). Aside from a brief increase at B0, the NoSpl animals saw no real change in their VO_2. Spl VO_2 had the increase at B0, but also showed a tendency to be increased after resuscitation that approached significance at B2 and B4 ($p=0.06$). Shortly after resuscitation, circulating glucose levels in the Spl group fell significantly below the NoSpl group, which stayed constant (**Figure 6E**).

Electrolytes

In both groups, there was a general downward trend in sodium ions and upward trend in potassium ions (**Figure 7A and B**). However, this trend was greater in the Spl animals than in the NoSpl animals (note, if we subtract baseline K^+ values, Spl is still significantly greater than NoSpl from B7 onward; not shown). Calcium ions also dropped to a greater degree in the Spl group (**Figure 7C**). Chloride ions dropped in both groups over time, and though there were significant differences between groups at nearly every time point, including baseline (**Figure 7D**), if we subtract baseline values, the *change* in the Spl group was significantly greater than the NoSpl group from B1 to B6 and also at B9 (not shown), suggesting an additional interaction between shock and splenectomy.

Inflammation and coagulopathy

Except for the lack of a boost from splenic contraction in the Spl group, changes in platelet concentration mirrored the changes in RBCs, including the dilution due to LR resuscitation (**Figure 8A**). White blood cells, in contrast, showed trends indicating changes

beyond those caused by movement of fluid into and out of the vasculature. Lymphocytes got a temporary boost in concentration during shock in the Spl group that did not occur in the NoSpl group (**Figure 8B**). Unlike platelets and RBCs, lymphocytes in both groups did not decrease at later time points. Circulating granulocytes increased sharply in both groups in response to shock, especially in the NoSpl group, and continued increasing even during the dilution caused by LR (**Figure 8C**). After granulocyte concentration peaked, it dropped off in both groups faster than the drop in platelets or RBCs. Monocytes, similar to granulocytes, appeared to increase in response to shock, especially in the NoSpl group (**Figure 8D**). The later trend of decreasing concentration, however, was more like the gradual drop seen in platelets and RBCs.

We measured the plasma concentration of a number of inflammatory and anti-inflammatory cytokines. We found no differences in IL-1β, IL-8, IL-12, and IL-18 (not shown). At the end of shock (B1), NoSpl animals had significantly elevated GM-CSF, IFN-γ, TNF-α, IL-1a, IL-1ra, IL-2, IL-4, IL-6, and IL-10 (**Table 1**). However, the situation reversed by the end of the experiment, such that the concentration of all of these cytokines were lower (closer to baseline) in the NoSpl group (significantly lower for IFN-γ, IL-1a, IL-1ra, IL-2, IL-4, and IL-10).

Thromboelastography was used to determine effects on coagulation. NoSpl animals tended to have faster split points (SP), and Spl animals slower ones, relative to baseline, resulting in significant differences between the groups at numerous time points (**Figure 9A**). There were no significant differences in R time (not shown), however, making Delta, the time between SP and R representing the thrombin burst, significantly shorter in the Spl group (**Figure 9B**). The rate of initial clot formation, Angle, was similar between groups and changed little over the course of the experiment (**Figure 9C**). There was a small but significant increase in maximum

clot strength (i.e. maximum amplitude, MA) in the NoSpl group in the earlier half of the experiment (**Figure 9D**). The Spl group was also a little slower to reach MA after shock, relative to baseline and to the NoSpl group (**Figure 9E**). There were no apparent differences in clot lysing, however, from either the shock or group effects based on the percent of clot lysis at 60 min (CL60; **Figure 9F**).

Discussion

Shock period

The Spl group tolerated significantly less hemorrhage before hitting a MAP of 30 mmHg, suggesting that the spleen may already have been acting as a volume sink. Alternatively, splenectomy may have kick-started the NoSpl compensatory mechanisms, giving that group a head-start on compensation. Despite the eventual significant difference in mortality, lactate and base excess were similar between groups at the end of shock, suggesting an equivalent degree of tissue under-perfusion. Total protein dropped the same amount in both groups during shock indicating a similar degree of auto-transfusion. Though not directly measured, given the similar blood loss this suggests their total vascular volumes were also similar at the end of shock. However, NoSpl subjects were better able to maintain DAP, the hemodynamic parameter most associated with vascular compensation (18). This suggests that a greater fraction of the NoSpl group's total vascular volume was stressed volume. This interpretation is supported by the greater cardiac output in the NoSpl group during shock, since greater stressed volume would increase the maximum possible venous return, and cardiac output should be operating near capacity during the shock period when the body cannot fully compensate for the hemorrhage. HR and PP, which reflects trends in stroke volume (19), were modestly increased in the NoSpl

group, as expected for the higher cardiac output. Combined, DAP and PP produced a significantly improved MAP in the NoSpl group. Overall, the evidence suggests that despite a similar severity of shock, the NoSpl group was able to maintain a greater level of hemodynamic compensation in the shock period.

Resuscitation and Intensive Care periods

One of the animals in the Spl group had decompensated to the point that fluid, beginning at T = 1.5 h, could not rescue it. Even in the remainder of Spl animals, the benefits of resuscitation were short-lived. In the first half hour of resuscitation, 39 ± 5 ml/kg of fluid raised the Spl MAP by approximately 20 mmHg to 58 ± 5 mmHg. In contrast, the following half hour of fluid (32 ± 4 ml/kg) brought no further improvement in MAP (61 ± 10 mmHg). This indicates that all of that later fluid (and likely some of the earlier fluid) contributed only to unstressed volume, i.e., the spleen/vasculature decreased constriction with fluid influx fluid rather than resisting it to create elastic stretch. This crystalloid volume diluted both RBCs and plasma proteins in the Spl group. This reduces the viscosity of the blood, lowering resistance to flow through blood vessels, and explains the post-resuscitation increase in cardiac output above baseline in the Spl group better than any putative benefit to cardiac health. Dilution also lowers plasma oncotic pressure. If this drops below hydrostatic pressure, fluid efflux through the capillary wall increases, even when permeability is unchanged (20). This flux is self-limiting (e.g., increases plasma protein concentration), unless the dilution is maintained exogenously. Therefore, for shock resuscitated with a limited volume of crystalloid, most, if not all, of the added volume will extravasate as edema, causing the vasculature to become hypovolemic once more and pressure to drop. This is exactly what we observed in the Spl group, as RBC

concentration rose over the next two hours following the end of resuscitation, indicating a loss of plasma volume, until RBC concentration was again significantly greater than that of the NoSpl group. The simultaneous fall in cardiac output supports the idea its increase was due to blood dilution.

Unlike the Spl group, NoSpl animals needed little fluid to maintain their target pressure, suggesting a greater portion of it went directly to stressed volume. There was no great dilution of the blood, and no big shifts in RBC concentration or cardiac output. Instead, we observed a slow, steady drop in RBC concentration over the entire time-course. This suggests that *loss of compensation starts early and is ongoing even in survivors*. Interestingly, once the effects of massive crystalloid resuscitation faded away, the survivors in the Spl group showed the exact same trend in RBC concentration, offset presumably by the influx of RBCs from the spleen during hemorrhage. Total protein concentration, which was nearly identical between groups from B4 on, did not show the same downward trend as RBC concentration. This could indicate that the plasma protein concentration dropped to the point it was no longer higher than the incoming lymph fluid. Alternatively, production and release of new plasma proteins may have been sufficient to match the deficit between the plasma and the incoming fluid.

Overall, resuscitation with crystalloid provided only short-term benefit to blood pressure. This may be sufficient when transit time to surgical care is short, but the benefit appears to be severely truncated in a prolonged field care scenario, as modeled here. These findings are in agreement with a recent report, describing equal benefit of pre-hospital crystalloid with plasma resuscitation only when pre-hospital transit times were under 20 minutes (21).

<u>Mortality</u>

We previously observed in a rat splanchnic arterial occlusion model of shock that decompensation came in two forms: 1) slow, in which – after being sympathetically stimulated by intestinal ischemia for hours – blood pressure began an hours-long decline leading to death, and 2) rapid, in which blood pressure spontaneously plummeted over the course of minutes, resulting in significantly earlier death (3). More recently, we observed the same dual forms in a rat hemorrhagic shock model and saw that, while acute decompensation could occur prior to resuscitation as one might expect, it could also occur *well after* the start of resuscitation and result in a fatal loss of pressure even while receiving fluid resuscitation (18, 22). Our hypothesis is that acute deaths are the result of failures in efferent sympathetic signaling, whereas slow deaths are the result of the vasculature losing the ability to respond to that signaling. In our rat models, we found factors that increased the actual or perceived need for a sympathetic response increased the odds of acute decompensation, while factors that made the response easier improved survival.

In this swine model, based qualitatively on the rate of blood pressure decline, we saw that of the 9 deaths in the Spl group, 5 were acute decompensations, 3 were slow decompensations, and one we could not confidently assign to either type, as blood pressure declined over an intermediate interval of ~35 minutes. The one death in the NoSpl group was a slow decompensation, though it appeared that another subject was also slowly decompensating and would likely have died within another hour or two. These results fit with theory, as any volume pooling in the spleen would lower blood pressure in the Spl animals, which would be sensed by sympathetic afferents and interpreted as a need for greater sympathetic output, increasing the odds of acute decompensation. Likewise, the need to constrict the venous vasculature more to make up for the loss of stressed volume suggests the Spl animals would experience a greater

expenditure of vascular energy and resources, increasing the odds of being unable to match need (slow decompensation). The benefit to survival seen here with splenectomy has also been seen in rats after resection of intestine (23), another location believed to "pool" blood in shock. The increase in hind-limb oxygen usage and decrease in glucose in the Spl group supports the idea that there was, indeed, more work being done in some tissues. Of note, the group increase in hind-limb oxygen usage vanished after the final acute decompensation between B8 and B9, supporting the idea that animals "working" hardest will be those most likely to acutely decompensate. The creep in plasma sodium and potassium ions could represent either a shift in resources away from the sodium-potassium pump or a depletion of energy sources.

Our findings suggest that splenectomy provides a method by which the mortality of most swine models can be tuned, if death is due to decompensation. However, a model with greater hemorrhage or other modifications that make compensation more difficult could potentially overwhelm the compensatory mechanisms, regardless of splenectomy. For example, in a pilot study, we saw that addition of a larger pre-surgical dose of opioid analgesic returned this model to high mortality outcome despite splenectomy (opioids interfere with vessel constriction (24)). Unfortunately, previous studies of splenectomy on hemorrhagic shock were performed at the opposite extreme, in models with no lethality and short duration (4, 6, 25, 26). Ultimately, the lack of lethality in these models may have limited their ability to detect differences caused by splenectomy.

Oxygen Transport

The consistent group difference in arterial oxygen saturation may have been due to differences in the FiO_2 settings between groups. Since it was lower in the group with higher

survival, it is unlikely it played an important role in mortality in our model. The steadiness of the values also suggests that the lung injury was not sufficient to impair primary lung function in the presence of mechanical ventilation. Despite the boost to RBC concentration during shock in the Spl group from splenic contraction, the rate of oxygen delivery was identical between groups during shock. After the start of resuscitation, greater cardiac output gave the Spl group a temporary boost to oxygen delivery.

However, VO_2 never matched DO_2 or dropped below baseline in either group at any time point, suggesting that oxygen delivery was not a limiting factor in perfused tissues. Increased lactate and decreased pH and base excess indicate instead that there were tissues not being reached. We saw no major change in these markers of ischemia in either group with resuscitation, beyond what is attributable to dilution, suggesting resuscitation did not result in much arterial opening or improvement in functional capillary density. These data support the idea that treatments that only increase oxygen delivery in the places blood is already going will be less beneficial than treatments that return flow to under-perfused tissues, after the danger of decompensation is past.

Inflammation and Coagulopathy

During the shock period, the NoSpl group entered a more inflammatory state than the Spl group, as indicated by early increases in concentrations of cytokines, granulocytes, and monocytes. Increased inflammation via priming (two-hit model) could be one of the potential effects of surgical trauma from splenectomy (27). It is not yet clear, however, why the inflammatory mediators appeared to resolve faster in the NoSpl group. In contrast, there were almost no group differences in leukocyte concentrations between groups following the shock

period, though we did see a sharp drop in granulocytes in both groups suggesting the cells were either extravasating or becoming trapped in the vasculature (e.g., the lungs). There was a temporary boost to circulating lymphocytes in the Spl group that did not fit the above patterns. Splenectomy resulted in minor increases in coagulability, including a little acceleration in the earliest part of the coagulation cascade, a small increase in clot strength, and less dilution of platelets at B2. Alternatively, the presence of the spleen (or release of sequestered RBCs) may have contributed to a minor coagulopathy.

Limitations

This study came about out of an opportunity to improve our animal model and employs comparisons to historical controls, rather than a prospective, randomized approach. This resulted in a number of limitations and factors that need to be weighed when considering the findings. First, though the swine were obtained from the same vendor, there is the possibility of differences between swine (Spl) received at the time of the initial study and those of this study (NoSpl). Another major concern is the difference in anesthetic agents (sufentanil/midazolam for Spl; propofol/buprenorphine for NoSpl). Though we maintained a similar depth of anesthesia and baseline hemodynamics were similar, it is not known what effects on compensation this difference may have caused. Lastly, though the total volume of blood removed was similar in both groups, the proportion removed by controlled hemorrhage was different. It is unclear, what, if any, effects this might have.

Conclusions

There is a controversy on whether or not to include splenectomy in large animal models of shock, but it has only been tested in brief, non-lethal models. To our knowledge, ours is the first study to look at the effects of splenectomy on hemorrhagic shock that was potentially lethal and observed for more than a few hours. Splenectomy had a profound protective effect against mortality from decompensation within the first 14 h after injury in our model, without affecting lactate levels or base excess, suggesting a similar level of "shock". Improved survival with splenectomy occurred despite similar or decreased oxygen delivery and usage lower than delivery, suggesting oxygen debt is not playing a large role in *early* mortality. Our results suggest that whether or not you should include splenectomy as part of your model of severe hemorrhagic shock should depend most on whether or you are studying "early" death from decompensation or later events. If you are interested in early death, your best choice may be to leave the spleen intact so that it may continue to play its role in cardiovascular collapse. In contrast, if you wish to study the effects of severe hemorrhagic shock on organ damage – or are trying a treatment to reduce such – splenectomy may be helpful to avoid early losses of animals that could skew data due to decompensation independent of organ damage. Experimental designs should also consider that splenectomy caused greater inflammation during the shock period but resolved it faster in the observation period. Trauma and hemorrhage can lead to mortality and morbidity by multiple pathways. Treatments that act to block one pathway may amplify another, therefore it is critical to know which mechanisms are being affected by treatments under evaluation in animal studies. We hope that our findings will provide the rationale for more judicious use of splenectomy in the future.

Acknowledgments

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Disclaimer: The views expressed are those of the authors and do not reflect the official views or policy of the Department of Defense or its Components. The experiments reported herein were conducted according to the principles set forth in the National Institute of Health Publication No. 80-23, Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act of 1966, as amended

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Figures and Tables

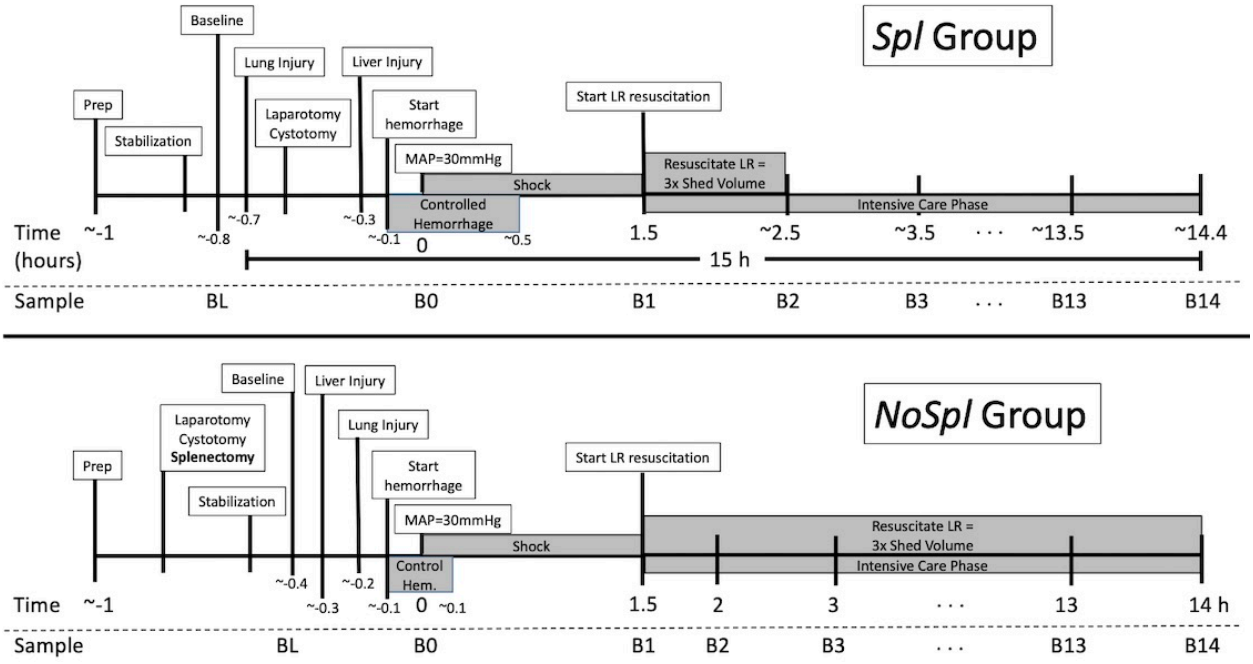

Figure 1 Experimental timelines of the historical group with spleens (Spl group; top) and the new study using splenectomy (NoSpl; bottom). The main differences are in the timing of the lung injury (prior to laparotomy in Spl versus after liver injury in NoSpl) and in the timing of the later blood draws (hourly after the end of resuscitation until the final draw 15 h after lung injury in the Spl group versus on the hour after the start of resuscitation in the NoSpl group). Splenectomy and/or these changes resulted in quicker completion of hemorrhage in the NoSpl grouper and quicker completion of resuscitation in the Spl group.

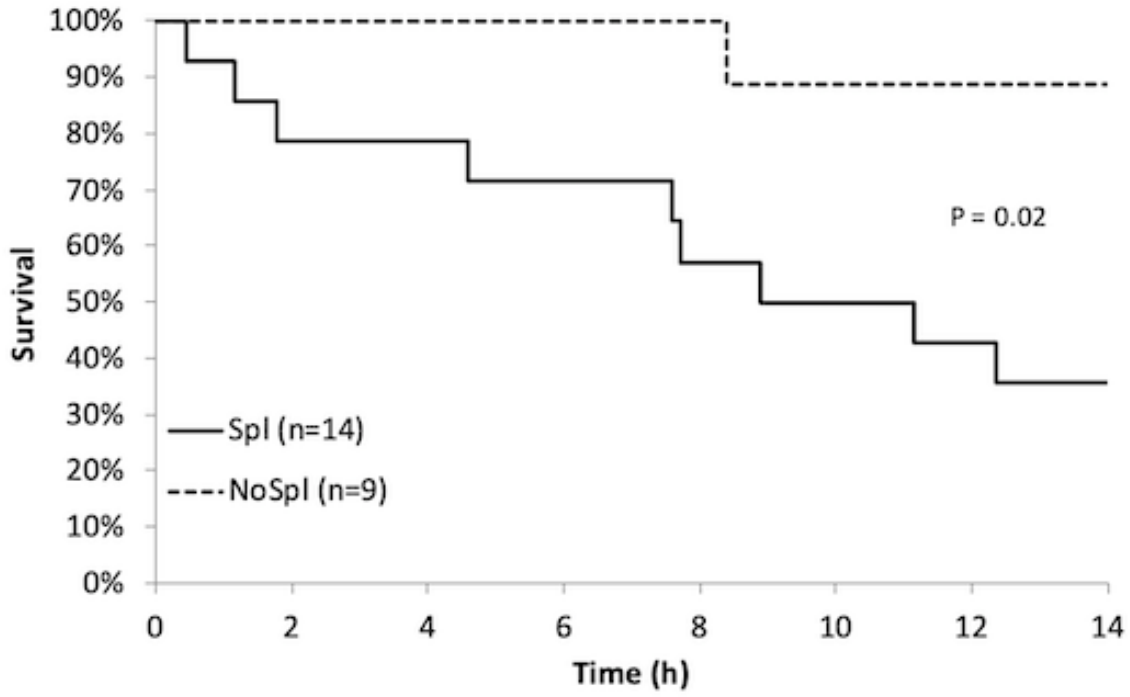

Figure 2 Effects of splenectomy on survival vs. time. Start of shock at T = 0 h. Start of resuscitation at T = 1.5 h. Nine animals of fourteen (57%) died in the Spl group compared to one of nine (11%) in the NoSpl group (p = 0.02 by log-rank analysis).

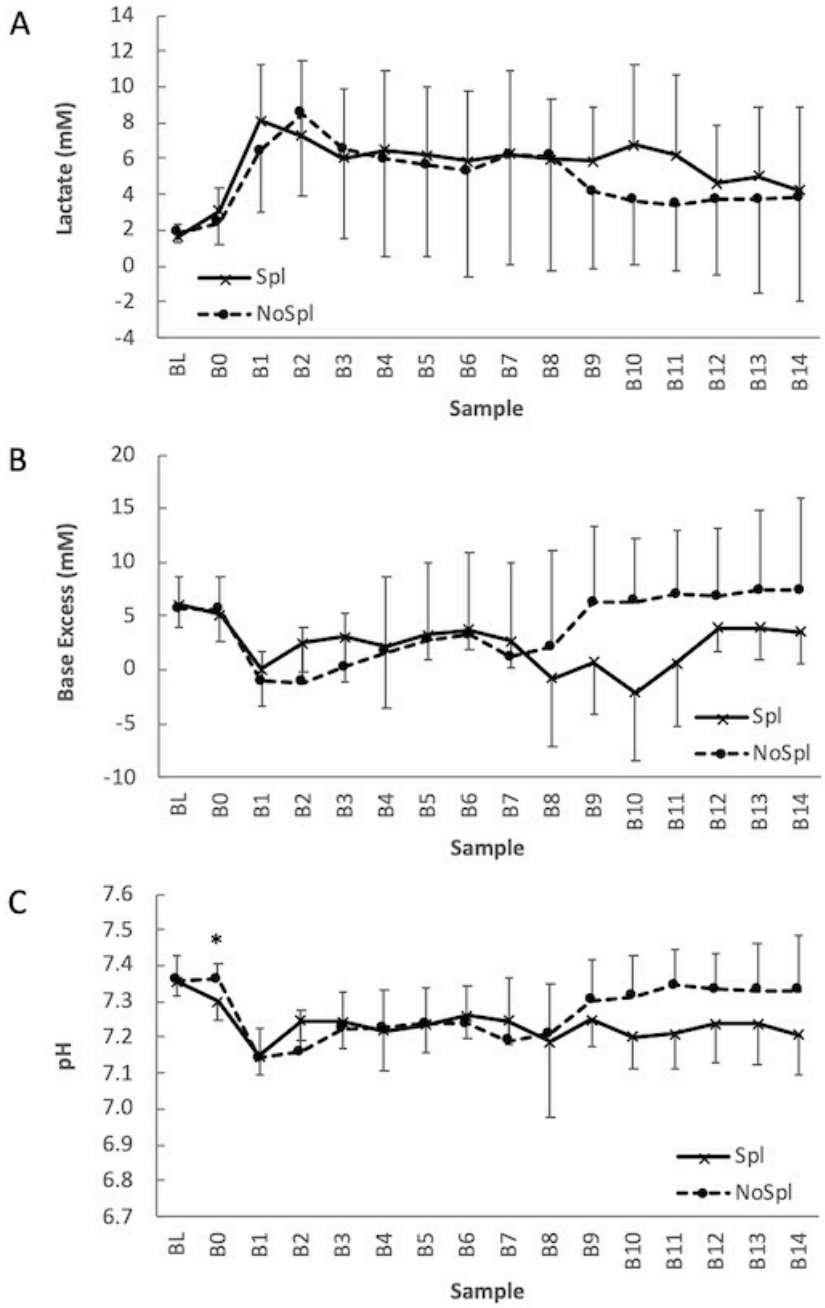

Figure 3 **Indices of shock severity**. Lactate (**A**), base excess (**B**), and venous pH (**C**). Data expressed as mean ± SD. $N_{Spl} = 14$ and $N_{NoSpl} = 9$ at baseline, decreasing to 6 and 8, respectively by B14. * $p < 0.05$ between groups.

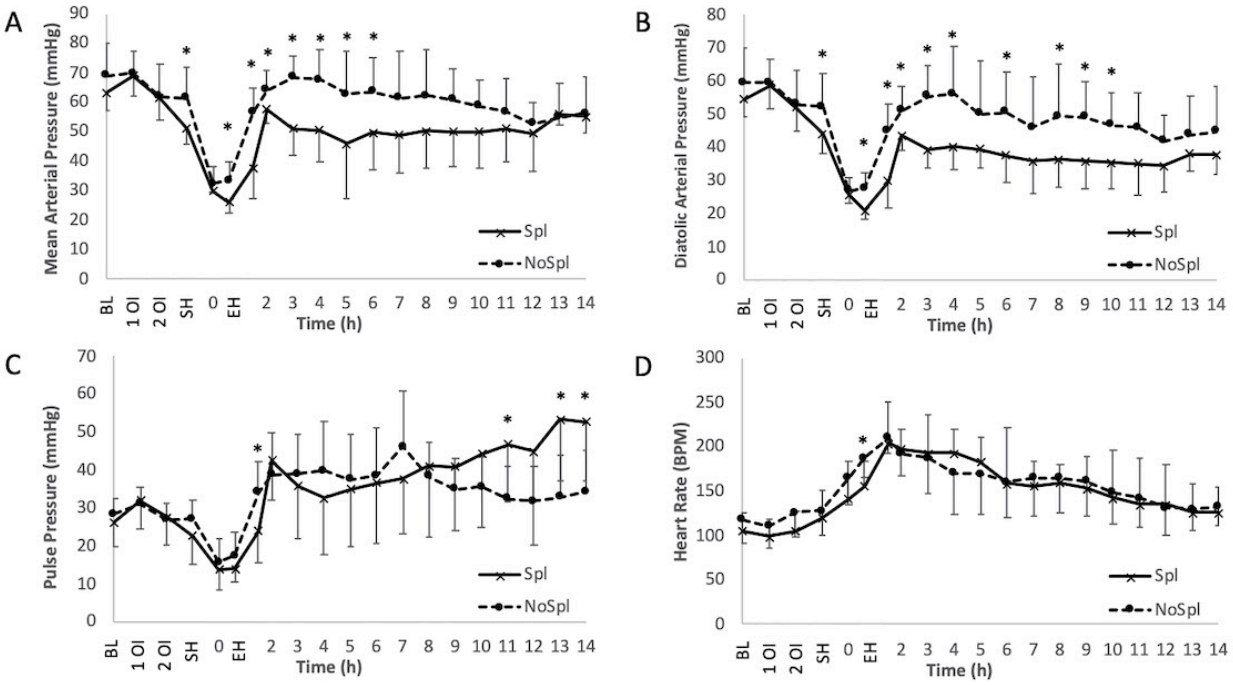

Figure 4 **Hemodynamic parameters.** Mean arterial pressure (**A**), diastolic arterial pressure (**B**), pulse pressure (**C**), and heart rate (**D**). Data expressed as mean ± SD. N_{Spl} = 14 and N_{NoSpl} = 9 at baseline, decreasing to 6 and 8, respectively by B14. * $p < 0.05$ between groups.

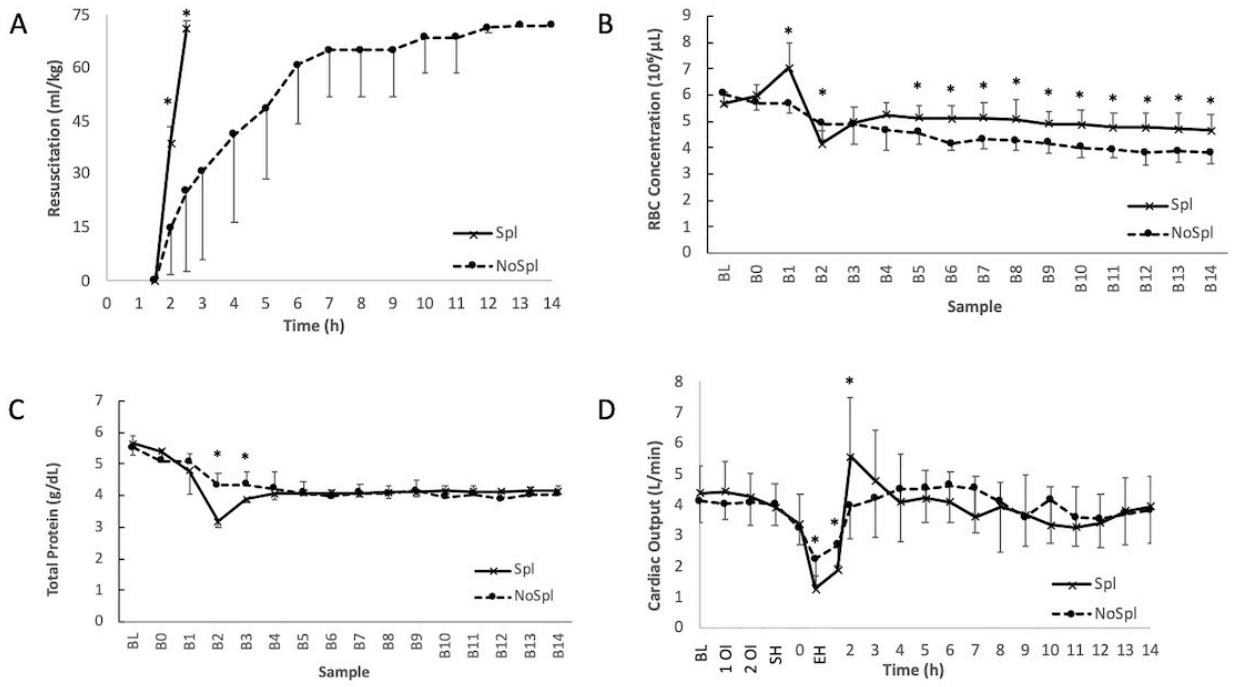

Figure 5 **Effects of resuscitation.** Resuscitation volume vs. time (A), red blood cell concentration in samples (B), plasma protein in samples (C), and cardiac output vs. time (D). Data expressed as mean ± SD. $N_{Spl} = 14$ and $N_{NoSpl} = 9$ at baseline. * $p < 0.05$ between groups. (A) Resuscitation was ended at 2.5 h in the Spl group (though two animals were still slightly shy of the 72 ml/kg maximum resuscitation volume). (D) Timing of baseline (BL), first organ (lung or liver) injury (1 OI), second organ (liver or lung) injury (2 OI), start of hemorrhage (SH), and end of hemorrhage (EH) for cardiac output are not to scale (see Figure 1 for approximate timing of these events).

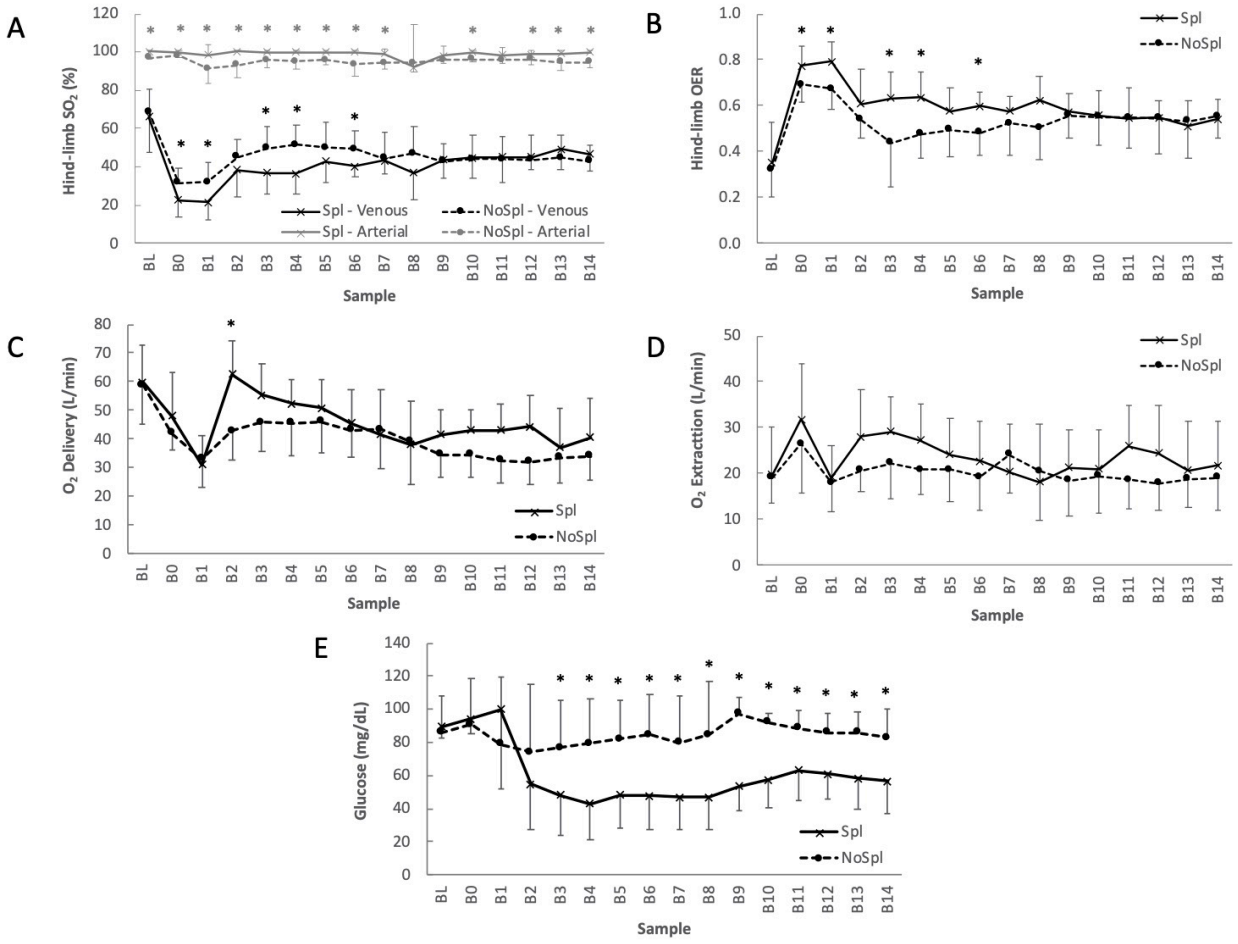

Figure 6 Oxygen and glucose dynamics. Femoral arterial and venous blood oxygen saturation (SO_2) (A), hind-limb oxygen extraction ratio (B), whole-body oxygen delivery (C), whole-body oxygen extraction (calculated using the mixed venous SvO_2 from the Swan-Ganz) (D), and blood glucose levels (E). Data expressed as mean ± SD. $N_{Spl} = 14$ and $N_{NoSpl} = 9$ at baseline, decreasing to 6 and 8, respectively by B14. * $p < 0.05$ between groups.

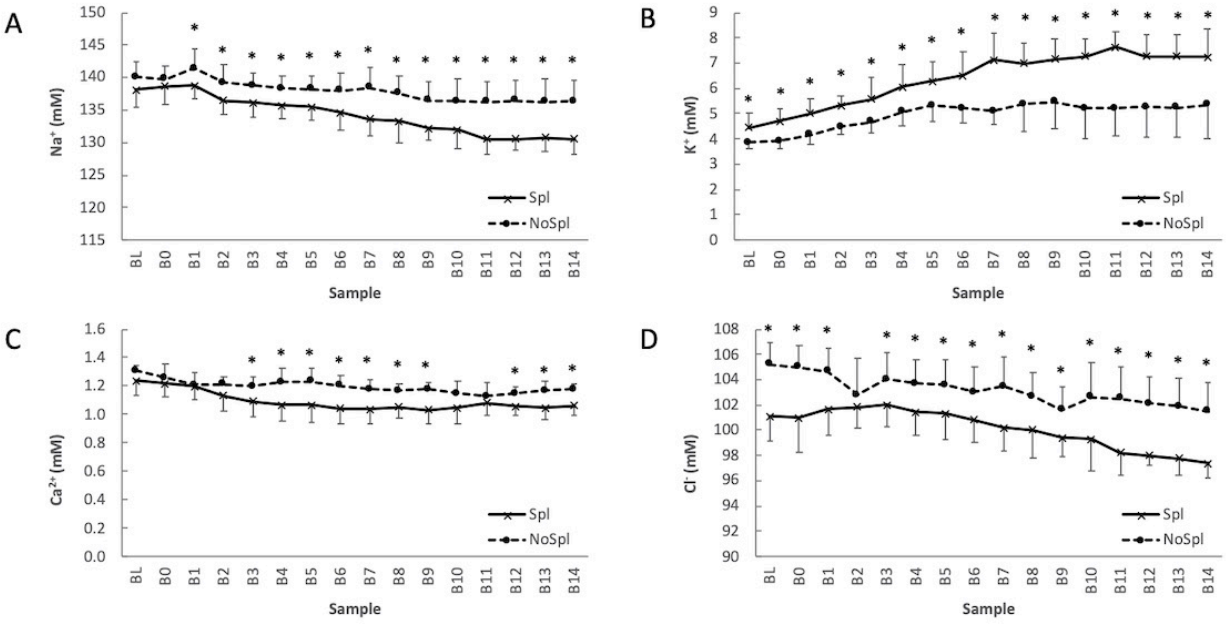

Figure 7 **Electrolytes.** Sodium (A), potassium (B), calcium (C), and chloride (D) ion concentrations. Data expressed as mean ± SD. $N_{Spl} = 14$ and $N_{NoSpl} = 9$ at baseline, decreasing to 6 and 8, respectively by B14. * $p < 0.05$ between groups.

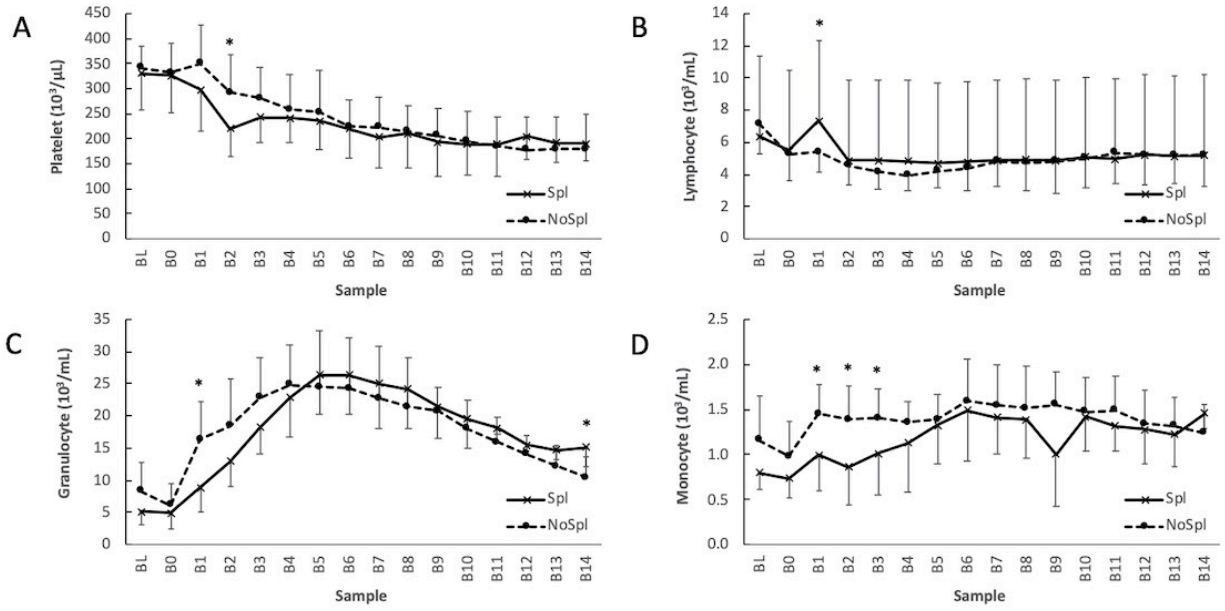

Figure 8 Blood platelet and leukocyte concentrations. Platelet (A), lymphocyte (B), granulocyte (C), and monocyte (D) concentrations. Data expressed as mean ± SD. $N_{Spl} = 14$ and $N_{NoSpl} = 9$ at baseline, decreasing to 6 and 8, respectively by B14. * $p < 0.05$ between groups.

Table 1 Cytokines

	GM-CSF			IFNg			TNFa		
	Spl	NoSpl		Spl	NoSpl		Spl	NoSpl	
B0	0.000 ± 0.013	0.002 ± 0.006		0.2 ± 0.5	0.7 ± 0.5	*	0.01 ± 0.05	0.01 ± 0.01	
B1	-0.006 ± 0.034	0.024 ± 0.018	*	2.2 ± 1.8	4.8 ± 1.6	*	0.02 ± 0.04	0.07 ± 0.04	*
B2	-0.001 ± 0.054	0.023 ± 0.010		5.6 ± 6.9	4.8 ± 1.7		0.06 ± 0.10	0.07 ± 0.03	
B7	0.021 ± 0.043	0.027 ± 0.017		8.0 ± 2.8	5.2 ± 1.8	*	0.09 ± 0.09	0.08 ± 0.04	
B14	0.030 ± 0.012	0.016 ± 0.018		7.2 ± 2.7	3.1 ± 2.8	*	0.10 ± 0.12	0.05 ± 0.05	

	IL-1a			IL-1ra			IL-2		
	Spl	NoSpl		Spl	NoSpl		Spl	NoSpl	
B0	0.000 ± 0.008	0.001 ± 0.002		1 ± 2	3 ± 3		-0.01 ± 0.07	0.00 ± 0.03	
B1	0.001 ± 0.013	0.011 ± 0.004	*	9 ± 9	23 ± 13	*	0.00 ± 0.10	0.12 ± 0.05	*
B2	-0.005 ± 0.022	0.010 ± 0.008		31 ± 26	31 ± 15		-0.02 ± 0.20	0.12 ± 0.10	
B7	0.017 ± 0.007	0.009 ± 0.007	*	59 ± 24	31 ± 16	*	0.21 ± 0.09	0.12 ± 0.10	
B14	0.011 ± 0.007	0.001 ± 0.009	*	53 ± 15	21 ± 20	*	0.18 ± 0.03	0.03 ± 0.13	*

	IL-4			IL-6			IL-10		
	Spl	NoSpl		Spl	NoSpl		Spl	NoSpl	
B0	-0.07 ± 0.26	0.01 ± 0.03		0.00 ± 0.03	0.02 ± 0.02	*	0.01 ± 0.14	0.01 ± 0.03	
B1	-0.17 ± 0.39	0.11 ± 0.08	*	0.15 ± 0.15	0.29 ± 0.10	*	0.05 ± 0.24	0.21 ± 0.05	*
B2	-0.35 ± 0.65	0.07 ± 0.10		0.33 ± 0.24	0.36 ± 0.11		-0.03 ± 0.31	0.20 ± 0.11	*
B7	0.13 ± 0.14	0.06 ± 0.11		0.41 ± 0.49	0.17 ± 0.09		0.38 ± 0.22	0.22 ± 0.14	
B14	0.08 ± 0.03	-0.02 ± 0.10	*	0.37 ± 0.20	0.16 ± 0.26		0.38 ± 0.11	0.10 ± 0.20	*

Values shown are change from baseline. All units are ng/ml. Measured but not shown (no significant differences) were IL-1b, IL-8, IL-12, and IL-18.

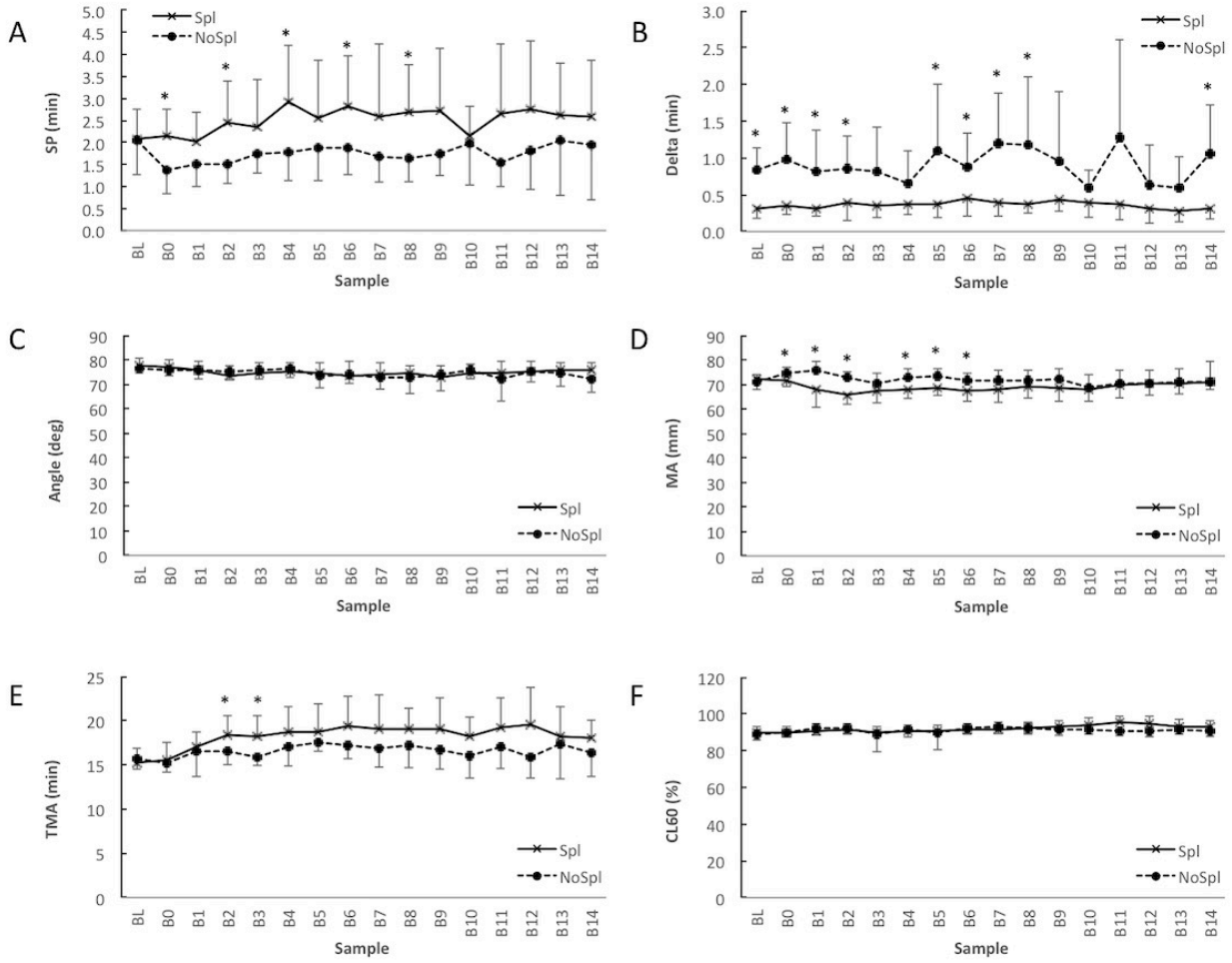

Figure 9 Coagulation parameters measured by thromboelastography. Split point (SP) (A), difference between the time amplitude = 2 mm ("R") and SP representing the thrombin burst (B), angle between the horizontal axis and the line formed by connecting the start of clot formation to the inflection point at which the first derivative starts decreasing (C), the maximum amplitude (MA) representing the maximum strength of the clot (D), the time to reach maximum amplitude (TMA) (E), and the amplitude (as a % of MA) 60 min after TMA (CL60) (F). Data expressed as mean ± SD. $N_{Spl} = 14$ and $N_{NoSpl} = 9$ at baseline, decreasing to 6 and 8, respectively by B14. * $p < 0.05$ between groups.