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An ovine model of haemorrhagic shock and resuscitation, to assess recovery of tissue oxygen delivery and oxygen debt, and inform patient blood management

Running head: Haemorrhagic shock & recovery of tissue oxygen delivery

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Abstract (256 words):

Background:

Aggressive fluid or blood component transfusion for severe haemorrhagic shock may restore macrocirculatory parameters, but not always improve microcirculatory perfusion and tissue oxygen delivery. We established an ovine model of haemorrhagic shock to systematically assess tissue oxygen delivery and repayment of oxygen debt; appropriate outcomes to guide patient blood management.

Methods:

Female Dorset-cross sheep were anaesthetised, intubated, and subjected to comprehensive macrohaemodynamic, regional tissue oxygen saturation (StO_2), sublingual capillary imaging and arterial lactate monitoring, confirmed by invasive organ-specific microvascular perfusion, oxygen pressure and lactate/pyruvate levels, in brain, kidney, liver and skeletal muscle. Shock was induced by stepwise withdrawal of venous blood until mean arterial pressure (MAP) was 30mmHg, mixed venous oxygen saturation (SvO_2) <60%, and arterial lactate >4mM. Resuscitation with PlasmaLyte® was dosed to achieve MAP >65mmHg.

Results:

Haemorrhage impacted primary outcomes between baseline and development of shock: MAP 89±5 to 31±5 mmHg ($p<0.01$), SvO_2 70±7 to 23±8% ($p<0.05$), cerebral regional tissue oxygen saturation (StO_2) 77±11 to 65±9% ($p<0.01$), peripheral muscle StO_2 66±8 to 16±9% ($p<0.01$), arterial lactate 1.5±1.0 to 5.1±0.8mM ($p<0.01$), and base excess 1.1±2.2 to -3.6±1.7mM ($p<0.05$). Invasive organ-specific monitoring confirmed reduced tissue oxygen delivery; oxygen tension decreased and lactate increased in all tissues, but moderately in brain. Blood volume replacement with PlasmaLyte® improved primary outcome measures toward baseline, confirmed by organ-specific measures, despite haemoglobin reduced from baseline 10.8±1.2 to 5.9±1.1g/dl post-resuscitation ($p<0.01$).

Conclusion:

Non-invasive measures of tissue oxygen delivery and oxygen debt repayment are suitable outcomes to inform Patient Blood Management of haemorrhagic shock, translatable for pre-clinical assessment of novel resuscitation strategies.

Keywords:

haemorrhagic shock, fluid resuscitation, Patient Blood Management, tissue oxygen delivery, microvascular function, haemodynamic recovery, anaemia compensation.

Background

Acute haemorrhage causes a blood pressure and flow-dependent decrease in tissue perfusion, progressing to shock and oxygen debt when tissue oxygen demand exceeds delivery. Crystalloids and blood are administered to restore intra-vascular volume, haemodynamic stability and oxygen carrying capacity of the macro-circulation, which is intended to restore tissue oxygen delivery and repay tissue oxygen debt. Without resuscitative interventions, the pathophysiology of haemorrhagic shock progresses rapidly from capillary collapse, anaerobic metabolism, endothelial glycocalyx degradation and coagulopathy, toward microvascular and endothelial dysfunction (1-3). Subsequent endothelial inflammation and leukocyte infiltration may result in tissue oedema, microvascular haemorrhage and further microvascular occlusion, culminating in multiple organ failure and death (1, 2, 4-6). Therefore, urgent effective treatment is critical in order to reduce haemorrhagic shock-associated mortality.

Clinical assessment of shock to guide treatment and monitor recovery is often limited to macrohaemodynamic parameters, while patient-relevant measures of tissue oxygen delivery are rarely used. Interventions to restore microcirculatory flow and tissue oxygen delivery may have greater relevance for organ survival and patient outcomes than addressing haemoglobin in the macrocirculation alone (1). In severe cases of shock, microvascular dysfunction and endothelial disruption may progress to organ failure despite intensive treatment with fluids and blood products (6, 7). Patient Blood Management is a multidisciplinary approach to managing a patient's own blood and haemostatic requirements. In principle, the decision to transfuse or use another treatment should be based not only on haemoglobin levels, but incorporate multiple clinical assessments to achieve the desired outcome of adequate tissue oxygen delivery. Therefore, confirmation and translation of reliable measurements of tissue oxygen delivery, and novel resuscitation strategies that restore microvascular function to prevent irreversible shock, may prove useful in guiding optimal treatment of haemorrhagic shock in the context of Patient Blood Management.

To advance patient outcomes, clinically-relevant animal models of haemorrhagic shock could provide insight into the efficacy of novel resuscitation strategies and the mechanisms involved (1, 3-5). Our group has previously developed clinically-relevant ovine models of trauma (8), haemorrhage (9), transfusion (10), TRALI (11) and septic shock (12), to investigate the pathophysiology of these conditions and to assess outcomes of transfusion or fluid resuscitation. To investigate tissue-specific pathophysiology of haemorrhagic shock, we developed an ovine model of massive haemorrhage with critically reduced tissue oxygen delivery and cumulative oxygen debt, confirmed by real-time organ-specific oxygen delivery, inflammatory markers and post-mortem histology. To demonstrate clinical utility of non-invasive measures of tissue oxygen delivery in guiding Patient Blood Management, we benchmarked these against invasive measures of tissue perfusion, oxygen delivery and oxygen debt in vital organs.

Methods

The Queensland University of Technology University Animal Ethics Committee approved this study (approval #1800000493). Experiments were conducted in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes (13).

Initial Instrumentation

To establish the model, six non-pregnant Dorset-cross ewes, <3-years old, determined healthy by veterinary and haematological assessment, were fasted overnight before the procedure. The operating facility, monitoring and data management systems (Figure S1, http://links.lww.com/SHK/B327) are described in detail elsewhere (14). A 7-Fr three-lumen central venous catheter and two 8-Fr venous sheaths (Arrow International Inc., PA, USA) were inserted under local anaesthesia and sutured in place in the left and right jugular veins for fluid infusion, blood sampling or haemorrhage, and central haemodynamic monitoring. After insertion of the first sheath, animals were pre-medicated with midazolam (3-6mg bolus). Anaesthesia was induced with propofol (3-4mg/kg), and maintained with midazolam 0.5-0.8mg/kg/hr, fentanyl 5-15µg/kg/hr and ketamine 2.5-7.5mg/kg/hr. An endotracheal tube (size 9, Smiths Medical, Australia) was inserted into the trachea through direct laryngoscopy, and after intubation the animal was placed on the lateral-left position on a heated operating table and connected to the ventilator (Galileo, Hamilton Medical, Switzerland). Initial mechanical ventilation parameters were 12 breaths/min, 8-10ml/kg tidal volume, 5cm H_2O positive end-expiratory pressure (PEEP), and 40% inspiratory fraction of oxygen (FiO_2). These settings were adjusted to maintain normocapnia and arterial oxygen saturation >92%. A 500-1000 ml bolus of Hartmann's solution was initially given to compensate for overnight fasting and potential dehydration; fluids were maintained at 1ml/kg/hr thereafter.

Systemic Monitoring

Basic monitoring included a pulse oximeter probe attached to the tongue, 3-lead electrocardiogram, invasive blood pressure monitoring via a 16Ga femoral artery line (Arrow International, PA, USA), and continuous waveform capnography. A 7.5-Fr Swan-Ganz catheter (Continuous Cardiac Output (CCO) VIP Pulmonary Artery Catheter, Edwards Life Science, CA, USA) was inserted into the right jugular sheath for continuous haemodynamic monitoring of pulmonary artery blood pressure, body temperature, and cardiac output. Stroke volume, mixed venous oxygen saturation (SvO_2), and systemic vascular resistance were measured at defined time points. A 12-Fr urinary catheter (Bard, GA, USA) and a 9-Fr nasogastric tube were inserted. Blood was sampled at major time points for arterial blood gas (ABG) analysis, 5-part differential blood counts (Mindray BC-5000 Vet), coagulation analysis using rotational thromboelastometry (ROTEM; Haemoview Diagnostics), and serum, plasma and urine stored for post-hoc tests and bio-banking. Additional ABG analyses were performed every 15min during haemorrhage and the first hour of resuscitation.

Non-invasive Tissue Monitoring

Non-invasive measures included continuous regional tissue oxygen saturation (StO_2) measured by near infer-red spectroscopy (NIRS; ForeSight Elite, Australia), with probes sutured to close-shaved skin over the frontal cortex (Figure S2,

http://links.lww.com/SHK/B327) and biceps femoris muscle. Sublingual microvascular blood flow was assessed hourly by incident dark-field (IDF) imaging (CytoCam, Braedius Medical, Netherlands), and data from videos were expressed as proportion perfused vessels (PPV), perfused vessel density (PVD), and average perfusion speed index (APSI).

Invasive Tissue Monitoring

Laser optical probes (Oxford Optronix, UK) were inserted into the liver, kidney, skeletal muscle and brain to invasively monitor microvascular perfusion (MNP probes; MSP for liver) and tissue oxygen partial pressure (LAS-8 probes); the monitoring system is illustrated in Figure S3, http://links.lww.com/SHK/B327. Interstitial glucose, lactate and pyruvate levels were sampled hourly using micro-dialysis probes (MD63; M Dialysis AB, Sweden) perfused at 0.3µl/min, and assessed in an ISCUS analyser (M Dialysis AB). Details on surgical insertion of probes into liver, kidney, muscle and brain are described and illustrated in Figures S4-S7, http://links.lww.com/SHK/B327, respectively.

Experimental timeline

Animals were instrumented then rested one-hour before the experimental protocol. At experimental baseline (T0), haemorrhage commenced and shock was induced within 90min. Resuscitation commenced at T1 followed by four hours assessment of recovery (T2-T5), as per the experimental timeline in Figure 1.

Haemorrhagic shock protocol

To minimise splenic auto-transfusion during haemorrhage (15), we infused adrenaline (0.001-0.1µg/kg/min) until splenic contraction was achieved after an elevated heart rate for 5min, confirmed by increased haemoglobin levels. Venous blood draws commenced immediately using an automated blood collector (T-RACII, Tumero BCT, Australia). The first 450ml blood was rapidly drawn within 10min. The next two draws of 225ml each continued if MAP was >40mmHg. Subsequent blood draws continued cautiously until targets of shock (SvO_2 <60%) and oxygen debt (arterial lactate >4mM) were achieved, while maintaining MAP around 30mmHg. Estimated iatrogenic blood loss from sampling and surgery before resuscitation was 300ml (8-12% TBV).

Resuscitation protocol

The target for fluid resuscitation was MAP >65mmHg, equivalent to 70-80% baseline MAP, and noradrenaline was given if MAP <50mmHg. An initial rapid bolus of PlasmaLyte® based on the haemorrhage volume of was administered within 10min, then tapered to 20ml/kg/hr. If MAP >65mmHg after 15min, infusion was decreased to 10ml/kg/hr. If MAP >65mmHg after 15min, fluids were reduced to maintenance dose of 1ml/kg/hr. Fluids were increased accordingly when MAP <65mmHg, and noradrenaline given if MAP <50mmHg. Noradrenaline was ceased if MAP could be maintained >50mmHg.

Outcome measures

The primary outcome measures were clinical markers of haemodynamic recovery and non-invasive measures of tissue oxygen delivery. Treatment targets were MAP >65mmHg, SvO_2 >65%, StO_2-brain >60% and StO_2-muscle >50%, and repayment of oxygen debt

defined by arterial lactate <2mM and positive base excess. Secondary outcome measures included heart rate, and less frequently used clinical and experimental variables, including cardiac output, systemic vascular resistance index (SVRI), PaO_2/FiO_2 (P/F ratio), urinary output, and sublingual capillary perfusion (IDF imaging). Investigational invasive organ-specific measures of oxygen delivery included microvascular perfusion and oxygen tension by laser probes, and lactate and lactate/pyruvate ratios by micro-dialysis.

Post-mortem assessment

Animals were euthanised with lethal dose pentobarbitone. Histopathological evidence of tissue damage induced by shock was determined in samples taken from brain (right frontal cortex), right kidney, right liver lobe, small intestine, heart (right and left ventricle), lung (right upper and middle lobes) and biceps femoris muscle. Tissues were fixed and stained with haematoxylin and eosin, or frozen for post-hoc analysis. A three-stage veterinary histopathology scoring system (Table S1, http://links.lww.com/SHK/B327) was established as described for lung (16), brain (17), kidney (18), heart (19), liver (20), intestine (21), and skeletal muscle (22).

Analysis of inflammatory markers

The concentration of inflammatory cytokines in serum (ovine IL-6, IL-8, IL-10 and IL-1β) was determined by ELISA using commercial antibodies (Abacus, Meadowbrook, QLD, Australia) based on previously published methods (23), and the endothelial glycocalyx component hyaluronan was assessed using an ELISA kit (R&D Systems, Minneapolis, USA), according to the manufacturer's protocol.

Statistical analysis

Summary statistics are presented as mean ± standard deviation. Changes in primary outcome measures and experimental variables from baseline through shock and recovery were assessed by 1-way ANOVA (Friedman's non-parametric test). Dunn's post-tests defined variance from baseline to hourly observation points to determine time to recovery in each outcome measure.

Results

Development of shock targets during haemorrhage, and resuscitation requirements

To establish our model, six animals (55±4kg) were bled until criteria of shock and oxygen debt were observed. Baseline characteristics and interventions are summarised in Table 1. Adrenaline-induced splenic contraction increased circulating haemoglobin by 29% from 8.4±1.9 to 10.8±1.2g/dL, before haemorrhage commenced. Combined haemorrhagic and iatrogenic blood loss was 1465±198ml (41% TBV). All animals met our criteria for haemorrhagic shock; MAP was 31±5mmHg, SvO_2 was 23±8%, and arterial lactate was 5.1±0.8mM. Resuscitation with 3707±1918ml PlasmaLyte® was 2.4±1.1 times total blood loss. Resuscitation concluded early in two sheep (35, 87min) because MAP rapidly increased and remained >65mmHg. Other animals required ongoing PlasmaLyte® throughout the recovery period. All six animals completed the experimental protocol.

Impact of shock and resuscitation on clinical haemodynamic markers and tissue oxygen delivery

All primary outcome measures, including shock criteria (MAP, SvO2 and arterial lactate), regional tissue oxygen delivery, and base excess were achieved with the haemorrhage protocol. Baseline levels, shock-associated nadir, and time to recovery of these primary outcome measures are reported in Table 2 and illustrated in Figure 2. MAP (Figure 2a) and SvO_2 (Figure 2b) were rapidly impacted by both haemorrhage and fluid resuscitation interventions. Regional tissue oxygen saturation in brain (Figure 2c) was relatively conserved during haemorrhage (16% decrease) compared with peripheral muscle (76% decrease; Figure 2d). The cerebral NIRS probe on animal PL2 had a weak signal due to poor skin contact, and was not included in the average. Critically-reduced tissue oxygen delivery was defined by decreased SvO_2 (23±8%) and skeletal muscle oxygen saturation (16±9%). All animals developed oxygen debt after achieving shock, measured by increased arterial lactate (Figure 2e) and base deficit (Figure 2f). Recovery from oxygen debt closely followed increased tissue oxygen delivery in 5 of 6 animals. Delayed peripheral tissue oxygen delivery in one animal (PL5) was associated with failure to repay oxygen debt during the observation period.

Clinical features of shock confirmed by secondary outcome measures

The secondary outcome measures identified additional features defining the clinical response to haemorrhagic shock (Table 2). The initial haemodynamic response to haemorrhage characterised by reduced MAP coincided with reduced cardiac output ($p<0.05$), partially compensated by increased heart rate ($p<0.05$) and/or SVRI ($p<0.05$). Reduced MAP also coincided with decreased pulmonary gas exchange (P/F ratio; $p<0.001$), urinary output ($p<0.05$), while sublingual microvascular perfusion, a non-invasive surrogate measure of microvascular perfusion for other vital organs, was not significantly reduced during haemorrhage.

In the resuscitation phase, all outcome measures recovered toward baseline within two hours, defined by $p>0.05$ in post-tests. MAP and urinary output recovered by three hours, but haemoglobin remained low due to haemodilution from resuscitation with PlasmaLyte (Table 2).

Impact of shock observed at an organ level

The impact of haemorrhage on organ-specific perfusion, oxygen tension and oxygen debt were measured in brain, kidney, liver and skeletal muscle (Figure 3). The brain was representative of other vital organs (e.g. heart), while peripheral muscle was considered a low priority of non-vital organ. While non-invasive sublingual perfusion was not significantly reduced during haemorrhage, invasive measures demonstrated reduced perfusion in brain and muscle, while perfusion in kidney and liver tended to increase between the end of haemorrhage (variable time between animals) and the start of resuscitation (T1; shock observation point), and was not significantly reduced. In agreement with non-invasive NIRS, tissue oxygen tension declined significantly in all organs except brain, confirming compensated oxygen delivery to this vital organ. Lactate increased in all tissues during shock, but to a lesser extent in brain as expected from oxygen levels. During resuscitation,

microvascular perfusion and oxygen tension increased in all tissues. Lactate declined earlier in highly vascularised kidney, peaked later in liver, and plateaued longer in muscle. The lactate/pyruvate ratio was significantly elevated in kidney and liver during shock, but metabolic recovery in liver was delayed in some animals.

To summarise the value of invasive measures in confirming organ-specific contributions to non-invasive measures of tissue oxygen delivery and debt, average changes in oxygen saturation in brain and muscle (Figure 4a) agreed with average changes in tissue oxygen tension (Figure 4b). These data confirmed relative preservation of oxygen delivery to brain compared to other organs. Arterial and tissue lactate levels were concordant between animals; failure to clear arterial lactate in animal PL5 (Figure 4c) was associated with elevated lactate levels in multiple organs (Figure 4d). Lactate from non-vital organs was closely associated with changes in arterial lactate

Inflammatory and tissue injury markers

Serum markers of inflammation, degradation of the endothelial glycocalyx, and changes in the circulating neutrophil count (Figure 5) demonstrated an inflammatory response to shock, and partial recovery during fluid resuscitation. The principal inflammatory cytokine, IL-6, increased during shock and remained high during resuscitation. Cytokines IL-8 and IL-10 increased during shock then declined during resuscitation, while endothelial glycocalyx breakdown product hyaluronan increased during initial bolus resuscitation then declined during tapered fluid treatment. IL-1β tended to decline throughout the protocol, while neutrophils gradually increased and remained in circulation.

Evidence of shock-associated inflammation and tissue injury was further confirmed by histopathological examination (Table S1, http://links.lww.com/SHK/B327). Examples of the following histopathological observations are illustrated in Figure 5. Mild to moderate injury was observed in lung (haemorrhage and neutrophilic infiltration of alveoli and interstitium, and bronchus-associated lymphoid tissue hyperplasia), brain (microvascular congestion and perivascular oedema), heart (occasional myocytolysis in the right ventricle, and infiltration of low numbers of neutrophils and necrosis of myocardiocytes in left and right ventricles), kidney (Bowman's capsule dilation, and proximal/distal tubule granular debris and hyaline casts), liver (microvascular congestion and neutrophilic infiltration), small intestine (abnormal villi structure and neutrophilic infiltration in the mucosa and lamina propria), while skeletal muscle showed no pathological effects.

Discussion

We developed an ovine model of controlled haemorrhage and shock, defined by reduced tissue oxygen delivery and oxygen debt. The primary strengths of the model are reproducible induction of clinically-relevant shock, confirmed by standard-of-care haemodynamic measures, surrogate and non-invasive measures of tissue oxygen delivery, supported by experimental invasive organ-specific measures. Intensive care and monitoring were designed on procedures, equipment and materials used in human intensive care management, supplemented with invasive methods, and protocols optimised for clinical translatability (14). To model physiological haemorrhagic shock, blood withdrawal was initially rapid, then withdrawal rate was reduced and guided by MAP to 30mmHg, until

oxygen debt accumulated to arterial lactate >4mM. The model replicated clinical progression of shock. Acute haemorrhage and hypovolemia defined by reduced MAP and cardiac output, was compensated by increasing heart rate and to a lesser extent systemic vascular resistance (24), despite potential attenuation of these compensatory mechanisms by anaesthesia (25). Onset of shock was defined by reduced SvO_2 and peripheral regional oxygen saturation; and finally the severity of shock defined by arterial lactate and base deficit, confirmed by invasive assessment of oxygen tension and lactate levels in vital and non-vital organs (1, 4, 5, 7, 26). Organ-specific effects of shock were confirmed by post-mortem histological analysis. Post-hoc tests confirmed a systemic inflammatory cytokine and neutrophil response to shock, and the endothelial glycocalyx experienced some degradation during shock and the initial rapid fluid bolus treatment, but soluble hyaluronan decreased during tapered fluid dosing. The primary treatment outcomes focussed on tissue oxygen delivery and repayment of oxygen debt (27), which are translatable as patient-outcome measures to guide Patient Blood Management. Although aggressive fluid resuscitation may adversely impact microvascular function via glycocalyx degradation, causing increased lung injury after severe shock (28, 29), histological assessment of lung tissue and the wet:dry ratio confirmed that fluid-associated lung injury was minimal. Importantly, the resuscitation protocol restored both macrocirculatory and microcirculatory parameters (6, 30-32).

In support of the model's clinical translatability and capacity to inform Patient Blood Management, reliable non-invasive diagnosis of tissue perfusion and oxygen delivery could guide the decision to transfuse or not when haemoglobin is at or below the transfusion threshold, and confirm efficacy when other treatment options are used (27, 33-38). Recovery of critical oxygen delivery, measured in our model by regional oxygen saturation and tissue oxygen pressure, did not depend on haemoglobin recovery, but on restoration of blood volume, cardiac output and perfusion pressure. The average reduction in haemoglobin to 5.9g/dL in sheep after resuscitation with PlasmaLyte® was equivalent to haemodilution to <7g/dL in humans; below the restrictive transfusion threshold. Haemoglobin was therefore considered a treatment variable applicable to transfusion, not a surrogate measure of tissue oxygen delivery.

Microvascular perfusion and tissue oxygen delivery are increasingly supported in evidence-based treatment of shock, although the ideal platforms for diagnosis and monitoring are subject to ongoing investigation (33, 38, 39). Reliability of sublingual capillary imaging was inconclusive in our study, because instrumentation procedures may have induced inflammation and impacted sublingual perfusion before haemorrhage, since post-resuscitation data were substantially higher than pre-haemorrhage levels. NIRS identified acute changes in tissue oxygen delivery, with vital organs represented by cerebral StO_2, and peripheral muscle StO_2 was a reliable measure for peripheral tissues and a surrogate for non-vital organ oxygen delivery. Arterial lactate and base excess are considered reliable surrogate measures of tissue oxygen delivery, predictors of severity of shock and oxygen debt, and measurements of efficacy to guide treatment (4, 5, 40). Our data suggested arterial lactate adequately represented oxygen debt across multiple organs during shock and recovery.

The primary objective in developing this model was to reliably induce clinically-relevant haemorrhagic shock, and demonstrate utility of technologies measuring tissue oxygen delivery, translatable to studies of novel treatment options incorporating outcomes centred on tissue oxygen delivery. We performed haemorrhage in a controlled ICU environment, with standardised ventilation parameters appropriate for anaesthetised sheep (14, 41, 42). Deep anaesthesia was mandatory for the extensive instrumentation procedures. Fully-preserved compensatory mechanisms in awake sheep may sustain perfusion pressure during early haemorrhage (15). Furthermore, the initial high haemorrhage rate in our anaesthetised sheep was key to early reductions in MAP and tissue oxygen delivery, and development of shock (25, 43, 44). Thereafter, the haemorrhage rate was reduced to maintain MAP ≥30mmHg, and continued until oxygen debt accumulated to arterial lactate >4mM, which peaked >5mM after initial blood volume replenishment. Oxygen delivery to vital organs (brain and heart) remained compensated after onset of systemic shock and oxygen debt, and recovered rapidly upon resuscitation. Our haemorrhage protocol was designed to induce treatable shock encountered in most clinical haemorrhage scenarios (45), whereas models that induce prolonged haemorrhagic shock with large base deficit often cause decompensation of vital organ perfusion and reduced survival (46). The haemodynamic targets during treatment were likewise appropriate for anaesthetised sheep.

This model of haemorrhagic shock is an improvement on models that haemorrhage to volume or pressure targets instead of shock and oxygen debt targets, and our comprehensive organ-specific assessments provide greater insight into the pathophysiology of shock (Table S2). In our earlier model of haemorrhage and transfusion (9), sheep were bled to a moderate volume target (30-35% TBV haemorrhage), resulting in moderate MAP nadir, and minimum haematocrit decrease because of splenic auto-transfusion. Another recent ovine model of haemorrhagic shock, designed to determine the impact of shock and resuscitation on haemodynamic microcirculatory coherence (47), did not induce substantial oxygen debt because haemorrhage ceased when MAP=30mmHg, not guided by lactate. Instead, our ovine shock model was comparable to benchmark models of severe haemorrhagic shock and critical oxygen debt developed in dogs (4) and swine (5, 44). These models used arterial lactate and base deficit to define a lethal-dose$_{50}$ for oxygen debt, but because we resuscitated animals with PlasmaLyte®, which contains bicarbonate equivalents, we chose lactate alone to define repayment of oxygen debt. We therefore redeveloped a benchmark haemorrhagic shock model, and superimposed comprehensive invasive organ monitoring procedures developed in our model of hyperdynamic septic shock (12). These technologies confirmed that currently-available clinical measures, in particular NIRS assessment of tissue oxygen saturation in central and peripheral tissues, provide appropriate advanced monitoring required by evidence-based Patient Blood Management programs.

In haemorrhagic shock, oxygen delivery to vital organs such as the brain is prioritised over that of non-vital organs such as the kidneys and skeletal muscle. Both non-invasive NIRS and invasive oxygen tension confirmed that oxygen delivery was relatively preserved in the brain during shock, and recovered earlier during resuscitation. The likely cause was vasoconstriction in peripheral tissues and lower priority organs to preserve blood flow to the brain (46, 48). Accordingly, tissue lactate accumulation was least in brain. The onset of

lactate clearance in other organs demonstrated repayment of oxygen debt earlier in kidney than in lower priority liver and muscle tissue. The lactate/pyruvate ratio defines tissue metabolic redox recovery (49, 50), which confirmed the priority for cerebral oxygen debt repayment.

The primary limitation of this study, in common with other similar studies (Table S2), was the relatively short-term assessment of post-resuscitation outcomes, because the highly invasive organ monitoring required deep anaesthesia until euthanasia, and it was not feasible to continue the experiment overnight. Our model instead focused on the restoration of haemodynamic variables, oxygen delivery and repayment of oxygen debt, which are critical short-term outcomes defining adequate resuscitation. To minimise impact on primary haemodynamic measures and oxygen exchange, we did not invasively monitor heart or lung tissue, apart from Swan-Ganz catheterisation. Real-time measures of organ dysfunction during shock and recovery were limited to urinary output. while the longer-term impacts of haemorrhagic shock and resuscitation on organ function and recovery could not be investigated. Histopathological evidence of tissue injury confirmed that our haemorrhage protocol resulted in clinically-relevant shock. Pulmonary inflammation (neutrophil infiltration and BALT hyperplasia) was the main form of lung injury associated with shock, while fluid-associated tissue damage in the form of alveolar oedema was mild, confirmed by normal lung wet/dry ratios. In other organs, very few histological changes were considered shock-specific (intestinal villi structural modifications) or treatment-specific (dilated Bowman's capsule in renal tissue associated with fluid loading).

Other limitations of this study were associated with animal variation, in particular the compensatory mechanisms in response to haemorrhage. We did not screen or exclude animals based on phase of oestrus cycle, whereby animals in proestrus may have exhibited reduced inflammatory responses and enhanced cardiovascular function and tissue perfusion after shock (51). Variations in heart rate and systemic vascular resistance impacted MAP, and since the ethics-approved protocol prohibited haemorrhage when MAP <30mmHg, TBV loss was lower in some animals, despite fulfilling criteria of shock, resulting in earlier haemodynamic recovery and completion of resuscitation in two animals. Our follow-on study comparing treatments will therefore require larger numbers of animals per treatment arm. Another negative consequence of invasive organ assessment is an increased inflammatory response which may have impacted microvascular perfusion. Furthermore, increasing the time in shock may reveal greater differences between investigational treatments. However, our choice of 90min haemorrhage time before resuscitation reflects the majority of clinical scenarios for both surgical and traumatic haemorrhagic shock.

This ovine model may inform current debate about appropriate fluid dosing for shock. Lung injury is a known outcome of shock exacerbated by aggressive fluid resuscitation in large animal models. Our ovine model of hyperdynamic endotoxemic shock demonstrated increased endothelial glycocalyx shedding, inflammatory cytokine levels and lung injury associated with high fluid dosing (28, 29). The same inflammatory effect was observed in a canine haemorrhagic shock model with high-dose fluids (52). Therefore, we used a conservative fluid resuscitation protocol to minimise lung injury. This was confirmed by

post-mortem lung wet/dry ratios (6.2±1.1) which were comparable with those previously reported in control anaesthetised sheep (5.7±0.4) (53). Although reduced PaO_2/FiO_2 ratios can indicate injury-associated impairment of oxygen extraction, the observed decrease in PaO_2/FiO_2 ratios during shock were likely caused by reduced pulmonary perfusion pressure. Ongoing studies of the effects of resuscitation regimens on inflammatory and endothelial glycocalyx markers in our model will provide further insight into appropriate treatments for shock.

Conclusions

We established an ovine model of haemorrhagic shock that reproduced the pathology observed clinically; hypotension, reduced tissue perfusion and oxygen delivery, accumulating oxygen debt, inflammation, compromised endothelial glycocalyx, and tissue damage. The model's design enables investigation of the clinical indicators of recovery from haemorrhagic shock, applicable for pre-clinical studies of novel resuscitation strategies. Invasive organ monitoring confirmed the utility of NIRS in characterising changes in oxygen delivery associated with haemorrhage and shock, and arterial lactate was representative of oxygen debt accumulation across multiple tissues. Further research in this area will help define optimal outcome measures and clinical options applicable to Patient Blood Management.

Declarations

Ethics Approval and Consent to participate:

The Queensland University of Technology University Animal Ethics Committee approved this study (approval #1800000493).

Consent for publication:

Not applicable.

Availability of data and materials:

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Conflict of interest:

All authors declare no conflict of interest.

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Authors' contributions:

WBD, JPT, GLB, AS, JYS, DOI and JFF designed the study. GLB, CW, JSJ, SC, SR and CA provided surgical and/or clinical oversight of experiments. WBD directed the protocol. WBD, JPT, GS, SC, FTT and TS performed scientific procedures and post-hoc analyses. CP performed histopathology assessments. WBD, JPT, GLB and JYS drafted the manuscript, and all other authors provided critical overview and approved the manuscript.

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Supplementary material

Figures S1-S7, Table S1 (Supplementary material.pdf).

Abbreviations

NIRS: near infra-red spectroscopy; MAP: mean arterial pressure; SvO2: mixed venous oxygen saturation; PRBC: packed red blood cells; ROTEM: rotational thromboelastometry; ABG: arterial blood gas, NIRS: near infra-red spectroscopy; IDF: incident dark field sub-lingual imaging; PPV: proportion perfused vessels; PVD: perfused vessel density; APSI: average perfusion speed index; TBV: total blood volume; SVRI: systemic vascular resistance index; P/F ratio: PaO2/FiO2 ratio; TRALI: transfusion-related acute lung injury; CCO: continuous cardiac output; PEEP: positive end-expiratory pressure.

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Figure legends:

Figure 1. Experimental time line.

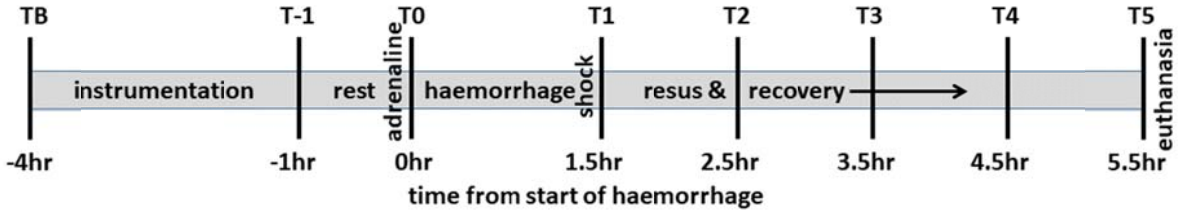

Figure 2. Primary outcome measures associated with shock and recovery.

Mean and individual animal data for (a) mean arterial pressure; (b) central venous oxygen saturation; (c) cerebral and (d) peripheral muscle regional tissue oxygen saturation (NIRS); (e) arterial lactate; and (f) base excess. Graph areas shaded green define treatment targets for the primary outcomes. MAP, SvO_2 and lactate acted as targets to guide haemorrhage. Time scale in hours from start of haemorrhage; haemorrhage and resuscitation indicated by H and R, respectively. Significance thresholds for variance from baseline: * $p<0.05$, † $p<0.01$, ‡ $p<0.001$ (Dunn's post-tests, Friedman's ANOVA).

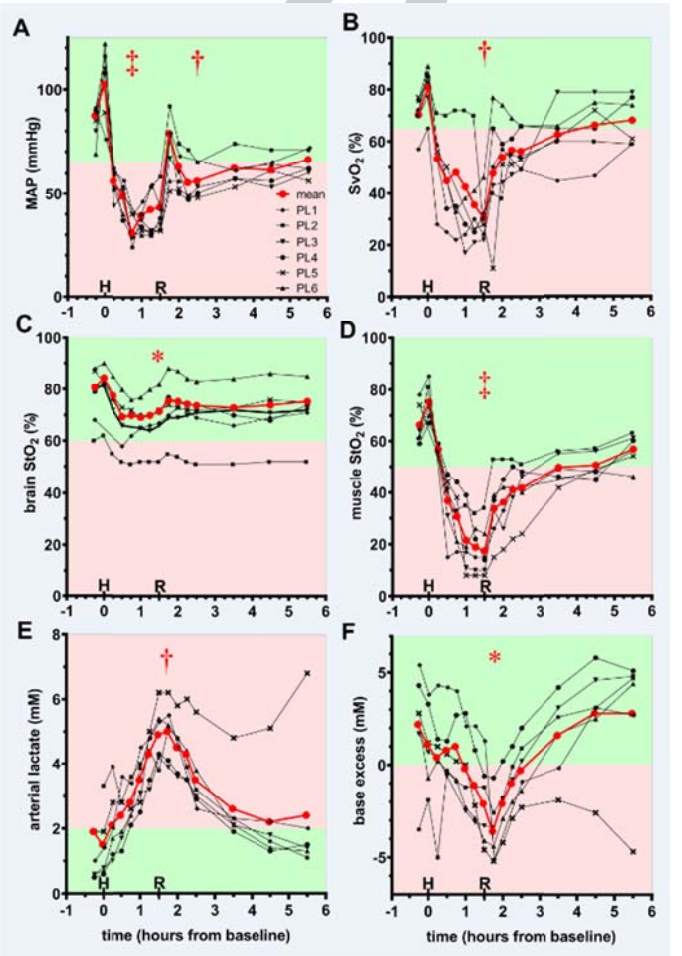

Figure 3. Invasive assessment of organ-specific microvascular flow, oxygen tension and oxygen debt during shock and recovery.

Time scale in hours from start of haemorrhage, with haemorrhage and resuscitation indicated by H and R, respectively. Mean ± SD. Significance thresholds for variance from baseline: * p<0.05, † p<0.01, ‡ p<0.001 (Dunn's post-tests, Friedman's ANOVA).

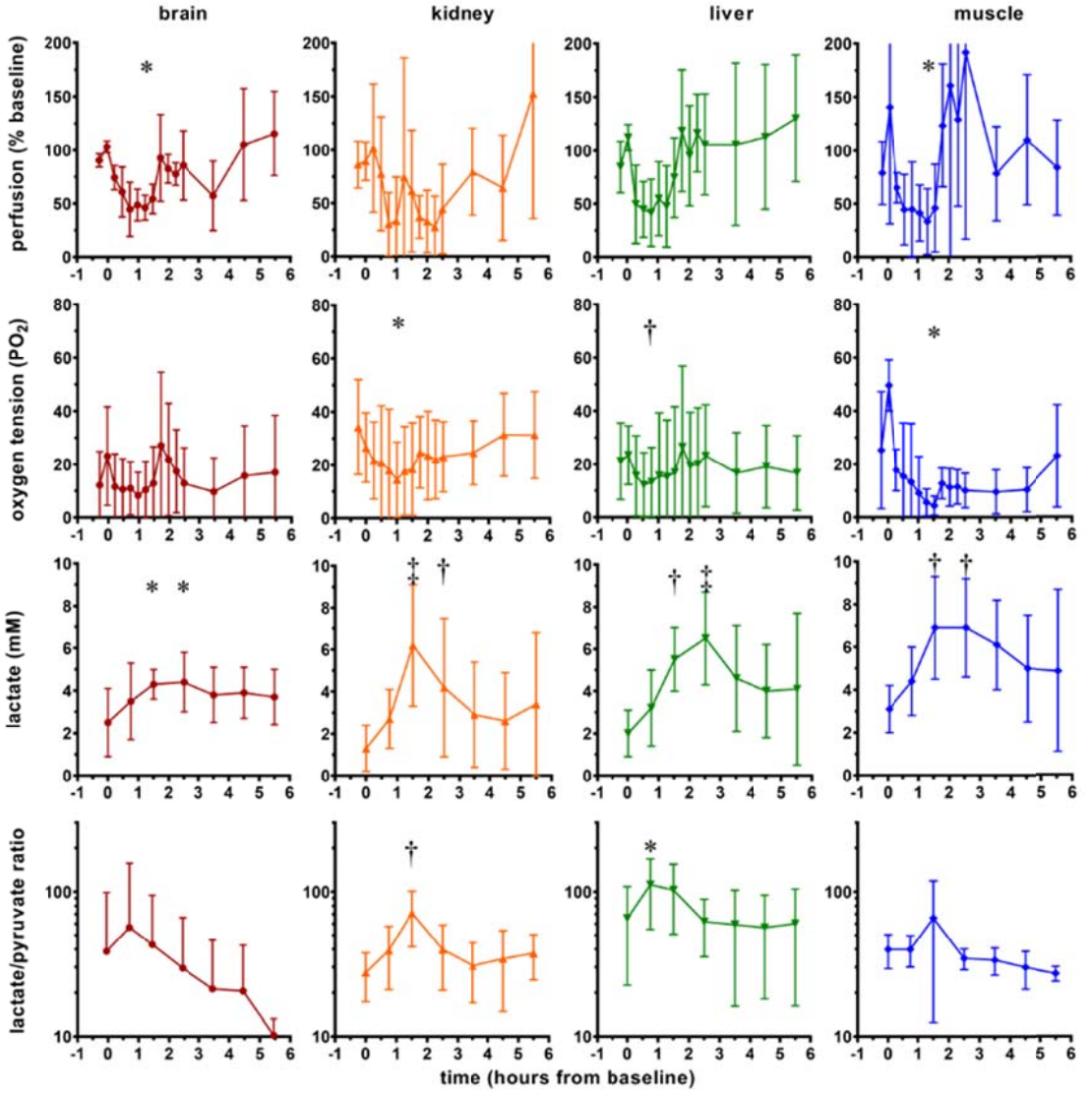

Figure 4. Clinical measures of tissue oxygen delivery and debt compared to organ-specific measures.

Mean and individual animal data for (a) cerebral and peripheral muscle regional tissue oxygen saturation (NIRS), compared to (b) organ-specific oxygen tension; and (c) arterial lactate, compared to (d) organ-specific lactate sampled by micro-dialysis.

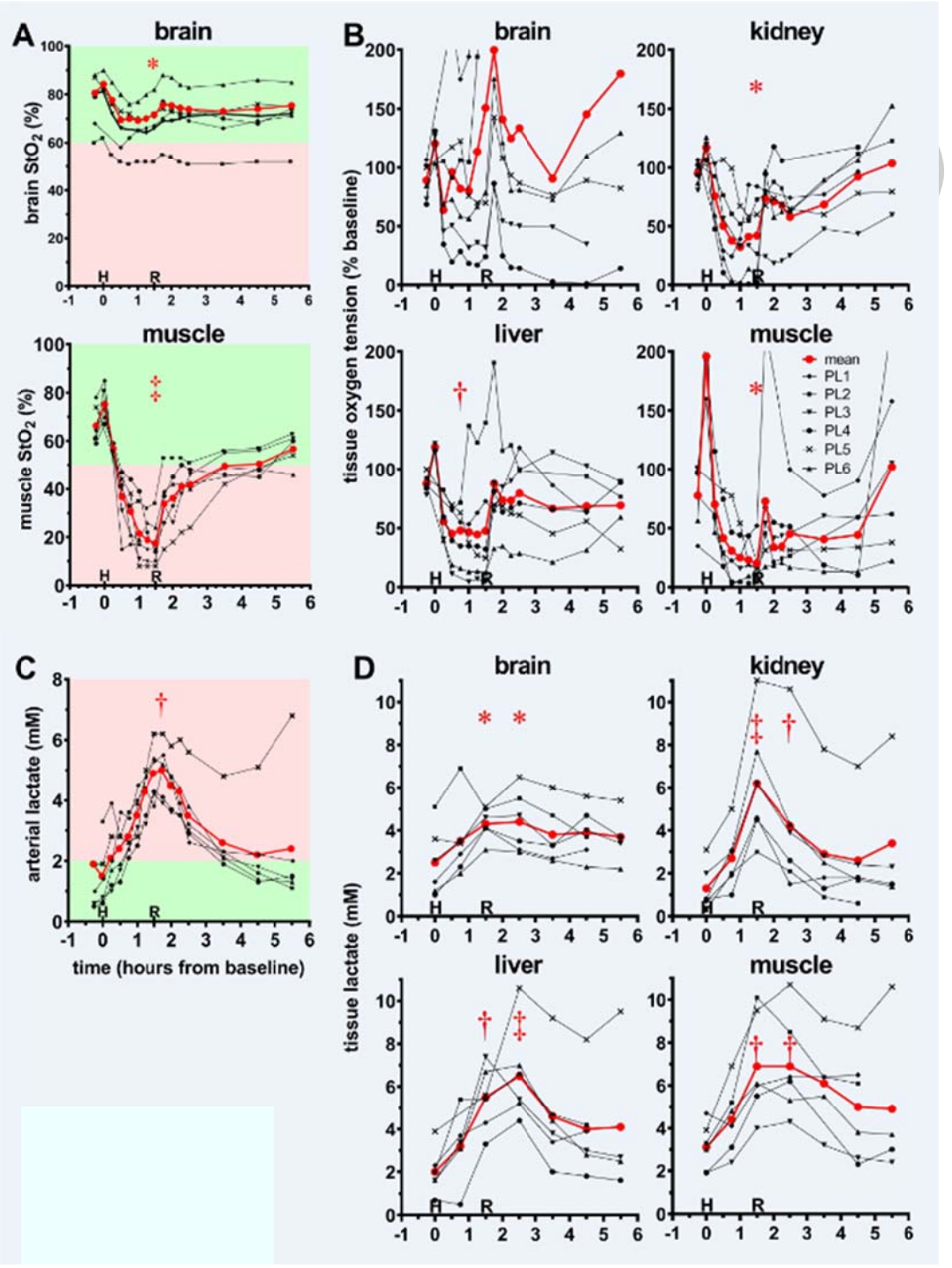

Figure 5. Impact of haemorrhagic shock and resuscitation on inflammatory markers. Changes in circulating inflammatory cytokines (a) IL-6, (b) IL-8, (c), IL-10, (d) IL-1β; shedding of endothelial glycocalyx component hyaluronan (e); and increase in circulating neutrophils (f).

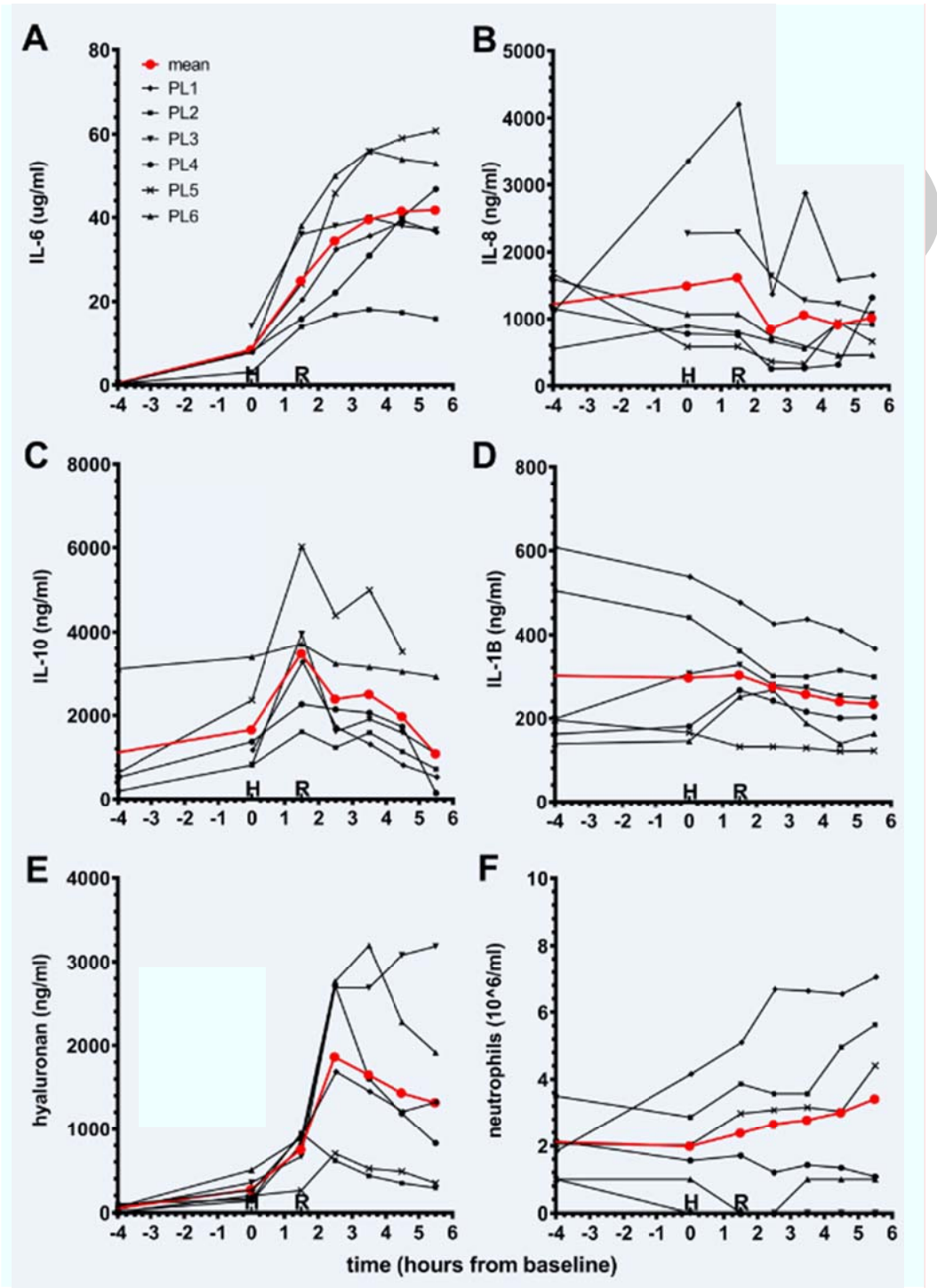

Figure 6. **Representative haematoxylin and eosin stained tissue sections demonstrating lesions associated with haemorrhagic shock.**
(a) Lung: multifocal alveolar oedema (asterisks) with diffuse congestion; (b) brain: perivascular oedema (arrows) and congestion; (c) heart: focal necrosis (arrow) and vacuolation (arrowhead) of myocardiocytes with infiltration of low numbers of neutrophils; (d) kidney: dilation of Bowman's capsules (arrows) with accumulation of eosinophilic material within the renal tubules (asterisks); (e) liver: infiltration of low numbers of neutrophils within the sinusoids (circles); (f) small intestine: sub-epithelial space with moderate lifting of the epithelial layer from the lamina propria (arrows).

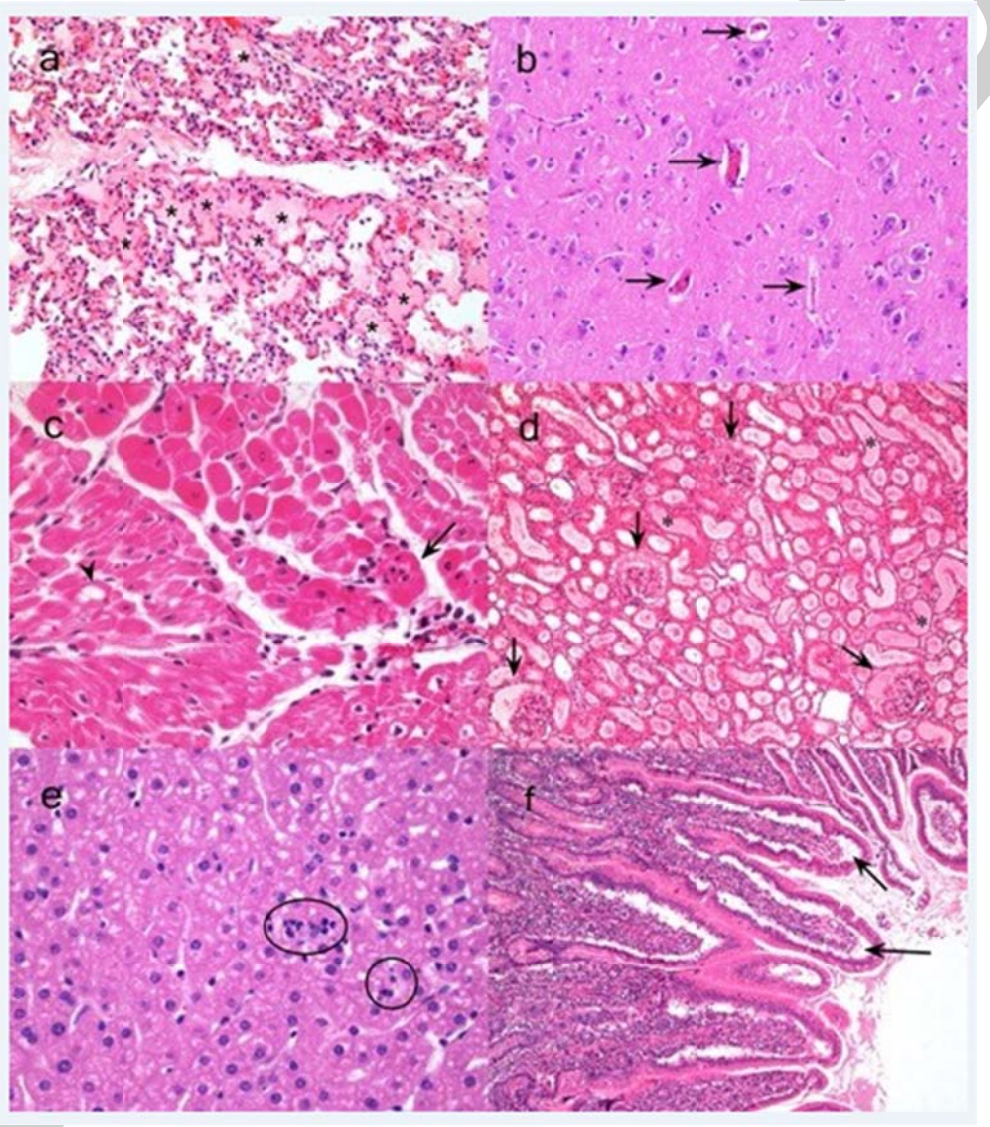

Table 1. Baseline characteristics, haemorrhage volumes and fluid requirements.

Animal ID	PL1	PL2	PL3	PL4	PL5	PL6	Mean ± SD
Weight (kg)	48	54	58	59	52	50	54.6 ± 3.8
Pre-adrenaline haemoglobin (g/dL)	8	5.1	10.3	8.6	8.1	10	8.4 ± 1.9
Post-adrenaline haemoglobin (g/dL)	10	9.5	11.5	10.6	10.4	12.7	10.8 ± 1.2
Total blood volume loss (ml)[1]	1200	1380	1500	1605	1756	1351	1465 ± 198
% total blood volume lost[1]	37%	38%	39%	41%	50%	40%	40.8 ± 4.7%
Haemorrhage time (min)	60	65	75	84	88	59	71.8 ± 12.4
Resuscitation volume (ml)	1320	1830	5410	5510	5254	2917	3707 ± 1918
Resuscitation time (min)	35[2]	87[2]	240	240	240	240	180 ± 94
Resuscitation : haemorrhage ratio	1.1	1.3	3.6	3.4	3.0	2.2	2.4 ± 1.1
Average resuscitation rate (ml/kg/hr)	47.1	23.4	23.3	23.3	25.3	14.6	26.2 ± 10.9

[1]Total blood loss at start of resuscitation included approximately 250ml iatrogenic loss from sampling and surgery.
[2]Resuscitation concluded early in the 4hr recovery period because MAP was sustained >65mmHg.

Table 2. Primary and secondary outcomes defining haemorrhagic shock and recovery after resuscitation with PlasmaLyte®.

	baseline (T0) unit	shock (nadir or peak) unit	[2]p value	study end unit	[3]recove time (hr)	[1]ANOVA
Primary outcomes:						
MAP (mmHg)	87 ± 4	31 ± 5	<0.01	64 ± 6	2	0.0001
SvO_2 (%)	70 ± 7	23 ± 8	<0.05	68 ± 10	1	0.0012
StO_2-brain (%)	77 ± 11	65 ± 9	<0.01	72 ± 11	1	0.0022
StO_2-muscle (%)	66 ± 8	16 ± 9	<0.01	57 ± 6	1	0.0001
arterial lactate (mM)	1.5 ± 1.0	5.1 ± 0.8	<0.01	2.4 ± 2.2	1	0.0014
base excess (mM)	1.1 ± 2.2	-3.6 ± 1.7	<0.05	2.8 ± 3.8	1	0.0018
Secondary outcomes:						
heart rate	101 ± 12	159 ± 45	<0.05	123 ± 31	1	0.076
cardiac index (l/min/m^2)	4.1 ± 1.6	1.1 ± 0.4	<0.05	4.3 ± 1.8	1	0.002
SVRI (dynes*sec/cm^5/m^2)	1642 ± 810	3274 ± 1654	<0.05	1059 ± 450	1	0.007
PaO_2/FiO_2 ratio	322 ± 62	212 ± 53	<0.001	275 ± 83	1	0.0059
urinary output (ml/hr)	80 ± 31	19 ± 11	<0.05	79 ± 68	2	0.0081
[4]IDF- Proportion Perfused Vessels	87 ± 13	56 ± 19	NS	93 ± 8	NA	0.8
IDF- Perfused Vessel Density	9.0 ± 2.1	4.9 ± 1.6	NS	14.4 ± 6.3	NA	0.0342
IDF-Av. Perfusion Speed Index	4.0 ± 0.6	3.2 ± 0.2	NS	4.8 ± 0.2	NA	0.19
haemoglobin (g/dl)	10.8 ± 1.2	6.0 ± 0.8	<0.05	5.9 ± 1.1	>4	0.0154

[1]One way repeated measures ANOVA (Friedman's test), with Dunn's post-tests comparing [2]shock (nadir) with baseline (T0), and [3]recovery time defined as first hourly observation similar to baseline ($p>0.05$). [4]IDF: sublingual capillary imaging using an incident dark field camera. Mean ± SD. NS: not significant. NA: not applicable.