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**DEVELOPMENT OF A SMALL-  
VOLUME RESUSCITATION FLUID  
FOR TRAUMA VICTIMS**

Thesis submitted by

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BSc (James Cook University)

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For the degree of

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in the College of Medicine and Dentistry

James Cook University

Townsville, AUSTRALIA

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***This thesis is dedicated to  
John Richard Letson (27/10/1944 – 19/9/2012)***

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**Chapter 2:** Reversal of acute coagulopathy during hypotensive resuscitation using small-volume 7.5% NaCl adenosine, lidocaine, and Mg<sup>2+</sup> (ALM) in the rat model of severe haemorrhagic shock (*Crit Care Med* 2012; 40:2417-2422)

Thesis Title	Development of a Small-Volume Resuscitation Fluid for Trauma Victims		
Name of Candidate	Publication	Nature and extent of the intellectual input of each author	I confirm the candidate's contribution to this paper and consent to the inclusion of the paper in the thesis
Hayley Letson	Letson HL*, Pecheniuk NM, Mhango LP, Dobson GP. <u>Reversal of acute coagulopathy during hypotensive resuscitation using small-volume 7.5% NaCl adenosine and Mg<sup>2+</sup> in the rat model of severe hemorrhagic shock.</u> 2012 Crit Care Med 40:2417-2422	Letson, Pecheniuk and Dobson co-developed the research question. Letson performed the experimental protocol and sample collection. Mhango performed coagulation testing. Letson performed all data analyses and developed figures and tables. Pecheniuk and Dobson assisted with data interpretation. Letson and Dobson wrote the manuscript with editorial input and revisions by Pecheniuk and Mhango.	Name: Natalie Pecheniuk Signature:  Name: Lebo Mhango Signature:  Name: Geoffrev Dobson Signature:

**Chapter 3:** Small-volume 7.5% NaCl adenosine, lidocaine, and Mg<sup>2+</sup> has multiple benefits during hypotensive and blood resuscitation in the pig following severe blood loss: Rat to pig translation (*Crit Care Med* 2014; 42(5): e329-344)

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**Chapter 4:** Correction of acute traumatic coagulopathy with small-volume 7.5% NaCl adenosine, lidocaine, and Mg<sup>2+</sup> occurs within 5 minutes: A ROTEM analysis (*J Trauma Acute Care Surg* 2015; 78(4): 773-783)

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Hayley Letson	<u>Letson HL*</u> , Dobson GP. <u>Differential contributions of platelets and fibrinogen to early coagulopathy in a rat model of hemorrhagic shock.</u> 2016 <i>Thromb Res</i> 141:58-65	Letson and Dobson co-developed the research question. Letson performed the experimental protocol and all data collection. Letson performed all data analyses and developed figures and tables. Letson and Dobson produced the manuscript.	Name: Geoffrey Dobson Signature:

**Chapter 6:** 7.5% NaCl adenosine, lidocaine, and Mg<sup>2+</sup> (ALM) resuscitation corrects fibrinolysis in the rat model of severe haemorrhagic shock

*Co-author:* Professor Geoffrey P. Dobson

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**Chapter 8:** Adenosine, lidocaine, and Mg<sup>2+</sup> (ALM): From cardiac surgery to combat casualty care – Teaching old drugs new tricks (*J Trauma Acute Care Surg* 2016; 80: 135-145)

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Hayley Letson	Dobson GP, <b>Letson HL*</b> . <u>Adenosine, lidocaine, and Mg<sup>2+</sup> (ALM): From cardiac surgery to combat casualty care – Teaching old drugs new tricks</u> . 2016 <i>J Trauma Acute Care Surg</i> 80:135-145	Letson and Dobson contributed equally to the design, implementation literature analysis, and writing of the manuscript.	Name: Geoffrey Dobson Signature:

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The studies presented in this thesis were completed in 2014. I had to delay writing the thesis and papers contained within because we were granted a contract from US Special Operations Command (USSOCOM) to further develop the small-volume ALM therapy for internal blood loss and traumatic brain injury (TBI) for far-forward use. These studies required new methodologies and have now been completed. Although the topics of internal blood loss and TBI were not part of the present thesis, I have provided a brief of the major findings in the General Discussion (Chapter 9).

## AWARDS/PRESENTATIONS

- 2015** Honourable Mention for “*Differential Contributions of Platelets and Fibrinogen to Early Hypocoagulopathy during Haemorrhage and Shock, and its Correction with 7.5% NaCl ALM*” Military Health System Research Symposium (MHSRS)
- 2015** Fresh Science Alumnus, Science in Public, Australia
- 2013** Best Abstract Award (Trauma) at the American Heart Association Resuscitation Science Symposium (ReSS) for “*Small-Volume 7.5% NaCl Adenocaine/Mg<sup>2+</sup> Preserves Cardiac Function During Hypotensive Resuscitation in the Pig Following Severe Haemorrhagic Shock*”
- 2013** Young Investigator Award for “*Rat To Pig Translation: Small-Volume 7.5% NaCl Adenocaine/Mg<sup>2+</sup> Has Multiple Physiological Benefits During Hypotensive And Blood Resuscitation In The Porcine Model Of Severe Haemorrhagic Shock*” presented at the Resuscitation Science Symposium (ReSS) (American Heart Association)
- 2012** “So You Think You Can Research” Winner for “*Development of a Revolutionary Small-Volume Resuscitation Fluid for Trauma Victims*” at North Queensland Festival of Life Sciences
- 2012** Young Investigator Award for “*Reversal of Acute Coagulopathy Using Small Volume 7.5% NaCl with Adenocaine and Mg<sup>2+</sup> Resuscitation in the Rat Model of Severe Haemorrhagic Shock*” being presented at the Resuscitation Science Symposium (ReSS) (American Heart Association)

## ABSTRACT

**Objective:** Traumatic haemorrhagic shock is a leading cause of mortality and morbidity on the battlefield and in civilian populations. In October 2016 the American College of Surgeons referred to it as ‘a neglected public health emergency’, highlighting the need for improved therapies in these environments. Small-volume 7.5% NaCl ALM had previously demonstrated some promising resuscitative capabilities in two rat models of haemorrhagic shock. The major aim of this thesis was to further investigate and develop this small-volume fluid following severe haemorrhagic shock in the rat and pig.

**Methods:** Haemorrhagic shock was induced in the rat by 20 min phlebotomy to decrease mean arterial pressure (MAP) to 35-40 mmHg followed by 60 min hypovolaemic shock. Small-volume (~0.7-1.0 ml/kg) 7.5% NaCl ± ALM resuscitation bolus was administered IV after 60 min shock, and haemodynamics were monitored for 60 min. Coagulopathy was assessed with PT, aPTT, ROTEM, and ELISAs. In the pig, phlebotomy reduced MAP to 35-40 mmHg, and 4 ml/kg 7.5% NaCl ± ALM resuscitation bolus was administered after 90 min shock. After 60 min single bolus resuscitation, shed blood volume was reinfused and pigs were monitored for 180 min. Metrics included haemodynamics, cardiac function, oxygen consumption, metabolic status, and kidney function.

**Results:** Small-volume 7.5% NaCl ALM induced and maintained a permissive hypotensive state (MAP 64-69 mmHg) with significantly higher pulse pressure in the rat after 41-42% blood loss and 60 min shock. ALM-treated animals also maintained significantly higher body temperature than saline controls (34°C vs. 32°C;  $p < 0.05$ ), and corrected haemorrhagic-shock induced hypocoagulopathy within 5 min of the single bolus administration with a reversal of PT and aPTT times to baseline, and restoration of ROTEM EXTEM, INTEM, and FIBTEM clots. ALM also reversed hyperfibrinolysis, and led to higher PAI-1 levels and lower D-dimers (8% of saline controls at 60 min) further supporting an ALM anti-fibrinolytic effect. Lower P-selectin in ALM-treated animals may indicate improved endothelial function, and reduced platelet dysfunction and inflammation, however this requires further investigation.

In the pig model of 75% blood loss and 90 min shock, 4 ml/kg 7.5% NaCl ALM had significantly higher MAP (48 mmHg vs. 33 mmHg;  $p<0.0001$ ), significantly higher cardiac index (76 ml/min/kg vs. 47 ml/min/kg;  $p<0.033$ ), significantly lower arterial lactate (7.1 mM vs. 11.3 mM), and higher base excess, compared to 7.5% saline controls. Higher cardiac index was associated with two-fold higher stroke volume and significantly increased left ventricular systolic ejection times. After return of shed blood, whole body oxygen consumption decreased in ALM-treated pigs from 5.7 ml O<sub>2</sub>/min/kg to 4.9 ml O<sub>2</sub>/min/kg. After 180 min blood resuscitation, pigs that received 7.5% NaCl ALM had three-fold higher urinary output (2.1 ml/kg/hr vs. 0.7 ml/kg/hr;  $p=0.001$ ), significantly lower plasma creatinine levels, and significantly higher creatinine clearance ratio, compared to saline controls.

**Conclusions:** In the rat model of severe haemorrhagic shock, a single small-volume 7.5% NaCl ALM bolus rescued the heart, resuscitated into the permissive hypotensive range, protected against hypothermia, corrected trauma-induced hypocoagulopathy within 5 min, reversed hyperfibrinolysis, and possibly protected endothelial protection. In the pig model, ALM fluid also significantly improved left ventricular-arterial coupling, reduced whole body oxygen consumption, restored acid-base balance, and protected renal function. These multi-factorial restorative and protective effects may be explained by the ALM resynchronization hypothesis, and may involve nitric oxide as a possible mediator, as well as modulation of autonomic nervous system outputs. Further investigations are required to elucidate the underlying mechanisms of small-volume ALM resuscitation, as well as translational studies for possible human use.

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## LIST OF ABBREVIATIONS

A: Adenosine  
A: Clot amplitude  
ACF: Actual clot firmness  
ACS: American College of Surgeons  
ACoT: Acute coagulopathy of trauma  
ACoTs: Acute coagulopathy of trauma/shock  
ADP: Adenosine diphosphate  
AITHM: Australian Institute of Tropical Health and Medicine  
AL: Adenosine + lidocaine  
ALM: Adenosine, lidocaine and magnesium  
ALT: Alanine aminotransferase  
ANOVA: Analysis of variance  
aPC: Activated protein C  
aPTT: Activated partial thromboplastin time  
ARDS: Acute respiratory distress syndrome  
AST: Aspartate aminotransferase  
ATC: Acute traumatic coagulopathy  
ATP: Adenosine triphosphate  
AV: Arterial-venous  
BH4: Tetrahydrobiopterin  
BOB: Blood on board  
BP: Blood pressure  
BT: Body temperature  
C: Oxygen content  
Ca<sup>2+</sup>: Calcium  
CABG: Coronary artery bypass graft  
CaCl<sub>2</sub>: Calcium chloride  
CBC: Complete blood count  
CCPA: 2-chloro-N<sup>6</sup>-cyclopentyladenosine  
CCVE: Central-CardioVascular-Endothelium  
CFR: Clot formation rate  
CFT: Clot formation time

CI: Cardiac index  
CI: Confidence interval  
Cl<sup>-</sup>: Chloride  
CLP: Caecal ligation and puncture  
CNS: Central nervous system  
CO: Cardiac output  
CPD: Citrate phosphate dextrose  
CRASH-2: Clinical Randomisation of an Antifibrinolytic in Significant Haemorrhage  
CRP: C-reactive protein  
CT: Clotting time  
CT: Component therapy  
CV: Coefficient of variation  
CVE: Cardiovascular-Endothelium  
CVP: Central venous pressure  
DIC: Disseminated intravascular coagulation  
DO<sub>2</sub>: Tissue oxygen delivery  
DP: Diastolic pressure  
 $dP/dt_{max}$ : Maximum positive rate of ventricular pressure development over time  
 $dP/dt_{min}$ : Maximum negative rate of ventricular pressure decrease over time  
ECG: Electrocardiogram  
EGF: Endothelial growth factor  
EF: Ejection fraction  
ELISA: Enzyme-linked immunosorbent assay  
EMA: European Medicines Agency  
eNOS: Endothelial nitric oxide synthase  
EPCR: Endothelial protein C receptor  
F<sub>1+2</sub>: Prothombin fragment 1+2  
FDA: Food and Drug Administration  
FDP: Fibrin degradation product  
FFP: Fresh frozen plasma  
FiO<sub>2</sub>: Fraction of inspired oxygen  
FWB: Fresh whole blood  
GFR: Glomerular filtration rate  
H<sup>+</sup>: Hydrogen



Hb: Haemoglobin  
HCl: Hydrochloride  
 $\text{HCO}_3^-$ : Bicarbonate  
Hct: Haematocrit  
HES: Hetastarch  
HF: High frequency  
HMGB1: High mobility group box protein 1  
HPA: Hypothalamic-pituitary-adrenal axis  
HR: Heart rate  
HRV: Heart rate variability  
HSD: Hypertonic saline dextran  
ICAM-1: Intracellular adhesion molecule 1  
ICP: Intracranial pressure  
ICU: Intensive care unit  
IFN- $\gamma$ : Interferon-gamma  
IL: Interleukin  
iNOS: Inducible nitric oxide synthase  
INR: International Normalized Ratio  
IO: Intraosseous  
IR: Ischaemia reperfusion  
ISTH: International Society for Thrombosis and Hemostasis  
IV: Intravenous  
JCU: James Cook University  
 $\text{K}^+$ : Potassium  
L: Lidocaine  
LF: Low frequency  
LI: Lysis index  
L-NAME:  $N^G$ -nitro-*L*-arginine methyl ester  
LOT: Lysis onset time  
LPS: Lipopolysaccharide  
LV: Left ventricle  
LVEDP: Left ventricular end diastolic pressure  
LVESP: Left ventricular end systolic pressure  
M: Magnesium

MAP: Mean arterial pressure  
MaxV: Maximum clot velocity  
MaxVt: Time to maximum clot velocity  
MCE: Maximum clot elasticity  
MCF: Maximum clot firmness  
MCFt: Time to maximum clot firmness  
Mg<sup>2+</sup>: Magnesium  
MgSO<sub>4</sub>: Magnesium sulphate  
MHSRS: Military Health System Research Symposium  
ML: Maximum lysis  
MPV: Mean platelet volume  
mTBI: Mild traumatic brain injury  
Na<sup>+</sup>: Sodium  
NaCl: Sodium chloride  
NAG: N-acetyl-β-D-glucosaminidase  
NATO: North Atlantic Treaty Organization  
NIH: National Institutes of Health  
NMRC: Naval Medical Research Center  
nNOS: Neuronal nitric oxide synthase  
NO: Nitric oxide  
NOS: Nitric oxide synthase  
NSE: Neuron specific enolase  
NTS: Nucleus tractus solitarius  
NY: New York  
P: Plasma creatinine concentration  
PaCO<sub>2</sub>: Partial pressure of carbon dioxide  
PAI-1: Plasminogen activator inhibitor-1  
PCr: Phosphocreatine  
PEEP: Positive end-expiratory pressure  
PF: Platelet factor  
PLT: Platelet  
PMNs: Polymorphonuclear neutrophils  
PO<sub>2</sub>: Partial pressure of oxygen  
PP: Pulse pressure

PRBC: Packed red blood cells  
P-selectin: Platelet-selectin  
PT: Prothrombin time  
PVN: Paraventricular nucleus  
QUT: Queensland University of Technology  
RANTES: Regulation on activation, normal T-cell expressed and secreted  
RBC: Red Blood Cell  
ReSS: Resuscitation Science Symposium  
ROC: Resuscitation Outcomes Consortium  
ROSC: Return of spontaneous circulation  
ROTEM: Rotational thromboelastometry  
SEM: Standard error of the mean  
SO<sub>2</sub>: Oxygen saturation  
SP: Systolic pressure  
SV: Stroke volume  
SVR: Systemic vascular resistance  
SVRI: Systemic vascular resistance index  
TAFI: Thrombin activatable fibrinolysis inhibitor  
TAT: Thrombin-antithrombin complex  
TBI: Traumatic brain injury  
TCCC: Tactical combat casualty care  
TEG: Thromboelastography  
TEM: Temogram  
TIC: Trauma-induced coagulopathy  
TF: Tissue factor  
Th: T-helper cell  
tHb: Total haemoglobin  
TM: Thrombomodulin  
TNF- $\alpha$ : Tumour necrosis factor-alpha  
tPA: Tissue plasminogen activator  
TTE: Transthoracic echocardiogram  
TXA: Tranexamic acid  
U: Urine creatinine concentration  
US: United States

USD: United States dollars

USSOCOM: United States Special Operations Command

V: Urine volume

VA: Ventricular-arterial

VF: Ventricular fibrillation

VO<sub>2</sub>: Oxygen consumption

vWF: Von Willebrand factor



## CHAPTER 1

### INTRODUCTION

*“The tragedies of life are largely arterial”*

Sir William Osler (1908) <sup>1</sup>

#### 1.1 THE MAJOR BURDEN OF TRAUMA

Traumatic injury is responsible for approximately six million or 10% of all deaths worldwide each year <sup>2</sup>, and is the leading cause of death in people aged 1-44 years <sup>3</sup>. In the United States, traumatic injury causes in aggregate the loss of more years of life than any other source of illness or disability <sup>4</sup>, and more than two million American citizens have died from trauma since 2001 <sup>5</sup>. In Australia, injury is the fourth most common cause of death with more than 10,000 deaths annually, and a mortality rate of 46.7 deaths per 100,000 population <sup>6-8</sup>. The burden of traumatic injury is even greater in regional and remote areas where one-third of Australia’s population resides <sup>7</sup>. Trauma is a significant burden to the health care system in both Australia and the U.S. with an annual cost to society in excess of \$4.1 billion and \$670 billion in these countries respectively <sup>9-11</sup>. This is an escalating problem with total health expenditure on injury anticipated to increase more than 100% over the next decade <sup>10</sup>.

Haemorrhage is responsible for 30-40% of civilian trauma deaths each year globally <sup>12</sup>, with one-third to one-half of these deaths occurring in the pre-hospital environment <sup>13</sup>. It has been estimated that as many as 20% of civilian trauma deaths may be prevented with optimal pre-hospital care, equating to nearly 30,000 preventable deaths in the United States, and more than 2,000 lives in Australia annually <sup>14</sup>. On the battlefield, catastrophic haemorrhage is responsible for up to 50% of trauma deaths, and up to 20% of these may also be salvageable <sup>15-17</sup>. A U.S. Joint Trauma System study reported 87% of the 4,596 combat deaths between 2001 and 2011 in Iraq and Afghanistan occurred before the casualty reached a medical treatment facility, and 24% of these deaths were potentially survivable <sup>4,18</sup>. This equates to approximately 1,000 American soldiers,

sailors, airmen, and marines dying of wounds they potentially could have survived <sup>18</sup>. Therefore, there is an urgent unmet need for improved methods to resuscitate and stabilise injured civilians and combatants in the pre-hospital environment, before transport to definitive care.

## 1.2 TRAUMA-HAEMORRHAGIC SHOCK

*‘Shock is a momentary pause in the act of death’*

John Collins Warren (1895) <sup>19</sup>

John Collins Warren’s description of shock as a ‘momentary pause in the act of death’ still remains accurate today if the diagnosis is missed or delayed. Haemorrhagic shock arises from insufficient cardiac output leading to systemic hypotension and widespread tissue hypoperfusion <sup>20</sup>. It can be considered as the final pathway through which a variety of pathological processes lead to cardiovascular collapse and death. While a large number of patients die early from exsanguination, approximately 20% of patients suffer late-stage mortality in the days to weeks after injury from the trauma-related secondary ‘hit’ complications arising from the host’s pathophysiological response to trauma-haemorrhage <sup>21</sup>. These secondary ‘hit’ complications include ischaemia and hypoxia <sup>22</sup>, acidosis <sup>23</sup>, hypothermia <sup>24</sup>, coagulopathy <sup>25</sup>, inflammation <sup>26</sup>, endothelial dysfunction <sup>27</sup>, and multiple organ failure <sup>28</sup>. In addition to restoration of cardiac output, optimal pre-hospital trauma care will address and prevent these secondary ‘hit’ complications as early as possible from the point of injury, thereby improving patient outcomes and reducing mortality and morbidity.

## 1.3 FLUID RESUSCITATION

*“While the widespread training of medics in tactical combat casualty care (TCCC) has clearly saved lives, the use of saline and colloid starch by medics on the battlefield does not represent a significant technological advance in ability since saline was first used for resuscitation in 1831”*

Blackbourne *et al.* (2010) <sup>29</sup>

This quote by Blackbourne and colleagues is alarming but true: there have been no new advances in fluid therapy for nearly 200 years. Many fluids, as we have argued in our previous publications<sup>30,31</sup>, shock the body a second time and new innovation is urgently required. Together with haemostasis, fluid resuscitation is one of the fundamentals of pre-hospital treatment for haemorrhagic shock and is designed to sustain tissue perfusion and protect organ function until definitive intervention<sup>32-34</sup>.

Despite a large number of preclinical and clinical studies, there is still no consensus on the optimal pre-hospital fluid resuscitation strategy for traumatic haemorrhagic shock<sup>32,35-41</sup>. Crystalloid fluids with or without colloids including albumin<sup>42</sup>, dextrans<sup>43-47</sup>, and hetastarches<sup>48-51</sup>, have either failed to translate to humans or been withdrawn from use due to safety concerns<sup>52-58</sup>. Other pharmacological adjuncts that have been investigated with limited success include calcium-channel blockers<sup>59</sup>, ATP-pathway modifiers<sup>60</sup>, pyruvate<sup>61,62</sup>, Na<sup>+</sup>/H<sup>+</sup> exchange inhibitors such as amiloride<sup>63</sup>, phosphodiesterase inhibitors such as pentoxifylline<sup>64-66</sup>, dehydroepiandrosterone<sup>67,68</sup>, and antioxidants such as platonin<sup>69</sup> and Tempol<sup>70</sup>. Valproic acid and 17 $\beta$ -oestradiol have shown a number of promising clinically-relevant attributes, including anti-inflammatory effects, endothelial protection, and multiple organ support including neuroprotection and improved cardiac contractility, and further translational studies are required<sup>71-79</sup>.

The aim of this thesis is the development of a new small-volume resuscitation fluid for treatment of trauma and haemorrhage on the battlefield and in the civilian sector, with important unmet need applications in rural and remote environments.

#### 1.4 WHAT CONSTITUTES AN 'IDEAL' RESUSCITATION FLUID

*'Although the use of resuscitation fluids is one of the most common interventions in medicine, no currently available resuscitation fluid can be considered to be ideal'*

Myburgh JA and Mythen MG (2013)<sup>38</sup>

What constitutes an ideal fluid therapy must rely on a strong understanding of the underlying pathophysiology of what you are attempting to reverse or treat. At the very



least the key is to resuscitate the heart, and all its coupling functions to various parts of the body, with the goal to ensure adequate tissue oxygenation including to the brain<sup>80</sup>. Table 1 lists some of the ‘ideal’ characteristics that may constitute an improved therapy for military and civilian environments. It is important to note these characteristics are not mutually exclusive.

Table 1.1: Some Key Properties of the ‘Ideal’ Resuscitation Fluid

<ul style="list-style-type: none"><li>• Safe, efficacious, and cost-effective</li><li>• Rescue and stabilise the heart</li><li>• Low cube weight to maximise benefit to casualty ratio</li><li>• Ability to resuscitate mean arterial pressure (MAP) into the permissive range to restore tissue perfusion without causing re-bleeding</li><li>• Correct trauma-induced coagulopathy</li><li>• Blunt the inflammatory cascade</li><li>• Prevent endothelial dysfunction</li><li>• Prevent multiple organ dysfunction and failure</li><li>• Prevent secondary brain injury</li><li>• Protect against hypothermia</li><li>• Restore acid-base balance</li><li>• Efficiently repay oxygen debt</li><li>• Stable in different environments</li><li>• Easy to administer (intravenous or intraosseous)</li><li>• Possess analgesic properties</li></ul>
--

#### 1.4.1 Safety, Efficacy, and Cost

First and foremost, safety and efficacy of any new resuscitation fluid must be established through preclinical animal studies followed by randomised controlled trials in patients with different trauma states and severities. Safety concerns surrounding Ringer’s lactate, a crystalloid solution, as well as the synthetic colloidal plasma volume expander hetastarch (HES), were not adequately addressed prior to widespread clinical use<sup>30,56,81-83</sup>. The composition of Ringer’s lactate was eventually changed from a racemic mix of D- and L-lactate isoforms to pure L-lactate when the D-isomer was found

to be neurotoxic, and associated with multiple cardiac complications including ventricular tachycardia, ventricular fibrillation, sinus bradycardia, third-degree heart block, and asystole <sup>84,85</sup>.

Hetastarch was first recommended in 2001 by a Joint Taskforce from the U.S. Army Medical Research and Material Command and the Office of Naval Research, and has also been used extensively in Level 1 Trauma Centres and peri-operatively in general surgery since that time <sup>86</sup>. The military adoption of HES for initial battlefield resuscitation was based on need and cube weight (see 1.4.3) to reduce the logistical burden of medics carrying large volumes of crystalloid solutions, and to avoid fluid overload <sup>49,87</sup>. Before 2012, Tactical Combat Casualty Care (TCCC) guidelines recommended Hextend® (6% HES 670/0.7 in Lactated Ringer's) for hypotensive resuscitation of haemorrhagic shock.

In 2010, one of the key proponents of HES, Dr. Joachim Boldt, was accused of misrepresenting clinical trial data <sup>88</sup>, and following further investigation nearly 90 refereed publications, many of which formed the basis of clinical guidelines for HES fluid therapy, were retracted from 11 leading journals. During a period of 10 years when Boldt and colleagues were claiming clinical safety and efficacy of hetastarches <sup>89,90</sup>, an increasing number of animal and clinical studies were showing adverse events including increased risk for bleeding complications, heart and kidney failure, shock, and death <sup>56,91,92</sup>.

Some of the key studies reporting adverse effects of HES include that of Bechir and colleagues who demonstrated fatal outcomes within the first 24 hours after severe burn injury in humans following the use of a second generation 10% hetastarch (HES 200/0.5) <sup>93</sup>. Similarly, a 2012 study comparing the effects of Voluven®, a third generation HES (HES 130/0.4), with Ringer's Acetate in 798 patients with sepsis, reported an increase in absolute risk of death <sup>94</sup>. Furthermore, a review of 56 randomized trials by Hartog and colleagues concluded that the safety of Voluven® had not adequately been addressed <sup>56</sup>. Ogilvie and colleagues examined the effect of 500 to 1000 ml Hextend® on 1714 patients (805 received Hextend®) at a Level 1 Trauma Centre in Florida <sup>95</sup>. Although the study was limited in not being blinded or randomised, the Hextend® group had significantly more intensive care unit admissions (41% vs.

35%), with a larger number of blood transfusions (34% vs. 20%) and plasma transfusions (20% vs. 12%), and significantly higher rates of septic shock and acute respiratory distress syndrome <sup>95</sup>.

In response to the growing number of experimental studies and randomised trials cautioning HES use, on June 24 2013 the U.S. Federal Drug Administration (FDA) issued a “black box warning” and concluded that HES solutions should not be used to treat hypovolaemic patients, the critically ill (including patients with sepsis), or patients undergoing cardiac surgery <sup>96</sup>. In the same month, the European Medicines Agency (EMA) formally suggested that HES be banned. Following on from this, the updated TCCC guidelines (June 2, 2014) revised their recommendations and stated that Hextend® was a less desirable option compared to whole blood, blood components, or dried plasma, and should only be used when preferred options are not available <sup>48</sup>.

Anaphylactic reactions should always be a consideration with any fluid being administered, particularly in far-forward and remote environments away from secondary or tertiary medical treatment facilities. Hetastarches and other colloids including dextrans can cause the release of vasoactive mediators and result in severe anaphylactoid reactions <sup>97-99</sup>.

Cost is obviously secondary to safety and efficacy but is also an important consideration because more than 90% of the 5.8 million civilian trauma deaths annually occur in low- and middle-income countries <sup>100,101</sup>, and it is in these areas where optimal point of injury fluid resuscitation could have the greatest impact in significantly reducing preventable and potentially survivable deaths. One of the disadvantages of colloid fluid therapy compared to crystalloids is the expense. A review of the per-patient cost of colloid therapy revealed it to be 30 times the cost of crystalloid therapy (\$1,176.21 vs. \$39.06) <sup>102</sup>. Average prices (USD) of commonly used resuscitation fluids per 1000 ml are \$6.97 for 0.9% saline, \$8.71 for Lactated Ringer’s, \$118.22 for Hextend, and \$56.70 for Dextran 40 solution <sup>103</sup>. Based on the cost-benefit ratio it can be concluded that colloid fluids are not ideal resuscitation fluids for treatment of traumatic haemorrhagic shock, particularly in developing nations.

#### 1.4.2 Cardiac Rescue and Stabilisation

Twenty years ago William Shoemaker and colleagues launched a global research challenge regarding the prevention of cardiac arrest in the bleeding patient<sup>104</sup>. The heart can be considered the “pressure generator”<sup>80</sup>, and therefore rescue and stabilisation of optimal heart function is critical for successful resuscitation from traumatic haemorrhagic shock. Cardiac dysfunction contributes to the high mortality of traumatic haemorrhagic shock<sup>105</sup>, with low cardiac index and stroke volume at hospital admission associated with worse outcomes in critically ill patients<sup>106</sup>. Myocardial depression associated with trauma and shock is multifactorial<sup>107,108</sup> involving pro-inflammatory signalling pathways<sup>109,110</sup>, toll-like receptors<sup>105</sup>, and gut-derived effector molecules carried in mesenteric lymph<sup>111</sup>.

There is a paucity of information on the effect of crystalloids and colloids on electrical stability of the compromised heart, which led to my first haemorrhagic shock study in 2011 described in more detail below (Section 1.5.1). Hypertonic saline with dextran (HSD) restored myocardial blood flow and corrected reductions in cardiac output and cardiac work in a sheep model of large burn injury<sup>112</sup>, and reductions in burn-related cardiac dysfunction with HSD were also seen in human patients with thermal injury<sup>113</sup>. However, in another study HSD only resulted in a temporary, short-lived increase in cardiac output with no augmentation of cardiac contractility<sup>114</sup>. Hypertonic saline alone is known to increase myocardial contractility<sup>115,116</sup>, and so it may be that these beneficial cardiac effects after resuscitation with HSD are not related to the colloid dextran, and further studies are required to elucidate the mechanism(s).

#### 1.4.3 Low Cube Weight

A low cube weight to maximise benefit to casualty is another important property of the ‘ideal’ resuscitation fluid and is a priority particularly in combat casualty care<sup>16,117</sup>. The capacity for fluid resuscitation at the point of injury depends on the amount of fluid that is available which, on the battlefield, is limited by the volume that can be carried by each medic and individual mission constraints. This restriction was perfectly illustrated by the case of R. Mabry who carried six litres of crystalloid fluid weighing 5.9 kg (13 lb.) while on assignment in Somalia in 1993. By the time the number of casualties

following heavy gunfire reached 12, the medic's intravenous fluids were depleted<sup>118</sup>. The logistical advantage afforded by a resuscitation fluid with a low cube weight would also be beneficial in rural and remote environments<sup>36</sup>. For example, in Australia, 40% of major trauma incidents occur in rural areas often some distance from secondary and tertiary care, and first responders and aeromedical retrievalists have limited resources<sup>119</sup>.

For many decades, traditional resuscitation involved the early administration of large volumes of crystalloids such as isotonic saline or Lactated Ringer's<sup>102,120-122</sup>. Given that one 1,000 ml bag of Lactated Ringer's weighs 1.1 kg or 2.4 lb. and has the capacity to expand the intravascular fluid volume by only approximately 250 ml<sup>50,123</sup>, weight and cube limitations restrict its use in far-forward environments<sup>124</sup>. In direct contrast, just 250 ml of hypertonic saline (7.5% NaCl) rapidly expands intravascular plasma volume by 1,000 ml<sup>102</sup>. In 1980, De Felippe and colleagues showed that small volumes (100-400 ml) of 7.5% NaCl successfully resuscitated 11 out of 12 patients in terminal hypovolaemic shock after larger volumes of isotonic crystalloids had failed<sup>125</sup>, and a multicentre trial in 1993 reported improved survival of patients during rapid urban transport when resuscitated with 250 ml hypertonic saline<sup>46</sup>. In addition to being low cube weight, hypertonic saline has many beneficial haemodynamic properties including increased myocardial contractility, positive microcirculatory effects including improved capillary patency and arteriolar flow, and decreased peripheral vascular resistance<sup>20,116,117,126-130</sup>. While small-volume hypertonic saline has not translated into clinical practice<sup>131,132</sup>, with multiple physiological and clinical benefits further research is necessary to evaluate its efficacy for resuscitation from traumatic haemorrhagic shock with other pharmacological adjuncts<sup>43,44</sup>.

As previously mentioned in 1.4.1, the recommendation for inclusion of the colloid Hextend® in the TCCC guidelines was largely based on cube weight. Hextend® and other hetastarches increase plasma volume between 1-1.5 times while expansion for Dextran-70 is 0.8, compared to only 0.25 times for Lactated Ringer's<sup>102</sup>. Notwithstanding that colloids are more efficient volume expanders than crystalloids, achieving resuscitation outcomes with smaller volumes; high costs, safety concerns (see 1.4.1), and a trend towards increased mortality preclude their use in critically ill patients.

#### 1.4.4 Permissive Hypotension

*“Injection of a fluid that will increase blood pressure has dangers in itself... If the pressure is raised before the surgeon is ready to check any bleeding that might take place, blood that is sorely needed may be lost”*

Walter B. Cannon (1918) <sup>133</sup>

An important characteristic of the ‘ideal’ resuscitation fluid is the smallest volume possible to raise mean arterial pressure to restore tissue perfusion without causing re-bleeding. That large volume intravenous fluid administration is not ‘ideal’ has been recognised since 1918 when Walter Cannon recommended a target systolic pressure of 70-80 mmHg in the bleeding combatant during World War I to avoid losing “more blood that is sorely needed” <sup>134</sup>. The limited fluid concept, termed permissive hypotension or hypotensive resuscitation, was further developed in World War II by Beecher and colleagues who suggested that, particularly in the instance of delayed surgical intervention, elevation of a patient’s systolic blood pressure to approximately 85 mmHg is all that is necessary <sup>135</sup>. However, since World War II, hypotensive resuscitation was not widely adopted by military services or civilian trauma centres in favour of traditional aggressive fluid resuscitation with large intravenous volumes targeting normal systolic pressures <sup>136,137</sup>. Renewed interest in hypotensive resuscitation occurred in the late 1980s and early 1990s following the Mattox trial of Bickell and colleagues demonstrating significantly improved survival (70% vs. 62%,  $p=0.04$ ), reduced post-operative complications, and shorter hospital stays, when pre-hospital fluid resuscitation was delayed in patients with penetrating torso injuries <sup>138</sup>.

Permissive hypotensive resuscitation is defined as the limited volume of intravenous (IV) or intraosseous (IO) fluid required to rescue a patient from haemorrhagic shock and raise mean arterial pressure (MAP) to ~50 to 60 mmHg with the goal to restore adequate tissue perfusion while preventing dislodgement of early thrombi <sup>137,139</sup>. Since the Mattox trial, subsequent preclinical studies and clinical trials have confirmed a role for permissive small-volume hypotensive resuscitation to treat severe trauma-haemorrhagic shock patients in out-of-hospital and in rural and remote environments where there is delayed access to surgical care <sup>52,140-145</sup>, and it has gained increasing acceptance in Level 1 Trauma Centres and on the battlefield <sup>15,40,48</sup>. The U.S. Military

Committee on Tactical Combat Casualty Care (TCCC) advocate the use of permissive hypotension to keep the severely wounded alive with a palpable pulse or consciousness and not to restore MAP to normal limits before definitive control of haemorrhage <sup>48</sup>.

#### 1.4.5 Correction of Trauma-Induced Coagulopathy

Coagulopathy resulting from severe trauma and tissue hypoperfusion was first reported in the Vietnam War <sup>146</sup> and confirmed by Brohi and colleagues in 2003 <sup>147</sup>. Acute traumatic coagulopathy (ATC), also known as trauma-induced coagulopathy (TIC), occurs early after injury, has been found to be present in up to 41% of severely injured adult patients on admission to hospital, and is independently associated with worse outcomes including a four-fold increase in mortality <sup>148-152</sup>. Two recent retrospective analyses of paediatric patients have demonstrated an association between elevated INR (International Normalized Ratio) and mortality in a diverse paediatric trauma population <sup>153</sup>, with presence of traumatic coagulopathy on hospital admission associated with a two- to four-fold increased risk of death <sup>154</sup>.

Coagulopathy, together with hypothermia (1.4.10) and acidosis (1.4.11), constitutes a condition associated with severe trauma and hypovolaemia known as “the lethal triad” or “the bloody vicious cycle” <sup>155-157</sup>. While coagulopathy, hypothermia, and acidosis individually can have lethal consequences; together they form a vicious cycle where each condition can significantly worsen the others rapidly leading to early mortality if the cycle remains unbroken <sup>158-160</sup>.

Fluid resuscitation can exacerbate TIC by damaging fibrinogen or fibrin, thereby weakening clots and leading to secondary bleeding <sup>161</sup>. Large aggressive resuscitation with crystalloids results in non-specific dilution of coagulation factors and platelets <sup>162</sup>, and coagulopathy <sup>163</sup>. Hypertonic crystalloid solutions also lead to imbalances in procoagulants and anticoagulants, and in turn worsen hypocoagulability and hyperfibrinolysis after traumatic haemorrhagic shock <sup>164</sup>. Colloids have adverse effects on systemic coagulation <sup>123</sup>, and have been associated with significant bleeding complications <sup>165,166</sup>, limiting their use in haemorrhaging patients. Hetastarches cause coagulopathy by reducing factor VIII and von Willebrand factor, inhibiting platelet function, and interfering with the interaction between activated factor XIII and fibrin

polymers<sup>38,167-170</sup>. Dextrans have powerful anticoagulant effects, reducing blood viscosity and platelet adhesiveness, decreasing factor VIII, and enhancing fibrinolysis<sup>171-174</sup>. Analysis of coagulation data from the Resuscitation Outcomes Consortium (ROC) trial showed patients administered a 250 ml bolus of 7.5% NaCl/6% Dextran 70 (HSD) in the pre-hospital environment were hypocoagulable with higher admission INR scores and evidence of hyperfibrinolysis<sup>164</sup>. Correction of trauma-induced coagulopathy is recognised as a key attribute of a first responder resuscitation fluid for haemorrhage and traumatic shock.

#### 1.4.6 Attenuation of Inflammation

A fluid that attenuates the systemic inflammatory response would also be of significant clinical benefit. Haemorrhagic shock leads to ischaemia-reperfusion (IR) injury, inflammation, coagulopathy, metabolic acidosis, hypothermia, multiple organ dysfunction and, if not treated, death<sup>175,176</sup>. The inflammatory response occurs from mobilization and activation of immune cells including neutrophils and lymphocytes which despite being vital for protecting against infectious agents as part of innate immunity<sup>177</sup>, can also inflict destructive organ damage following ischaemia-reperfusion<sup>178</sup>. Even short periods of low- or no-flow ischaemia can trigger complex signalling cascades resulting in a detrimental inflammatory attack and coagulopathy<sup>179</sup>.

Crystalloids, including Lactated Ringer's, and artificial colloids, have potent immune activation and pro-inflammatory properties, and are known to upregulate cellular injury markers<sup>36,180,181</sup>. In contrast, small animal studies have demonstrated positive immunomodulatory properties of hypertonic saline with effects on various functions including expression of cytokines and adhesion molecules, degranulation, and production of reactive oxygen species<sup>130,180,182</sup>, and a reduction in the incidence of acute lung injury and infectious complications following haemorrhagic shock<sup>183</sup>. The 'ideal' resuscitation fluid will halt the exaggerated inflammatory response and possess positive immune modulatory effects in order to prevent multiple organ dysfunction and secondary infection.



#### 1.4.7 Protection of the Endothelium

Also listed as one of the ‘ideal’ properties of resuscitation fluids in Table 1 is the ability to prevent endothelial dysfunction. The vascular endothelium is vital to the outcome of traumatic haemorrhagic shock because it covers a surface area of 4000-7000 m<sup>2</sup> in the body and exchanges gases, fuels, hormones, nutrients and wastes between the blood and 10<sup>14</sup> of the body’s cells<sup>184</sup>. It is the master integrator and regulator of vascular tone, inflammation and coagulation, vascular permeability, blood fluidity and lymphatic function<sup>185-187</sup>. Endothelial health depends on maintaining the integrity of the luminal glycocalyx mesh which becomes pro-inflammatory, coagulopathic, and leaky following a breach<sup>188-192</sup>. Traumatic injury and shock leads to a catecholamine surge and consequently endothelial glycocalyx shedding<sup>192,193</sup>, which can contribute to trauma-induced coagulopathy through thrombin generation, activation of protein C, and activation of hyperfibrinolysis<sup>194</sup>.

Hypertonic saline has positive microcirculatory effects and attenuates endothelial cell swelling<sup>128</sup>, whereas Lactated Ringer’s has shown microcirculatory impairments and negative effects on endothelial function in preclinical studies<sup>195</sup>. Since the endothelium is a major target of damage from trauma- and haemorrhage-induced hypoperfusion and reperfusion injury<sup>161</sup>, a resuscitation fluid that preserves endothelial integrity and function would be of great benefit.

#### 1.4.8 Prevention of Multiple Organ Dysfunction and Failure

The type of resuscitation fluid can exacerbate cellular injury caused by trauma, haemorrhage and shock<sup>36</sup>. As early as 1911 Evans noted the untoward effects of 0.9% saline IV fluid<sup>196</sup>. In the Vietnam War, aggressive crystalloid fluid replacement therapy led to secondary complications including shock lung<sup>52,197</sup>, a fatal lung condition characterised by pulmonary congestion, respiratory distress and hypoxaemia which is today called acute respiratory distress syndrome (ARDS). ARDS and multiple organ dysfunction are major causes of delayed mortality following traumatic injury<sup>21,198</sup>.

Large aggressive resuscitation with crystalloids results in interstitial oedema<sup>102,199</sup>. Microvascular integrity is compromised due to trauma and shock resulting in increased

vascular permeability and significant vascular leakage<sup>200</sup>. The subsequent oedema impairs wound healing, and can adversely affect organ functions including gas exchange in the lung, cognitive status, and significantly increase bacterial translocation in the gut<sup>201</sup>. Decreased tissue oxygenation following high-volume crystalloid administration further leads to increased organ dysfunction and failure<sup>12,202-204</sup>. Compared with small-volume hypertonic saline, resuscitation with Lactated Ringer's following haemorrhagic shock in a rat model resulted in an upregulation of apoptosis in multiple tissues, including intestinal mucosa, smooth muscle, lung, and liver<sup>205,206</sup>. However, small-volume hypertonic saline resuscitation has also been associated with organ failure and death in burns patients<sup>207</sup>.

The gut and splanchnic circulation is particularly vulnerable to hypovolaemia, and intestinal ischaemia may fuel sepsis and multiple organ failure<sup>172</sup>. Development of gut dysfunction correlates with increased intensive care unit stays, and increased mortality<sup>208,209</sup>. Renal dysfunction is another common adverse effect associated with commonly used resuscitation fluids. Preclinical studies showed afferent renal artery vasoconstriction due to hyperchloraemia following isotonic saline resuscitation<sup>123</sup>, and there are documented cases of renal injury and failure following hypertonic saline administration in burns and trauma patients<sup>164,210</sup>. Resuscitation with dextran has been linked with acute renal failure as a result of dextran molecules accumulating in the renal tubules and causing plugging<sup>211,212</sup>. Lissauer and colleagues demonstrated a correlation between 6% hetastarch and renal dysfunction and death in a retrospective analysis of 2,225 critically ill trauma patients<sup>213</sup>. There is now extensive clinical evidence showing hetastarches and Hextend® to be nephrotoxic and associated with the development of acute kidney injury and chronic renal failure<sup>123,214</sup>. In addition to accumulating in the kidney, HES can also accumulate in the skin, liver and bone marrow, consequently causing pruritus, worsening hepatic dysfunction, ascites and anaemia<sup>215</sup>. HES can be detected in skin for up to 54 months after administration<sup>216</sup>.

The 'ideal' resuscitation fluid will not exacerbate traumatic shock-induced cellular and tissue injury, but prevent further dysfunction in the lungs, mesentery, and kidneys, to reduce the likelihood of organ failure and sepsis.

#### 1.4.9 Prevention of Secondary Brain Injury

Prevention of secondary brain injury at hypotensive pressures is vitally important because the combination of traumatic haemorrhage and brain injury is highly lethal<sup>73,217</sup>, and the use of permissive hypotension is contentious when head injury is suspected because of reduced cerebral perfusion pressure<sup>218-221</sup>. Traumatic brain injury (TBI) is often associated with haemorrhagic shock<sup>78,218</sup>, and mild-TBI (mTBI; concussion/sub-concussion) was the ‘signature injury’ of the Iraq and Afghanistan conflicts, affecting over 325,000 military personnel, since 2000<sup>222-225</sup>.

The ‘ideal’ small-volume hypotensive fluid will maintain sufficient cerebral perfusion pressure and brain function, reduce neuroinflammation, and prevent secondary ischaemic injury that can exacerbate TBI<sup>161,226</sup>. Preclinical data and clinical trials support the use of hypertonic solutions for traumatic brain injury due to a demonstrable ability to lower intracranial pressure (ICP) and improve cerebral perfusion<sup>227-230</sup>. However, a recent review of 1,820 patients across 11 studies demonstrated no mortality benefit or effect on ICP control with hypertonic saline, and further research is required<sup>231</sup>. Prevention of secondary brain injury at hypotensive pressures is one of the greatest challenges in the development of the ‘ideal’ resuscitation fluid.

#### 1.4.10 Protection against Hypothermia

Hypothermia following traumatic injury is a serious complication associated with a three-fold independent risk of death<sup>232,233</sup>, with 100% mortality if core body temperature drops below 32°C (90°F)<sup>234</sup>. Hypothermia defined by a core body temperature of  $\leq 35^{\circ}\text{C}$  (95°F) is found in up to 40% of trauma patients due to the nature of the injury, exposure, and altered thermoregulation<sup>234-237</sup>.

The consequences of accidental hypothermia include decreased myocardial contractility and increased incidence of arrhythmias<sup>238,239</sup>, trauma-induced coagulopathy including reduced platelet aggregation<sup>23</sup> and increased fibrinolysis<sup>158,240,241</sup>, as well as immunosuppression<sup>159,242</sup>. Analysis of 15,320 polytrauma patients with accidental hypothermia ( $\leq 33^{\circ}\text{C}/91^{\circ}\text{F}$ ) showed an increased incidence of sepsis and multiple organ

failure<sup>243</sup>. Shivering associated with hypothermia in the trauma patient can further compound lactic acidosis<sup>172</sup>. Since fluid warming devices are not generally available in far-forward and remote environments, the ‘ideal’ resuscitation fluid would have thermoregulatory properties to protect against hypothermia.

#### 1.4.11 Restoration of Acid-Base Balance

Acidosis occurs in a haemorrhaging patient due to hypercapnia, end-organ hypoperfusion, cellular hypoxia, and lactic acid production<sup>244</sup>, and the associated mortality rate can exceed 50%<sup>245-248</sup>. Acidosis impairs perfusion of the kidneys, and can have negative inotropic effects and promote arrhythmogenicity<sup>204,249</sup>. Profound lactic acidosis develops with severe hypovolaemia<sup>172</sup>, and serum lactate levels have been shown to correlate with injury severity and outcome, and have therefore been used to guide resuscitation<sup>250,251</sup>.

Traditional large-volume resuscitation with crystalloid fluids can further worsen acidosis and worsen the patient condition<sup>252</sup>. Rapid administration of large volumes of isotonic saline can lead to hyperchloraemic acidosis due to the higher than physiologic chloride concentration<sup>102,123,253,254</sup>. While Lactated Ringer’s does not cause metabolic acidosis, patients have exhibited a respiratory acidosis with large volume use<sup>204</sup>. A resuscitation fluid with pH buffering capabilities may help to restore acid-base balance following severe traumatic haemorrhagic shock.

#### 1.4.12 Repayment of Oxygen Debt

Haemorrhagic shock leads to hypovolaemia and a decrease in blood flow and oxygen delivery to vital organs resulting in hypoperfusion at the cellular level<sup>255</sup>. There is a mismatch between delivery of oxygen to tissues ( $DO_2$ ) and tissue oxygen consumption ( $VO_2$ ), which reaches a critical threshold (critical  $DO_2$ ) when  $DO_2$  falls below  $VO_2$ . At this point there is a metabolic transition from aerobic to anaerobic metabolism and oxygen extraction at the level of the microcirculation becomes directly dependent on  $DO_2$ <sup>255,256</sup>. Tissues become ischaemic and an oxygen deficit is incurred. The accumulation of multiple oxygen deficits over time represents the total oxygen debt<sup>161,255,256</sup>.

The development of multiple organ failure after traumatic haemorrhagic shock is strongly influenced by the level of accumulated oxygen debt, and cells most vulnerable are those with the greatest oxidative requirements including the brain, liver, kidney, and myocardium<sup>256</sup>. Just this year Bjerkvig and colleagues suggested that the endothelium and blood are also very sensitive to oxygen debt, and introduced the concept of ‘blood failure’, as a result of accumulated oxygen deficits<sup>161</sup>.

Preclinical and clinical investigations have established oxygen debt as a key predictor of late outcome from shock<sup>256</sup>, therefore prevention of further debt accumulation and timely debt repayment commencing in the pre-hospital environment should be a primary goal of the ‘ideal’ resuscitation fluid. For maintenance of patients at hypotensive pressures, the resuscitation fluid will need to reduce tissue oxygen requirements to mitigate debt accumulation (possibly by reducing whole body metabolism), and improve microcirculatory blood flow to enhance debt repayment<sup>255,257</sup>.

#### 1.4.13 Stability

A resuscitation fluid should have a long shelf-life and be resilient to environmental extremes including changes in temperature and ambient pressure<sup>80</sup>. Stability in different clinical environments is an important characteristic of the ‘ideal’ fluid, particularly on the battlefield and for aeromedical retrieval from rural and remote environments. During Operation Enduring Freedom NATO forces encountered temperature extremes ranging from as low as -9°C (15°F) to as high as 49°C (120°F)<sup>258</sup>, as well as varying altitudes in the mountainous regions of Afghanistan. Fluid stability is particularly critical during prolonged retrievals and evacuations of casualties which may exceed six hours in remote areas of Australia<sup>119,259,260</sup>, and ranged from four to 15 hours in recent military operations in the Middle East and Somalia<sup>48</sup>.

#### 1.4.14 Ease of Administration

First responders on the battlefield and in rural and remote civilian environments are challenged to perform under extreme conditions often with minimal equipment, and therefore the ‘ideal’ resuscitation fluid should be easy to administer with simple

procedures <sup>161</sup>. Administration of resuscitation fluids is typically via intravenous (IV) access that is obtained on any casualty with significant injury in the pre-hospital environment as standard of care <sup>50,199</sup>. In addition to being suitable for IV delivery, resuscitation fluids should also suit intraosseous (IO) administration, particularly in critically ill patients where vascular access is difficult <sup>50,261</sup>. The IO route is an established method used in paediatric emergency care <sup>262</sup> and increasingly in adults <sup>263</sup>, and intraosseous fluid delivery systems have been used in military operations since World War II <sup>264</sup>.

#### 1.4.15 Analgesic Properties

Finally, analgesic effects would be a useful adjunct in the ‘ideal’ pre-hospital resuscitation fluid. The prevalence of pain in trauma patients has been estimated to be as high as 91% at the time of hospital admission <sup>265</sup>. Early treatment of acute pain associated with injury could improve long-term outcomes <sup>266,267</sup>, and adequate analgesia will also prevent tachycardia secondary to pain being misinterpreted as a sign of hypovolaemia <sup>268</sup>.

#### 1.4.16 The “Ideal” Resuscitation Fluid Summary

In summary, the ‘ideal’ pre-hospital resuscitation fluid for critically injured and haemorrhaging battlefield and civilian patients should rescue and stabilise the heart; be low cube weight to hypotensively resuscitate into the permissive range; correct trauma-induced coagulopathy; blunt inflammation; prevent endothelial dysfunction, multiple organ failure, and secondary brain injury; protect against accidental hypothermia; restore acid-base balance; and mitigate oxygen debt and efficiently repay accumulated debt. Ideally a pre-hospital resuscitation fluid will also be safe, efficacious, cost-effective, stable, and easy to administer, and have analgesic properties. It is apparent large volume crystalloids or colloids such as hetastarches and dextrans are not ‘ideal’ pre-hospital resuscitation fluids, and in light of the evidence presented here regarding safety concerns, costs, coagulation effects, and organ dysfunction and injury, new fluid therapies are urgently required.

## 1.5 THE USE OF BLOOD AND BLOOD PRODUCTS

A comprehensive review of the ideal resuscitation fluid for traumatic haemorrhage would not be complete without consideration of whole blood and blood products. Ever since 25 year old Cpl Henri Legrain, of the French Army's 45<sup>th</sup> Infantry Regiment, received the first recorded fresh whole blood transfusion directly from 23-yr old Pte Isidore Colas in World War I <sup>269</sup>, blood has been used to resuscitate wounded soldiers <sup>270,271</sup>. Whole blood was the primary resuscitation fluid during the first half of the 20<sup>th</sup> century until the development of fractionation techniques and component therapy <sup>272,273</sup>. The rationale for separate packed red blood cells (PRBC), fresh frozen plasma (FFP), and platelets (PLT), was to reduce disease transmission and improve resource utilization <sup>274</sup>. Together with permissive hypotension (see 1.4.4) and minimization of fluid volume (see 1.4.3), early administration of RBCs, FFP, and PLT formed the basis of damage control resuscitation adopted in both military and civilian trauma <sup>275,276</sup>. Extensive pre-clinical and clinical research in recent decades has focussed on the optimum ratio of the various blood components <sup>277-289</sup>, and led to adoption of a 1:1 PRBC:FFP transfusion strategy <sup>274,282,290</sup>. However there remains a lack of evidence from prospective randomised controlled trials, and the retrospective observational studies this strategy was based on are limited because of design flaws, and survival and selection biases <sup>291-294</sup>. Furthermore, transfusion is not without risk and PRBC, FFP, and PLT administration is associated with complications including non-hemolytic transfusion reactions, TRALI (transfusion-related acute lung injury), multiple organ failure, hypothermia, coagulopathy, systemic inflammatory response syndrome, and infectious transmission <sup>271,280,281,291,295-298</sup>.

The far-forward and austere theatres of war in recent times where the ability to store blood components was restricted or unavailable necessitated a return to the use of fresh whole blood. Platelets, which have the shortest shelf life of all blood components <sup>299</sup>, are impossible to transport and use in far forward environments <sup>300,301</sup>, and were not available for forward surgical teams in Afghanistan <sup>302</sup>. In addition to the impracticality of blood component use in remote settings, improved survival outcomes in combat casualties receiving FWB compared to component therapy (CT) <sup>297,302,303</sup>, led to the Committee on Tactical Combat Casualty Care prioritizing whole blood over blood components in the most recent guidelines <sup>48</sup>. In 2009, Spinella and colleagues reported

higher 24-hour and 30-day survival in US Military combat casualty patients transfused with warm FWB compared with CT patients<sup>303</sup>. A larger 2013 retrospective analysis of transfusions to 488 wounded soldiers by six forward surgical teams in Afghanistan showed that FWB was safe and was independently associated with improved survival to discharge compared to resuscitation with red blood cells or fresh frozen plasma alone<sup>302</sup>. These results were not surprising given that whole blood provides a balanced amount of red blood cells, plasma, and platelets with 30% higher oxygen-carrying capacity than component therapy<sup>297,300,301</sup>.

While the use of FWB is well-established in military settings with >9000 units of whole blood transfused safely during Operation Iraqi Freedom and Operation Enduring Freedom in Afghanistan<sup>271,304</sup>, civilian use has been limited due to the unrestricted availability of individual blood components<sup>305</sup>. Currently, physicians and nurses on Royal Caribbean cruise ships are trained in fresh whole blood transfusion for the treatment of critically ill passengers at sea<sup>306</sup>. Establishing protocols for a FWB and/or cold-stored whole blood program may be of benefit in mass casualty situations, natural disasters, and rural and remote areas. Despite concerns over efficacy of platelets after cold storage, *in vitro* testing has established preservation of the haemostatic properties of cold-stored whole blood<sup>273,297,307-309</sup>. ROTEM investigations by the Norwegian Naval Special Operation Commando unit demonstrated fibrinogen and platelet function of whole blood was maintained after 14-days cold storage<sup>310</sup>. Based on evidence supporting the maintenance of both procoagulant and anticoagulant proteins, including protein S, for up to 11 days in cold leukodepleted blood, the London Air Ambulance service has introduced pre-hospital transfusion of male type O-negative cold whole blood, referred to as cold 'blood on board' (BOB), to patients suffering uncontrolled haemorrhage<sup>311</sup>. A pilot study of 47 hypotensive bleeding male trauma patients at a level 1 trauma centre demonstrated feasibility and safety of transfusion of cold-stored uncross-matched leuko-reduced whole blood with no adverse reactions<sup>309</sup>. This was supported by a separate study showing no clinical transfusion reactions following administration of cold, uncross-matched, low-titre, group O-positive whole blood in bleeding civilian trauma patients<sup>312</sup>. A multi-centre randomised clinical trial is required to demonstrate clinical efficacy of cold-stored whole blood in pre-hospital and in-hospital trauma settings for bleeding trauma patients.



## 1.6 DEVELOPMENT OF A NEW SMALL-VOLUME RESUSCITATION FLUID: ALM

The adenosine and lidocaine with magnesium (ALM) concept was initially developed in 1998 by my primary supervisor, Professor Geoffrey Dobson, as the world's first low potassium polarizing cardioplegia<sup>313</sup>, and has since shown superiority in two randomised controlled trials of low-risk and high-risk emergency patients undergoing coronary artery bypass graft (CABG) surgery with improved cardiac function, significantly lower lactates in the coronary sinus at reperfusion and during the first post-operative day, and one to two full days less in the intensive care unit (ICU) and hospital<sup>314,315</sup>. The original objective was to borrow from the tricks of natural hibernators who do not use high potassium to slow their hearts during hibernation, and arrest the human heart at more natural resting 'polarised' membrane potentials (-80 mV). This was achieved by inhibiting the voltage-dependent Na<sup>+</sup> fast channels responsible for the phase O upstroke of the action potential with lidocaine, and simultaneously decreasing the duration of the action potential by opening K<sup>+</sup><sub>ATP</sub> channels with adenosine. Magnesium was added to reduce Ca<sup>2+</sup> entry and protect the heart from ischaemia-reperfusion injury and arrhythmias<sup>80,316</sup>. After witnessing the heart spontaneously reanimating after cardiac surgery with little inotropic support, Professor Dobson questioned whether lower, non-arresting, doses of ALM could resuscitate the heart after major trauma or haemorrhagic shock.

### 1.6.1 ALM in Traumatic Haemorrhagic Shock

The first haemorrhagic shock study that I designed and conducted using the rat model of severe, pressure-controlled blood loss (~40% total blood volume), confirmed that colloids 6% and 10% hetastarch, and 6% dextran had adverse effects on haemodynamics and cardiac stability with increased mortality<sup>30</sup>. By applying lower "non-arresting" concentrations of ALM with 7.5% hypertonic saline as the vehicle, the combination of adenosine, lidocaine, and magnesium was found to rescue and resuscitate the heart after severe to catastrophic blood loss. A ~0.7-1.0 ml/kg (0.3 ml) intravenous bolus of 7.5% NaCl ALM resuscitated mean arterial pressure (MAP) into a permissive hypotensive range in rat models of severe pressure-controlled (~40% blood loss) and catastrophic volume-controlled (60% blood loss) haemorrhagic shock with no

mortality<sup>31,317</sup>. The haemodynamic rescue potential of small-volume 7.5% NaCl ALM was also demonstrated by Granfeldt and colleagues who showed that a 20 ml bolus of 7.5% NaCl ALM (0.5 ml/kg) significantly reduced crystalloid fluid requirement by 40% to maintain a target MAP of 50 mmHg in the pig following ~75% blood loss<sup>318</sup>.

## 1.7 THESIS AIMS

The major aim of this thesis was to further develop small-volume 7.5% NaCl ALM resuscitation fluid and to investigate its capabilities in the context of improving cardiac and whole body function. Seven specific objectives of the thesis are outlined below:

- 1) Investigate the ability of small-volume 7.5% NaCl ALM fluid (0.7-4 ml/kg) to resuscitate into the permissive hypotensive range over 60 min following severe haemorrhagic shock in the rat (Chapters 2, 4, and 6) and pig (Chapter 3).
- 2) Study the metabolic outcomes and organ-protective properties including cardiac rescue and function, after ALM resuscitation in the large animal porcine model of ~75% blood loss and 90 min haemorrhagic shock (Chapter 3).
- 3) Develop a rat model of trauma-induced coagulopathy with hyperfibrinolysis to study coagulopathy correction following severe haemorrhagic shock (Chapter 5).
- 4) Investigate the ability of small-volume 7.5% NaCl to correct trauma-induced coagulopathy and hyperfibrinolysis, including timing and mechanism of correction (Chapters 2, 4, and 6).
- 5) Contribute to the current literature on trauma-induced coagulopathy and its pathophysiology (Chapters 5 and 7).
- 6) Review the development and translation of ALM therapy from cardioplegia to small-volume treatment for haemorrhagic shock, cardiac arrest, endotoxaemia, and polymicrobial sepsis, and outline future directions (Chapters 8 and 9).
- 7) Describe two hypotheses of ALM's mechanism of action involving nitric oxide and the autonomic nervous system and current investigations to date (Chapter 9).

## CHAPTER 2

### **REVERSAL OF ACUTE COAGULOPATHY DURING HYPOTENSIVE RESUSCITATION USING SMALL-VOLUME 7.5% NaCl ADENOSINE, LIDOCAINE, AND Mg<sup>2+</sup> (ALM) IN THE RAT MODEL OF SEVERE HAEMORRHAGIC SHOCK**

Previous studies showed that a small intravenous bolus (0.3 ml/300-400 g rat) of hypertonic saline (7.5% NaCl) with adenosine, lidocaine, and magnesium (ALM) resuscitated mean arterial pressure (MAP) into a hypotensive range with improved cardiac stability and 100% survival in rat models of severe (~40%) to catastrophic (60%) blood loss and haemorrhagic shock<sup>31,317</sup>. The objective of this study was to examine the effect of small-volume 7.5% NaCl with adenosine (A), lidocaine (L) and Mg<sup>2+</sup> (M), on hypotensive resuscitation and coagulopathy in a rat model of severe pressure-controlled haemorrhagic shock. Trauma-induced coagulopathy occurs early in haemorrhagic trauma and is a major contributor to mortality and morbidity<sup>146,149,319</sup>. Small bolus volumes and the ability to hypotensively resuscitate and correct trauma-induced coagulopathy (TIC) are three of the major properties that constitute the ‘ideal’ resuscitation fluid identified in Chapter 1.

Haemorrhagic shock was induced in male Sprague-Dawley rats by 20 min phlebotomy (41% blood loss) followed by 60 min shock. Animals were randomly assigned to one of five resuscitation treatment groups: 1) Untreated; 2) 7.5% NaCl; 3) 7.5% NaCl AL; 4) 7.5% NaCl M; or 5) 7.5% NaCl ALM. ALM comprised 1 mM Adenosine, 3 mM Lidocaine, and 2.5 mM MgSO<sub>4</sub>. The concentrations were determined from pilot studies and previously shown to resuscitate MAP into a permissive hypotensive range in rat models of severe pressure-controlled (~40% blood loss) and catastrophic volume-controlled (60% blood loss) haemorrhagic shock<sup>31,317</sup>. Small-volume (0.3 ml) resuscitation bolus was administered after 60 min shock, and haemodynamics were monitored for a further 60 min. Coagulation was assessed in plasma using activated partial thromboplastin time (aPTT) and prothrombin time (PT).

The study showed a pronounced hypocoagulopathy in this model, evidenced by over 10-fold increases in aPTT and PT times during the bleed and shock periods. Trauma-

induced coagulopathy was reversed in the 7.5% NaCl ALM treatment group only, with aPTT and PT times returned to baseline levels after 60 min resuscitation. In addition to correcting coagulopathy, small-volume hypertonic saline with ALM induced and maintained permissive hypotensive resuscitation.

# Reversal of acute coagulopathy during hypotensive resuscitation using small-volume 7.5% NaCl adenocaine and Mg<sup>2+</sup> in the rat model of severe hemorrhagic shock\*

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**Objective:** Acute traumatic coagulopathy occurs early in hemorrhagic trauma and is a major contributor to mortality and morbidity. Our aim was to examine the effect of small-volume 7.5% NaCl adenocaine (adenosine and lidocaine, adenocaine) and Mg<sup>2+</sup> on hypotensive resuscitation and coagulopathy in the rat model of severe hemorrhagic shock.

**Design:** Prospective randomized laboratory investigation.

**Subjects:** A total of 68 male Sprague Dawley Rats.

**Intervention:** Post-hemorrhagic shock treatment for acute traumatic coagulopathy.

**Measurements and Methods:** Nonheparinized male Sprague-Dawley rats (300–450 g, n = 68) were randomly assigned to either: 1) untreated; 2) 7.5% NaCl; 3) 7.5% NaCl adenocaine; 4) 7.5% NaCl Mg<sup>2+</sup>; or 5) 7.5% NaCl adenocaine/Mg<sup>2+</sup>. Hemorrhagic shock was induced by phlebotomy to mean arterial pressure of 35–40 mm Hg for 20 mins (~40% blood loss), and animals were left in shock for 60 mins. Bolus (0.3 mL) was injected into the femoral vein and hemodynamics monitored. Blood was collected in Na citrate (3.2%) tubes, centrifuged, and the plasma snap frozen in liquid N<sub>2</sub> and stored at –80°C. Coagulation was assessed using activated partial thromboplastin times and prothrombin times.

**Results:** Small-volume 7.5% NaCl adenocaine and 7.5% NaCl adenocaine/Mg<sup>2+</sup> were the only two groups that gradually

increased mean arterial pressure 1.6-fold from 38–39 mm Hg to 52 and 64 mm Hg, respectively, at 60 mins (*p* < .05). Baseline plasma activated partial thromboplastin time was 17 ± 0.5 secs and increased to 63 ± 21 secs after bleeding time, and 217 ± 32 secs after 60-min shock. At 60-min resuscitation, activated partial thromboplastin time values for untreated, 7.5% NaCl, 7.5% NaCl/Mg<sup>2+</sup>, and 7.5% NaCl adenocaine rats were 269 ± 31 secs, 262 ± 38 secs, 150 ± 43 secs, and 244 ± 38 secs, respectively. In contrast, activated partial thromboplastin time for 7.5% NaCl adenocaine/Mg<sup>2+</sup> was 24 ± 2 secs (*p* < .05). Baseline prothrombin time was 28 ± 0.8 secs (n = 8) and followed a similar pattern of correction.

**Conclusions:** Plasma activated partial thromboplastin time and prothrombin time increased over 10-fold during the bleed and shock periods prior to resuscitation, and a small-volume (~1 mL/kg) IV bolus of 7.5% NaCl AL/Mg<sup>2+</sup> was the only treatment group that raised mean arterial pressure into the permissive range and returned activated partial thromboplastin time and prothrombin time clotting times to baseline at 60 mins. (Crit Care Med 2012; 40:2417–2422)

**Key Words:** adenocaine; adenosine; coagulopathy; hemorrhagic; hypertonic saline; hypocoagulopathy; lidocaine; magnesium; military; prehospital; resuscitation; shock; small volume; trauma

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\*See also p. 2519.

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Dr. Dobson consulted for Hibernation Therapeutics and has equity interest and stock ownership in the

company. He is also the inventor of Adenocaine Technology for organ protection and preservation, including trauma. The remaining authors have not disclosed any potential conflicts of interest.

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## CHAPTER 3

### **SMALL-VOLUME 7.5% NaCl ADENOSINE, LIDOCAINE, AND Mg<sup>2+</sup> HAS MULTIPLE BENEFITS DURING HYPOTENSIVE AND BLOOD RESUSCITATION IN THE PIG FOLLOWING SEVERE BLOOD LOSS: RAT TO PIG TRANSLATION**

In rats, small-volume resuscitation with 7.5% NaCl adenosine, lidocaine, and Mg<sup>2+</sup> (ALM) gently raises and maintains mean arterial pressure (MAP) into the permissive hypotensive range following severe to catastrophic blood loss and shock with a full correction of coagulopathy<sup>31,317,345</sup>. In pigs, small-volume ALM was also shown to reduce crystalloid fluid requirements by 40% and improve cardiac function during hypotensive resuscitation in a porcine model of severe haemorrhagic shock<sup>346</sup>. The aim of this study was to translate small-volume 7.5% NaCl ALM resuscitation from the rat to a pig model of 75% blood loss and 90 min shock, and investigate haemodynamic rescue and multiple organ protection.

Female pigs were randomly assigned to 7.5% NaCl control group, or 7.5% NaCl ALM treatment group. After 90 min haemorrhagic shock, animals were resuscitated with 4 ml/kg bolus for 60 min, followed by re-infusion of shed blood and 180 min monitoring. ALM bolus comprised 0.54 mg/kg adenosine, 1.63 mg/kg lidocaine, and 0.6 mg/kg MgSO<sub>4</sub> and was equivalent to previous rat studies<sup>31,317,345</sup>, however a larger dose of 4 ml/kg was administered compared to ~0.7-1.0 ml/kg in rats after pilot studies demonstrated a need for a larger volume bolus to resuscitate MAP. After 60 min hypotensive resuscitation, ALM-treated animals had significantly higher MAP, cardiac output and oxygen delivery, significantly lower blood lactate, and significantly higher arterial pH and base excess compared to saline controls. Whole body oxygen consumption decreased in ALM pigs following return of shed blood, and ALM treatment improved and protected renal function with a significant three-fold higher urine output and significantly lower plasma creatinine levels.

This chapter concludes that the resuscitation benefits of small-volume 7.5% NaCl ALM translated from the rat to the pig with superior haemodynamic, acid-base and metabolic benefits, and improved cardiovascular and renal function compared to hypertonic saline

alone. In addition to previous studies, ALM therapy in this porcine study also demonstrated: 1) low cube weight (2L blood removed and 140-160 ml bolus fluid administered), 2) resuscitation into the permissive range (MAP ~50 mmHg), 3) rescue and stabilisation of the heart with significantly improved left ventricular-arterial coupling, 4) protection against acidosis, 5) prevention of multiple organ dysfunction, 6) improved oxygen delivery, and 7) a reduction in whole body oxygen metabolism, which may support timely repayment of oxygen debt and mitigate further debt accumulation. In conclusion, small-volume 7.5% NaCl ALM therapy conferred whole body protection after 75% blood loss and 90 min shock in the pig model.

# Small-Volume 7.5% NaCl Adenosine, Lidocaine, and Mg<sup>2+</sup> Has Multiple Benefits During Hypotensive and Blood Resuscitation in the Pig Following Severe Blood Loss: Rat to Pig Translation

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**Objectives:** Currently, there is no effective small-volume fluid for traumatic hemorrhagic shock. Our objective was to translate small-volume 7.5% NaCl adenosine, lidocaine, and Mg<sup>2+</sup> hypotensive fluid resuscitation from the rat to the pig.

**Design:** Pigs (35–40 kg) were anesthetized and bled to mean arterial pressure of 35–40 mm Hg for 90 minutes, followed by 60 minutes of hypotensive resuscitation and infusion of shed blood. Data were collected continuously.

**Setting:** University hospital laboratory.

**Subjects:** Female farm-bred pigs.

**Interventions:** Pigs were randomly assigned to a single IV bolus of 4 mL/kg 7.5% NaCl + adenosine, lidocaine and Mg<sup>2+</sup> ( $n = 8$ ) or

4 mL/kg 7.5% NaCl ( $n = 8$ ) at hypotensive resuscitation and 0.9% NaCl ± adenosine and lidocaine at infusion of shed blood.

**Measurements and Main Results:** At 60 minutes of hypotensive resuscitation, treatment with 7.5% NaCl + adenosine, lidocaine, and Mg<sup>2+</sup> generated significantly higher mean arterial pressure (48 mm Hg [95% CI, 44–52] vs 33 mm Hg [95% CI, 30–36],  $p < 0.0001$ ), cardiac index (76 mL/min/kg [95% CI, 63–91] vs 47 mL/min/kg [95% CI, 39–57],  $p = 0.002$ ), and oxygen delivery (7.6 mL O<sub>2</sub>/min/kg [95% CI, 6.4–9.0] vs 5.2 mL O<sub>2</sub>/min/kg [95% CI, 4.4–6.2],  $p = 0.003$ ) when compared with controls. Pigs that received adenosine, lidocaine, and Mg<sup>2+</sup>/adenosine and lidocaine also had significantly lower blood lactate (7.1 mM [95% CI, 5.7–8.9] vs 11.3 mM [95% CI, 9.0–14.1],  $p = 0.004$ ), core body temperature (39.3°C [95% CI, 39.0–39.5] vs 39.7°C [95% CI, 39.4–39.9]), and higher base excess (–5.9 mEq/L [95% CI, –8.0 to –3.8] vs –11.2 mEq/L [95% CI, –13.4 to –9.1]). One control died from cardiovascular collapse. Higher cardiac index in the adenosine, lidocaine, and Mg<sup>2+</sup>/adenosine and lidocaine group was due to a two-fold increase in stroke volume. Left ventricular systolic ejection times were significantly higher and inversely related to heart rate in the adenosine, lidocaine, and Mg<sup>2+</sup>/adenosine and lidocaine group. Thirty minutes after blood return, whole-body oxygen consumption decreased in pigs that received adenosine, lidocaine, and Mg<sup>2+</sup>/adenosine and lidocaine (5.7 mL O<sub>2</sub>/min/kg [95% CI, 4.7–6.8] to 4.9 mL O<sub>2</sub>/min/kg [95% CI, 4.2–5.8]), whereas it increased in controls (4.2 mL O<sub>2</sub>/min/kg [95% CI, 3.5–5.0] to 5.8 mL O<sub>2</sub>/min/kg [95% CI, 4.9–5.8],  $p = 0.02$ ). After 180 minutes, pigs in the adenosine, lidocaine, and Mg<sup>2+</sup>/adenosine and lidocaine group had three-fold higher urinary output (2.1 mL/kg/hr [95% CI, 1.2–3.8] vs 0.7 mL/kg/hr [95% CI, 0.4–1.2],  $p = 0.001$ ) and lower plasma creatinine levels.

**Conclusion:** Small-volume resuscitation with 7.5% NaCl + adenosine, lidocaine, and Mg<sup>2+</sup>/adenosine and lidocaine provided superior cardiovascular, acid-base, metabolic, and renal recoveries following severe hemorrhagic shock in the pig compared with 7.5% NaCl alone. (*Crit Care Med* 2014; 42:e329–e344)

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**Key Words:** adenosine; hemorrhagic; hypertonic saline; hypotensive resuscitation; lidocaine; magnesium; military; prehospital; shock; small volume; trauma

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## CHAPTER 4

### **CORRECTION OF ACUTE TRAUMATIC COAGULOPATHY WITH SMALL-VOLUME 7.5% NaCl ADENOSINE, LIDOCAINE, AND Mg<sup>2+</sup> OCCURS WITHIN 5 MINUTES: A ROTEM ANALYSIS**

In Chapter 2 it was shown that 0.7-1.0 ml/kg bolus 7.5% NaCl with adenosine, lidocaine and Mg<sup>2+</sup> (ALM) led to a reversal of traumatic haemorrhage-induced coagulopathy with full correction of activated partial thromboplastin time (aPTT) and prothrombin time (PT) at 60 min resuscitation<sup>345</sup>. Given the clinical significance of early detection and treatment of trauma-induced coagulopathy, the aim of Chapter 4 was to use rotational thromboelastometry (ROTEM) to investigate the timing of this correction in the rat model of severe haemorrhagic shock and perform a detailed examination of clot initiation, kinetics, propagation, stability and lysis.

Haemodynamics and coagulopathy (aPTT, PT, ROTEM EXTEM, INTEM, and FIBTEM) were assessed at baseline, after 20 min pressure-controlled bleeding, after 60 min haemorrhagic shock, and after 5 min and 60 min resuscitation with 0.3 ml bolus 7.5% NaCl ± ALM. ROTEM indicated a progressive hypocoagulopathy during bleeding and shock, with no sustainable clots after 60 min shock. In contrast to 7.5% NaCl alone which failed to resuscitate and correct coagulopathy, 7.5% NaCl ALM resuscitated mean arterial pressure into the permissive hypotensive range and corrected coagulopathy within 5 min with a reversal of fibrinolysis. This chapter addresses the potential significance of this early correction of coagulopathic bleeding in the development of the small-volume 7.5% NaCl ALM resuscitation fluid, and introduces possible mechanisms for the correction.

# Correction of acute traumatic coagulopathy with small-volume 7.5% NaCl adenosine, lidocaine, and Mg<sup>2+</sup> occurs within 5 minutes: A ROTEM analysis

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- BACKGROUND:** Acute traumatic coagulopathy is a major contributor to mortality and morbidity following hemorrhagic shock. Our aim was to examine the effect of small-volume 7.5% NaCl with adenosine, lidocaine, and Mg<sup>2+</sup> (ALM) resuscitation on the timing of correction of coagulopathy in the rat model of severe hemorrhagic shock using ROTEM.
- METHODS:** Male rats (300–450 g, n = 64) were randomly assigned to (1) baseline, (2) sham, (3) bleed, (4) shock, (5) 7.5% NaCl for 5 minutes, (6) 7.5% NaCl with ALM for 5 minutes, (7) 7.5% NaCl for 60 minutes, or (8) 7.5% NaCl with ALM for 60 minutes (all n = 8). For resuscitation, 0.3-mL intravenous bolus of 7.5% NaCl was administered with and without ALM (n = 8 each group). Hemodynamics and coagulopathy were assessed.
- RESULTS:** After hemorrhage, prothrombin time (PT) and activated partial thromboplastin time (aPTT) increased approximately four to six times, and ROTEM indicated hypocoagulopathy. After 60-minute shock, no sustainable clots could form. 7.5% NaCl increased mean arterial pressure (MAP) to 46 ± 2 mm Hg at 5 minutes and generated a weak clot in EXTEM with hyperfibrinolysis in all tests. At 60 minutes, 7.5% NaCl failed to sustain MAP (43 ± 5 mm Hg) and generate a viable clot. In direct contrast, 7.5% NaCl with ALM at 5 minutes resuscitated MAP to 64 ± 3 mm Hg, corrected PT and aPTT, and generated fully formed EXTEM and FIBTEM clots. At 60 minutes, MAP was 69 ± 5 mm Hg, PT and aPTT were fully corrected, and α angle, clot amplitudes (A10, A30), as well as clot firmness and elasticity were not significantly different from baseline. ALM clot lysis at 60 minutes was significantly less than bleed, shock, or 7.5% NaCl, indicating protection against hyperfibrinolysis.
- CONCLUSION:** Small-volume 7.5% NaCl failed to resuscitate and correct coagulopathy. In contrast, 7.5% NaCl with ALM resuscitated MAP and corrected coagulopathy at 5 minutes, with further improvements at 60 minutes in clot kinetics, propagation, and firmness. ALM fully reversed hyperfibrinolysis to baseline. The possible mechanisms are discussed. (*J Trauma Acute Care Surg.* 2015;78: 773–783. Copyright © 2015 Wolters Kluwer Health, Inc. All rights reserved.)
- KEY WORDS:** Resuscitation; coagulopathy; shock; hemorrhage; ROTEM; rats.

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TABLE 2 (Supplemental Content) Clot lysis parameters from EXTEM, INTEM, and FIBTEM tests for baseline, sham, bleed, shock, and after 5 and 60 min resuscitation with hypertonic saline alone and hypertonic saline with ALM. Number of animals shown in parentheses; if not shown n=8. HFL = hyperfibrinolysis, indicated by clot lysis >15% MCF, and presented as number of animals and percentage. LOT = lysis onset time (sec), time from clot initiation until HFL.

Test	Group	LI30(%)	LI45(%)	LI60(%)	ML (%)	ACF (mm)	HFL (#, %)	LOT (s)
EXTEM	Baseline	99.6±0.3	97.9±0.5	95.3±0.6	<b>16±1</b>	62±1	5/8 (62.5)	5752±529 (n=5)
	Sham	98.9±1.0	96.8±1.7	93.8±2.1	<b>17±2</b>	61±2	4/8 (50)	5351±944 (n=4)
	Bleed	99±1.0 (n=3)	93.7±4.5 (n=3)	88±3.5 (n=3)	<b>24±3<sup>o</sup></b> (n=3)	17±9 <sup>#</sup>	3/3 (100)	4466±878 (n=3)
	Shock	100±0 (n=2)	99.5±0.5 (n=2)	98±2.0* (n=2)	<b>8±6</b> (n=2)	12±7 <sup>#</sup>	0/3 (0)	-
	5 min Resus							
	7.5% NaCl	82.6±13.2 (n=5)	75±17.2 (n=5)	74.2±18.6 (n=5)	<b>31±18</b> (n=5)	27±11 <sup>#</sup>	2/5 (40)	1311±431 (n=2)
	7.5% NaCl ALM	91.6±7.3 (n=2)	86±9.6 (n=2)	83.9±10.4 (n=2)	<b>21±11</b> (n=2)	56±7	2/8 (25)	1336±701 (n=2)
	60 min Resus							
	7.5% NaCl	100±0 (n=3)	99.7±0.3* (n=3)	99.3±0.7* (n=3)	<b>36±31</b> (n=3)	20±10 <sup>#</sup>	1/3 (33)	128 (n=1)
	7.5% NaCl ALM	99.6±0.4 (n=2)	99.5±0.4* (n=2)	99±0.6 <sup>£</sup> (n=2)	<b>7±2<sup>£</sup></b> (n=2)	61±3	0/8 (0) <sup>£</sup>	-
INTEM	Baseline	100±0	98.8±0.3	96.8±0.5	<b>15±4</b>	67±1	0/8 (0)	-
	Sham	100±0	99.4±0.2	98.4±0.3*	<b>8±1</b>	70±2	0/8 (0)	-
	Bleed	88±12 (n=4)	83.5±16.5 (n=4)	84.5±14.5 (n=4)	<b>42±22</b> (n=4)	29±15 <sup>#</sup>	2/4 (50)	-
	Shock	50±50 (n=2)	50±50 (n=2)	50±50 (n=2)	<b>50±50</b> (n=2)	6±4 <sup>#</sup>	1/2 (50)	126 (n=1)
	5 min Resus							
	7.5% NaCl	62.5±23.9 (n=4)	59.8±23.7 (n=4)	61±22.2 (n=4)	<b>52±21</b> (n=4)	27±15 <sup>#</sup>	3/6 (50)	61±1 (n=3)
	7.5% NaCl ALM	97.7±1.7 (n=7)	93.7±3.4 (n=7)	91±4.7 (n=7)	<b>15±6<sup>o</sup></b> (n=7)	61±6	2/8 (25)	-

	60 min Resus							
	7.5% NaCl	22.3±15.3 <sup>#</sup> (n=3)	32±13 <sup>#</sup> (n=2)	24±8 <sup>#</sup> (n=2)	<b>74±13<sup>#</sup></b> (n=3)	2±1 <sup>#</sup>	4/4 (100)*	641±309 (n=4)
	7.5% NaCl ALM	99.5±0.5	98±1.7	96.5±2.7	<b>11±5</b>	59±5	1/8 (13)	2859 (n=1)
FIBTEM	Baseline	99.8±0.3	99.9±0.1	100±0	<b>2±1</b>	16±1	0/8 (0)	-
	Sham	100±0	100±0	100±0	<b>2±1</b>	24±7	0/8 (0)	-
	Bleed	100±0 (n=2)	100±0 (n=2)	100±0 (n=2)	<b>22±22</b> (n=2)	5±3 <sup>#</sup>	1/3 (33)	563 (n=1)
	Shock	64.4±21.2 (n=5)	33.6±17.2 <sup>¥</sup> (n=5)	64.3±32.2 <sup>#</sup> (n=3)	<b>77±18<sup>#</sup></b> (n=5)	2±2 <sup>#</sup>	4/5 (80) <sup>#</sup>	338±142 (n=4)
	5 min Resus							
	7.5% NaCl	88±12 (n=6)	77.3±6 (n=6)	77.2±6.1 (n=6)	<b>22±16<sup>†</sup></b> (n=6)	8±2 <sup>#</sup>	1/6 (17)	483 (n=1)
	7.5% NaCl ALM	99.6±0.3	100±0	100±0	<b>3±2</b>	16±1	1/8 (13)	551 (n=1)
	60 min Resus							
	7.5% NaCl	57.5±19.2 <sup>#</sup> (n=6)	51.6±20.2 <sup>#</sup> (n=5)	67.5±13.6 <sup>#</sup> (n=5)	<b>70±19<sup>^</sup></b> (n=6)	5±3 <sup>#</sup>	4/6 (67) <sup>#</sup>	609±71 (n=4)
	7.5% NaCl ALM	99.9±0.1	99.8±0.4	100±0	<b>1±1</b>	15±2	1/8 (13)	1234 (n=1)

<sup>#</sup>  $p < 0.05$  compared to Baseline, Sham, 7.5% NaCl ALM 5 min, and 7.5% NaCl ALM 60 min groups

\*  $p < 0.05$  compared to Baseline, Sham, and 7.5% NaCl ALM 60 min groups

<sup>∞</sup>  $p < 0.05$  compared to Baseline group

<sup>†</sup>  $p < 0.05$  compared to 7.5% NaCl ALM 60 min group

<sup>‡</sup>  $p < 0.05$  compared to Baseline, Sham and Bleed groups

<sup>^</sup>  $p < 0.05$  compared to Baseline and 7.5% NaCl ALM 60 min groups

<sup>¥</sup>  $p < 0.05$  compared to all groups except 7.5% NaCl 60 min

## CHAPTER 5

### **DIFFERENTIAL CONTRIBUTIONS OF PLATELETS AND FIBRINOGEN TO EARLY COAGULOPATHY IN A RAT MODEL OF HAEMORRHAGIC SHOCK**

Hyperfibrinolysis is recognised as a key pathological mechanism of trauma-induced coagulopathy and is a strong predictor of mortality and increased transfusion requirements<sup>374-376</sup>. Chapter 4 showed a profound hypocoagulopathy developed during the 20 min bleeding phase and worsened during the 60 min shock phase in the rat model of pressure-controlled haemorrhagic shock. Interestingly, after 60 min shock there was an apparent switch to a pro-fibrinolytic state indicated by maximum lysis values >15%<sup>377</sup>. The aim of Chapter 5 was to use ROTEM tests EXTEM, FIBTEM and APTEM to examine the contribution of hyperfibrinolysis to the hypocoagulopathy demonstrated in this model after haemorrhage and shock.

The progressive hypocoagulopathy involved a two-step process of platelet dysfunction during active bleeding, followed by hyperfibrinolysis associated with severe hypoperfusion after 60 min shock at MAP 35-40 mmHg. A significant four-fold increase in plasma levels of P-selectin after 60 min shock further indicated endothelial dysfunction associated with the coagulopathy.

In addition to hypocoagulopathy, this model of 20 min pressure-controlled bleeding and 60 min hypovolaemic shock resulted in hypothermia and metabolic acidosis. It appears that small-volume ALM therapy improved temperature control but further studies are required to investigate the underlying mechanisms.



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Full Length Article

## Differential contributions of platelets and fibrinogen to early coagulopathy in a rat model of hemorrhagic shock



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## ABSTRACT

**Background:** The mechanisms of early traumatic-induced coagulopathy are not well understood. Our aim was to examine the role of platelets and fibrinogen to early coagulopathy in the rat after hemorrhagic shock.

**Methods:** Adult Sprague-Dawley rats were anesthetized and randomly assigned to: 1) Baseline, 2) Hemorrhage or 3) Shock (n = 10 each). Controlled phlebotomy occurred over 20 min and animals were left in shock 60 min. Coagulation was assessed using PT, aPTT, ROTEM and ELISAs.

**Results:** PT and aPTT increased 5 to 7 times following hemorrhage and shock. Prolongation of EXTEM and INTEM clotting times, lower clot elasticity and increased EXTEM lysis index (LI) indicated a hypocoagulopathy. After 20 min hemorrhage, LI(30–60) in FIBTEM was ~100%, EXTEM 83–87% and APTM 80–82% indicating a platelet contribution to the coagulopathy with no hyperfibrinolysis. After 60 min shock, the situation was reversed with fibrinogen loss being a contributor. This apparent switch from a platelet- to a fibrinogen-based coagulopathy, with fibrinolysis, was supported by  $\geq 15\%$  in maximum lysis (ML), a threefold increase in plasma PAI-1 after hemorrhage, and undetectable levels after shock. Curiously, the relative contribution of fibrinogen/platelet ratio to clot amplitude, determined from FIBTEM/EXTEM A10 ratio (and MCF), remained unchanged at ~1:5 for baseline, hemorrhage and shock despite a progressive hypocoagulopathy. Significant increases in P-selectin, acidosis and lactate indicated systemic endothelial damage and tissue hypoperfusion.

**Conclusions:** Hypocoagulopathy following severe hemorrhage and shock in the rat appeared to involve a two-step process of platelet dysfunction followed by fibrinogen impairment, possibly linked to progressive endothelial dysfunction.

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## CHAPTER 6

### **7.5% NaCl ADENOSINE, LIDOCAINE, AND Mg<sup>2+</sup> (ALM) RESUSCITATION CORRECTS HYPERFIBRINOLYSIS IN THE RAT MODEL OF SEVERE HAEMORRHAGIC SHOCK**

Chapter 5 showed the presence of hyperfibrinolysis after 60 min severe haemorrhagic shock in the rat, which was associated with tissue hypoperfusion and widespread endothelial damage <sup>419</sup>. Small-volume 7.5% NaCl ALM resuscitation was shown in Chapter 4 to correct hypocoagulopathy within 5 min after 20 min pressure-controlled bleeding and 60 min shock <sup>377</sup>. The aim of Chapter 6 was to investigate if small bolus 7.5% NaCl ALM corrects hyperfibrinolysis, and assess systemic coagulation in real-time by analysing ROTEM EXTEM, INTEM, FIBTEM, and APTEM at 5-min and 15-min intervals.

It was found that 7.5% NaCl ALM was shown to: 1) correct shock-induced coagulopathy, and 2) correct hyperfibrinolysis. This was further supported by higher levels of the key fibrinolytic inhibitor, PAI-1, and lower levels of D-dimers. Thus it appears that small-volume ALM therapy not only corrects coagulopathy, but also is a potential anti-fibrinolytic. Lower levels of P-selectin in ALM-treated animals also provided some evidence for: 1) reduced platelet dysfunction, and 2) improved endothelial function; along with improved cardiac and haemodynamic stability compared to hypertonic saline controls. Further studies are required to quantitate other markers of endothelial injury including E-selectin, von Willebrand factor (vWF), intracellular adhesion molecule-1 (ICAM-1), and syndecan, to investigate whether ALM has an endothelial protective effect.

## (Original Article)

7.5% NaCl adenosine, lidocaine, and Mg<sup>2+</sup> (ALM) resuscitation corrects hyperfibrinolysis in the rat model of severe haemorrhagic shock

by

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## **Abstract**

**Objective:** Hyperfibrinolysis is a common complication of haemorrhagic shock. Our aim was to examine the effect of small-volume 7.5% NaCl adenosine, lidocaine, and Mg<sup>2+</sup> (ALM) on hyperfibrinolysis in the rat model of haemorrhagic shock.

**Methods:** Anaesthetised rats (n=112) were randomly assigned to: 1) Baseline, 2) Shock, 4) 7.5% NaCl controls, or 5) 7.5% NaCl ALM. Animals were bled for 20 min (42% blood loss) and left in shock for 60 min before resuscitation with 0.3 ml IV bolus 7.5% NaCl ± ALM. Rats were sacrificed at 5, 10, 15, 30, 45 and 60 min for ROTEM, and 15 and 60 min for ELISA analyses.

**Results:** Significant prolongation of EXTEM and INTEM clot times and reduced clot firmness suggest early hypocoagulopathy after shock, and correction of APTEM lysis indices indicate hyperfibrinolysis. Small-volume 7.5% NaCl failed to resuscitate and exacerbated hypocoagulopathy with hyperfibrinolysis at 15 min. In contrast, 7.5% NaCl ALM resuscitated haemodynamics, corrected coagulopathy at 5 min and hyperfibrinolysis at 15 min. Correction was associated with lower plasma TF, higher PAI-1, and lower D-Dimers (8% of controls at 60 min), indicating an anti-fibrinolytic effect. P-selectin fell to undetectable levels in 7.5% NaCl ALM animals indicating improved endothelial function, and less platelet dysfunction and inflammation during resuscitation.

**Conclusions:** Small-volume 7.5% NaCl resuscitation exacerbated coagulopathy and hyperfibrinolysis. In contrast, 7.5% NaCl ALM rapidly corrected both coagulopathy and hyperfibrinolysis, and improved endothelial function. ALM's antifibrinolytic effect, together with its resuscitation capability, may have clinical significance after severe trauma.

**Key Words:** fibrinolysis; hyperfibrinolysis; coagulopathy; shock; haemorrhage; ROTEM; resuscitation; endothelial

## Introduction

Traumatic-induced coagulopathy (TIC) begins as an acute impairment of haemostasis that appears in 25–40% of severe trauma patients at hospital admission and is independently associated with a two- to four-fold increased risk of death in both adult and paediatric patients<sup>152,154,320,420,421</sup>. Early TIC is linked to the severity and extent of the primary anatomical injury, haemorrhage, shock, tissue hypoperfusion, endothelial dysfunction, inflammation, platelet dysfunction, type and volume of pre-hospital fluid administration, and degree of hypothermia<sup>420,422-427</sup>. The nature and extent of TIC-induced hyperfibrinolysis and hypofibrinogenaemia is a major concern clinically because it leads to significant morbidity and mortality<sup>194,424,428</sup>. Mortality rates of between 60% and 100% have been reported in severely injured patients with hyperfibrinolysis<sup>429-431</sup>.

Recently we showed in the rat model of severe haemorrhagic shock that small-volume 7.5% NaCl adenosine, lidocaine and Mg<sup>2+</sup> (ALM) resuscitated mean arterial pressure (MAP) into a hypotensive range<sup>432,433</sup>, and reversed early coagulopathy at 5 min<sup>434</sup>. The rapid reversal of coagulopathy implies that the extrinsic and intrinsic clotting pathways, clotting factors and post- shock platelets secondary to blood loss were all present and fully operational at this time<sup>434</sup>. More recently, we reported that early TIC in the rat involved a platelet defect after controlled bleeding and a fibrinogen defect with hyperfibrinolysis after shock<sup>435</sup>. In both these studies, we did not examine the ability of 7.5% NaCl ALM and saline controls to correct or exacerbate this platelet or hyperfibrinolytic defect. The aim of the present study was to investigate the hypothesis that small bolus 7.5% NaCl ALM corrects hyperfibrinolysis in the rat model of severe haemorrhagic shock by analysing ROTEM and coagulation and fibrinolysis indices at 5-min and 15-min intervals for a real-time assessment of systemic coagulation.

## Methods

### Animals and Reagents

Male Sprague-Dawley rats (300-400g) were obtained from James Cook University's Breeding Colony, Townsville, Australia. All animals were housed in a 14-10 hr light-dark cycle with free access to food and water, and were anaesthetised intraperitoneally with 100 mg/kg sodium thiopentone (Thiobarb). Anaesthetic was administered as required throughout the protocol. The study conforms to the *Guide for Care and Use of Laboratory Animals* (NIH, 8<sup>th</sup> Edition, 2011) and was approved by JCU Animal Ethics Committee, No. A1646. Adenosine (>99%) and other chemicals were obtained from Sigma Aldrich (Castle Hill, Australia). Thiobarb and lidocaine hydrochloride (20% w/v solution) were purchased from Lypard (Townsville, Australia).

### Surgical Protocol

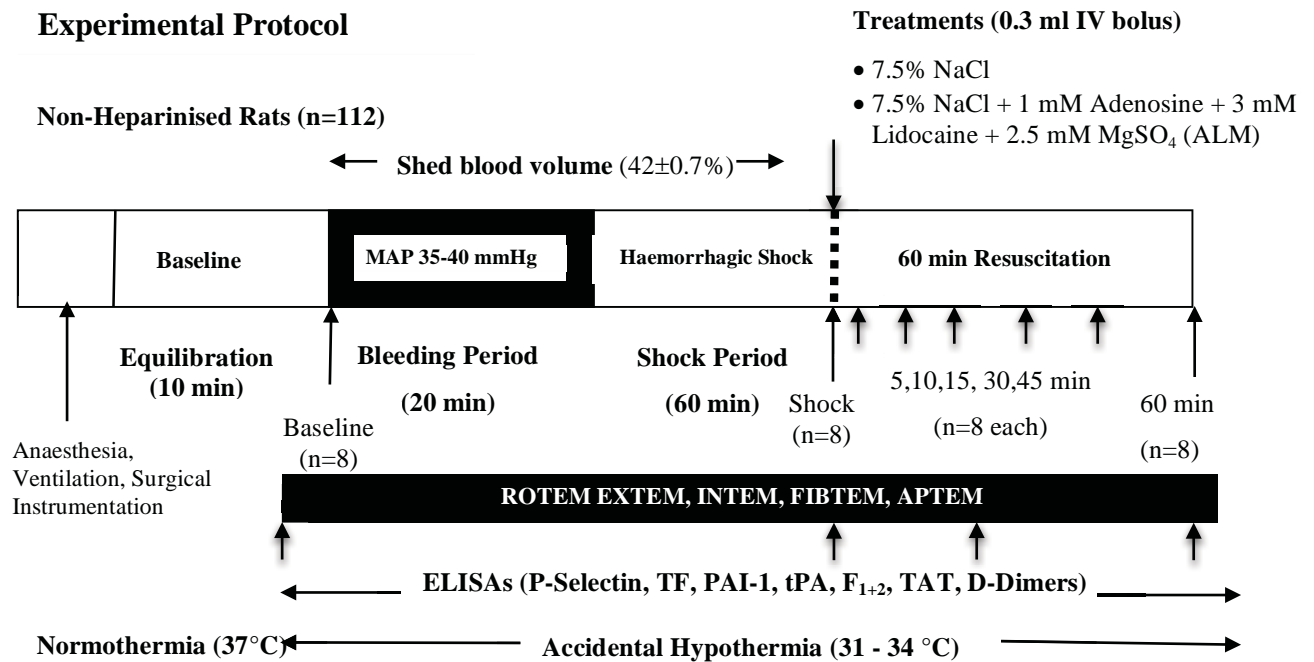
Surgical protocol has been described previously<sup>92,432</sup>. Briefly, following anaesthesia, a tracheotomy was performed and animals ventilated on humidified room air at 90 strokes/min with positive end expiratory pressure (PEEP) of 1 cm and 5 ml/kg tidal volume (Harvard Small Animal Ventilator, Holliston, USA). Temperature was monitored with a rectal probe (T-type Pod, ADInstruments, Bella Vista, Australia). No thermal support was provided during surgery, haemorrhagic shock, or resuscitation. The left femoral vein and artery were cannulated using PE-50 tubing for infusions and haemodynamic monitoring (Powerlab, ADInstruments)<sup>433</sup>, and the right femoral artery was cannulated for blood withdrawal and sampling<sup>436</sup>. Animals were non-heparinised and cannula patency was maintained with citrate-phosphate-dextrose (Sigma Aldrich).

### Experimental Design

Rats were randomly assigned to: 1) Baseline, 2) Shock, 3) 7.5% NaCl controls, and 4) 7.5% NaCl ALM (all n=8) (Fig. 1). Animals were sacrificed: i) following surgical intervention and 10 min stabilisation period prior to bleed (Baseline), ii) after 60 min shock period (Shock), or iii) after 5 min, 10 min, 15 min, 30 min, 45 min, or 60 min resuscitation. For resuscitation, rats received 0.3 ml bolus IV 7.5% NaCl ± ALM administered via the left femoral vein (Fig. 1). ALM comprised 1 mM Adenosine, 3 mM Lidocaine, and 2.5 mM MgSO<sub>4</sub>.



## Experimental Protocol



**Figure 1.** Schematic of the pressure-controlled *in vivo* rat model of severe haemorrhagic shock.

## Shock Protocol

Haemorrhagic shock was induced by withdrawing blood from the femoral artery to reduce MAP to 35-40 mmHg, and as animal compensated more blood was removed to maintain MAP in this range and this process was continued over 20 min (bleed period). Shed blood was kept at room temperature (22°C) in the presence of 0.7 ml CPD/10 ml blood for reinfusion if required. Rats were left in shock for 60 min, and blood was withdrawn or re-infused in 0.1 ml increments to ensure MAP remained between 35-40 mmHg. Average shed volume was 12.3±0.4 ml and represented an average blood loss of 42±0.7% over the bleed and shock periods. After 60 min shock, rats in resuscitation groups received 0.3 ml fluid (~3% shed volume) (Fig. 1).

## Rotation Thromboelastometry (ROTEM®)

ROTEM® (Tem International, Munich, Germany) was conducted according to manufacturer's instructions<sup>435</sup>. Whole blood collected in 3.2% sodium citrate tubes was warmed to 37°C. Four assays were performed: EXTEM (extrinsically-activated test using tissue factor), INTEM (intrinsically-activated test using ellagic acid), FIBTEM (fibrin-based EXTEM-activated test with 50 µg/ml cytochalasin D, to inhibit platelet contribution to clot formation) and APTEM (activation as for EXTEM with aprotinin to inhibit plasmin activation of fibrinolysis)<sup>435,437</sup>. All assays were run for 120 min.

ROTEM parameters include clotting time (CT, sec); clot formation time (CFT, sec); alpha angle ( $\alpha^\circ$ ); clot amplitude (A, mm); maximum clot firmness (MCF, mm); and maximum clot elasticity (MCE). Clot lysis parameters include maximum lysis (ML, %) and lysis index (LI, %). LI30, 45 and 60 represent the percentage of remaining clot firmness in relation to the MCF value at 30, 45 and 60 min after CT. Hyperfibrinolysis was defined as a decrease in percentage lysis  $\geq 15\%$  in the LI(30-60) index and confirmed with APTEM test<sup>435</sup>. The APTEM test, run simultaneously alongside the EXTEM test, has been validated for diagnosis of hyperfibrinolysis in trauma patients and other patient populations<sup>430,438,439</sup>. Quality control measurements (ROTROL-N and ROTROL-P) were performed weekly<sup>435</sup>.

### **ELISAs**

Plasma levels of Platelet selectin (P-selectin), Tissue factor (TF), Plasminogen activator inhibitor (PAI-1), Tissue-type plasminogen activator (tPA), Prothrombin Fragment 1+2 (F<sub>1+2</sub>), Thrombin-Antithrombin Complex (TAT), and D-dimers, were quantitated with rat-specific ELISA kits (MyBiosource, Resolving Images, Thomastown, Australia). ELISA kits were performed according to manufacturer's instructions. Detection range, sensitivity, and intra- and inter-assay CVs for each assay were as follows: P-selectin: 0.5-200 ng/ml, 0.21 ng/ml, <10%, <12%; TF: 0-10 ng/ml, 0.1 ng/ml, <10%, <10%; PAI-1: 0-10 ng/ml, 0.1 ng/ml, <9%, <10%; tPA: 0-2500 pg/ml, 1.0 pg/ml, <9%, <10%; F<sub>1+2</sub>: 62.5 pmol/L-4000 pmol/L, 15.6 pmol/L, <8%, <10%; TAT: 0-25 ng/ml, 0.1 ng/ml, <10%, <10%; D-dimers: 0-2500 pg/ml, 1.0 pg/ml, <9%, <10%.

### **Statistical Analysis**

*A priori* power analysis was conducted using G-power<sup>3</sup> program to determine sample size to minimize Type 1 errors (MAP 60 min resuscitation; n=8). SPSS Statistical Package 21 was used for all statistical analysis (IBM). All values are expressed as mean  $\pm$  SEM. Data normality was assessed with Shapiro-Wilks and Levene's test was used to determine equality of variances. Data were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's Honestly Significant Difference or Dunnett's post-hoc test. Two-way ANOVA comparison was used for within group comparisons. Non-parametric data was evaluated using Kruskal-Wallis test. ELISA data was analysed using Graphpad Prism 7 4-parameter-logistic curve fitting. Statistical significance was defined as  $p < 0.05$ .

## Results

### Haemodynamics During Shock and Resuscitation

There were no significant differences in haemodynamics between the groups at baseline or after 60 min haemorrhagic shock (Table 1). Heart rate (HR) decreased by 20% from baseline during shock which was associated with over 60% falls in systolic pressure, diastolic pressure and mean arterial pressure (MAP), and similar to results from previous studies (Table 1)<sup>419,434</sup>. Rectal body temperature fell by 2.4°C to 3.9°C from baseline during the 60 min shock period (Table 1).

At 5 min, 7.5% NaCl-treated rats significantly increased pulse pressure (PP), an index of stroke volume, 1.6-fold from shock, and MAP from 34 mmHg to a peak of 45 mmHg. MAP then steadily decreased to 40 mmHg over 60 min, PP decreased from 28 to 25 mmHg, and body temperature fell to 31.8°C at 60 min. In contrast, a 0.3 ml bolus of 7.5% NaCl ALM at 5 min significantly raised MAP to 64 mmHg, which stabilized then increased to 68 mmHg at 60 min. This steady increase in MAP was associated with significant increases in PP, and the maintenance of significantly higher body temperature (34°C at 60 min) (Table 1).

### ROTEM Parameters after 60 min Haemorrhagic Shock

ROTEM data after 60 min haemorrhagic shock were consistent with a hypocoagulable state (Table 2; Fig. 2-3) and support previous findings in this rat model<sup>377,419</sup>. Clot times (CT) in EXTEM significantly increased three times from baseline, 16 times in INTEM ( $p<0.05$ ), and 19 times in FIBTEM (Table 2). Clot formation time (CFT) was also prolonged 9-fold in EXTEM after shock, and 6-fold in INTEM accompanying falls in alpha angle and decreases in clot formation rate (CFR) and maximum clot elasticity (MCE) (Table 2). Three of eight animals were unable to initiate clot formation after 60 min shock. EXTEM amplitude at 10 min (A10) decreased by 50% after 60 min shock, INTEM by 75%, and FIBTEM by 56%, indicating a significant loss of clot strength (Fig. 2). A representative schematic of the ROTEM profile after 60 min haemorrhagic shock compared to baseline is shown in Figure 4.

TABLE 1 Haemodynamics before and after shock and during 60 min resuscitation with hypertonic saline alone and hypertonic saline with ALM

Time	HR (bpm)		SP (mmHg)		DP (mmHg)		MAP (mmHg)		PP (mmHg)		Temp (°C)	
	NaCl	ALM	NaCl	ALM	NaCl	ALM	NaCl	ALM	NaCl	ALM	NaCl	ALM
Baseline	356±16	357±17	131±7	132±11	97±5	98±9	108±5	110±10	34±5	34±4	36.9±0.1	36.6±0.3
60 min Shock	287±23	280±15	50±2	56±2	32±1	32±1	34±4	40±1	18±3	24±3	33.1±0.5	34.2±0.3
5 min Resuscitation	274±16	277±13	64±3 <sup>†</sup>	92±4*	36±2 <sup>†</sup>	50±2*	45±2 <sup>†</sup>	64±2*	28±3 <sup>†</sup>	42±4 <sup>†</sup>	33.0±0.5 <sup>†</sup>	34.2±0.3
10 min Resuscitation	273±14	288±15 <sup>§</sup>	64±3 <sup>†</sup>	88±6*	35±2	50±3*	44±3	63±4*	28±4 <sup>†</sup>	38±4 <sup>¶</sup>	32.9±0.5 <sup>¶</sup>	34.0±0.3 <sup>†</sup>
15 min Resuscitation	275±14	289±16 <sup>§</sup>	60±4 <sup>†</sup>	86±5*	34±2	49±3*	42±3	61±3*	26±3 <sup>†</sup>	37±4 <sup>†</sup>	32.8±0.5 <sup>¥</sup>	34.0±0.3 <sup>†</sup>
30 min Resuscitation	294±18 <sup>°</sup>	293±17	61±5	86±4*	32±3	49±3*	42±3	61±3*	28±4 <sup>†</sup>	36±4 <sup>¶</sup>	32.5±0.5 <sup>¥</sup>	33.9±0.3*
45 min Resuscitation	294±18 <sup>^</sup>	298±19 <sup>¶</sup>	59±5	92±5*	32±3	53±4*	41±3	66±4*	27±5 <sup>†</sup>	39±6 <sup>†</sup>	32.2±0.5 <sup>£</sup>	33.9±0.4 <sup>Ω</sup>
60 min Resuscitation	296±20 <sup>^</sup>	300±20 <sup>¶</sup>	57±7	97±7*	32±5	55±5*	40±5	68±5*	25±5	42±7 <sup>†</sup>	31.8±0.5 <sup>∞</sup>	34.0±0.4 <sup>Ω</sup>

HR = heart rate; SP = systolic pressure; DP = diastolic pressure; MAP = mean arterial pressure; PP = pulse pressure = SP – DP; Temp = rectal temperature. All parameters for both treatment groups at 60 min shock, and throughout resuscitation, are significantly different to baseline, except PP from 10-60 min resuscitation. <sup>Ω</sup> $p < 0.05$  compared to 7.5% NaCl treatment group; \* $p < 0.05$  compared to 7.5% NaCl treatment group and 60 min shock; <sup>†</sup> $p < 0.05$  compared to 60 min shock; <sup>¶</sup> $p < 0.05$  compared to 60 min shock and 5 min resuscitation; <sup>¥</sup> $p < 0.05$  compared to 60 min shock and 5-15 min resuscitation; <sup>£</sup> $p < 0.05$  compared to 60 min shock and 5-30 min resuscitation; <sup>∞</sup> $p < 0.05$  compared to 60 min shock and 5-45 min resuscitation; <sup>§</sup> $p < 0.05$  compared to 5 min resuscitation; <sup>°</sup> $p < 0.05$  compared to 10 min resuscitation; <sup>^</sup> $p < 0.05$  compared to 10 min and 15 min resuscitation

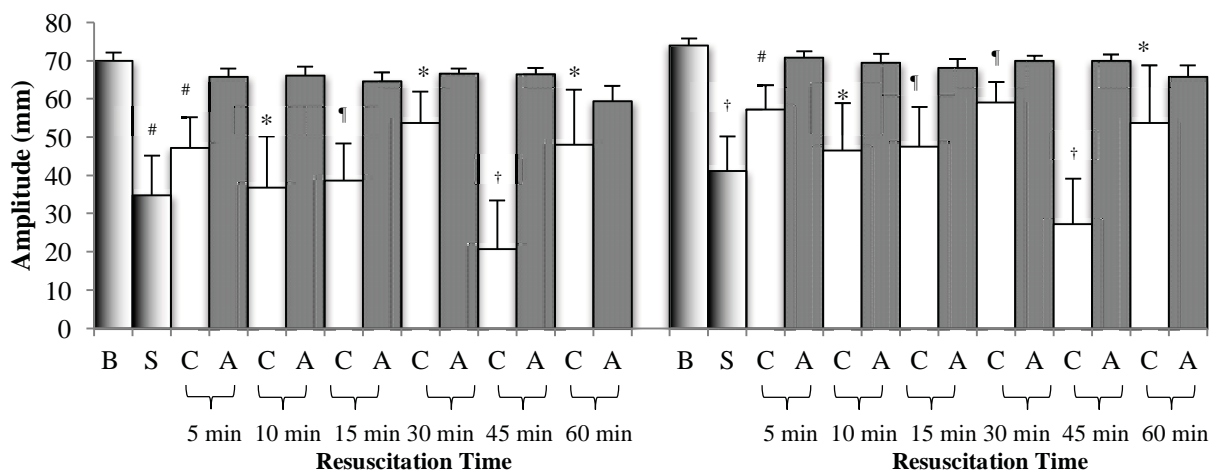
TABLE 2: ROTEM parameters from EXTEM, INTEM, and FIBTEM tests for baseline, shock, and after 5, 10, 15, 30, 45, and 60 min resuscitation with hypertonic saline alone and hypertonic saline with ALM

Parameter	Baseline	Shock	5 min		10 min		15 min		30 min		45 min		60 min		
			NaCl	ALM	NaCl	ALM	NaCl	ALM	NaCl	ALM	NaCl	ALM	NaCl	ALM	
EXTEM	CT (s)	<b>41±9</b>	<b>127±37*</b> (n=5)	<b>253±114*</b> (n=5)	<b>47±7</b>	<b>241±149</b> (n=5)	<b>40±4</b>	<b>118±31*</b> (n=5)	<b>47±10</b>	<b>89±32#</b> (n=4)	<b>39±4</b>	<b>1251±537#</b> (n=4)	<b>36±3</b>	<b>115±37*</b> (n=3)	<b>53±11</b>
	CFT (s)	42±13	365±203*	210±90#	33±2	156±99#	35±6	1065±808	38±3	102±52#	33±2	1134±990#	34±3	1066±999	63±27
	α (°)	82±3	68±11	59±10#	83±1	70±9#	83±1	60±11	82±1	73±7#	83±0.5	35±27#	78±5	77±4	78±4
	CFR (°)	83±2	75±7	71±5#	84±0.5	65±10#	84±1	74±6	83±1	77±6	84±0.5	70	84±1	80±4	80±3
	MCE	294±22	109±50*	153±31#	247±17	134±54*	238±21	119±37#	255±26	154±28#	273±33	60±48#	239±17*	153±76	220±17*
			(n=5)	(n=5)		(n=5)		(n=7)		(n=4)		(n=3)		(n=3)	
INTEM	CT (s)	<b>145±36</b>	<b>2318±1789*</b> (n=5)	<b>1728±734#</b> (n=6)	<b>172±32</b> (n=7)	<b>547±403</b> (n=2)	<b>943±756</b> (n=7)	<b>913±548</b> (n=5)	<b>85±28</b> (n=7)	<b>173±40</b> (n=4)	<b>251±139</b> (n=5)	<b>1294±301#</b> (n=6)	<b>135±31</b> (n=5)	<b>2689±913#</b> (n=4)	<b>147±35</b>
	CFT (s)	55±27	347±224	176±123	57±16	468±431	35±6	1933±1081	40±9	193±113*	704±542*	-	35±4	-	100±39*
	α (°)	80±4	51±17	62±19	79±3	50±32	70±13	39±17#	82±2	66±8*	57±15*	-	83±1	-	73±5*
	CFR (°)	82±4	57±7*	71±5#	81±2	55±28	73±8	66±12	83±1	69±8*	63±12*	65±5*	84±1	70±5	76±5
	MCE	309±33	42±28*	70±51#	259±27	166±101	176±54	78±59*	232±23	146±32*	176±74	5±1#	271±31	9±4#	229±41
			(n=5)	(n=5)	(n=7)	(n=2)	(n=7)	(n=5)	(n=7)	(n=4)	(n=5)	(n=6)	(n=5)	(n=4)	
FIBTEM	CT (s)	<b>42±8</b>	<b>801±503</b> (n=5)	<b>838±408#</b> (n=6)	<b>49±8</b>	<b>1454±836</b> (n=6)	<b>41±8</b>	<b>676±592</b> (n=5)	<b>49±6</b>	<b>304±164#</b> (n=4)	<b>47±4</b>	<b>158±41#</b> (n=4)	<b>36±3</b>	<b>1154±770#</b> (n=6)	<b>57±17</b>
	CFT (s)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	
	α (°)	79±1	80	72±7	73±4	80	81±1	62±19	69±4	65	76±3	-	77±3	-	68±7
	CFR (°)	80±1	78±2	57±16	76±3	72±6	82±1	82±1	73±3	67	78±2	67	80±2	68	78±2
	MCE	18±1	10±4	11±2#	16±1	9±2#	18±2	8±2#	14±2	9±4#	17±1	7±1#	17±2	5±1#	15±2
			(n=5)	(n=6)		(n=6)		(n=5)		(n=4)		(n=4)		(n=4)	

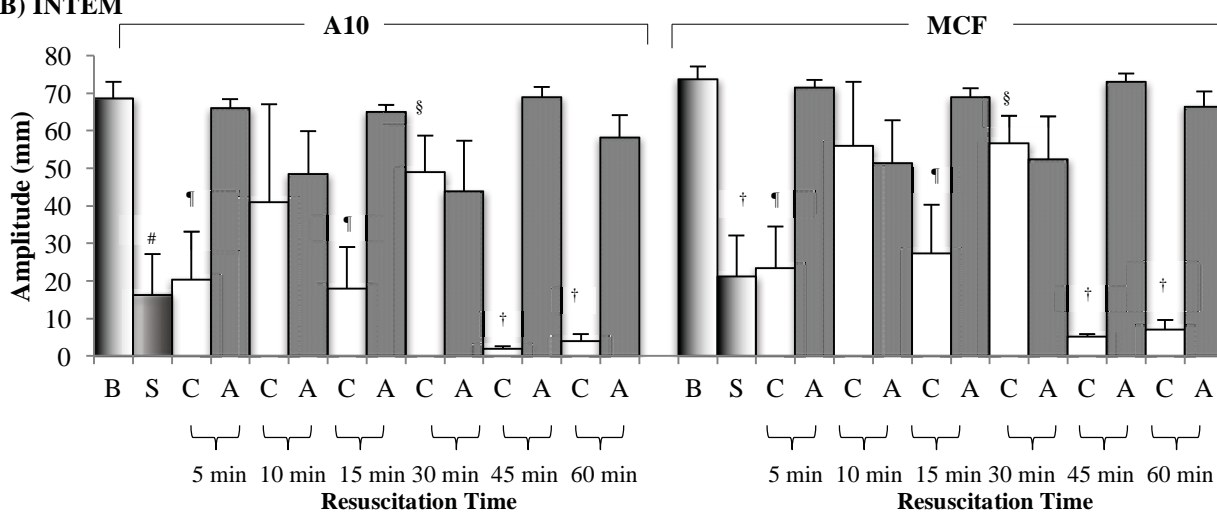
Number of calculated values in parentheses; if not shown n=8. CT = clot time (sec); CFT = clot formation time (sec); α = alpha angle (°); CFR = clot formation rate (°); MCE = maximum clot elasticity. CT and MCE recorded with initiation of 2 mm clot; CFT requires formation of 20 mm clot. NA = not applicable. \*p<0.05 compared to Baseline group; #p<0.05 compared to Baseline and 7.5% NaCl ALM group at same resuscitation time

Figure 2

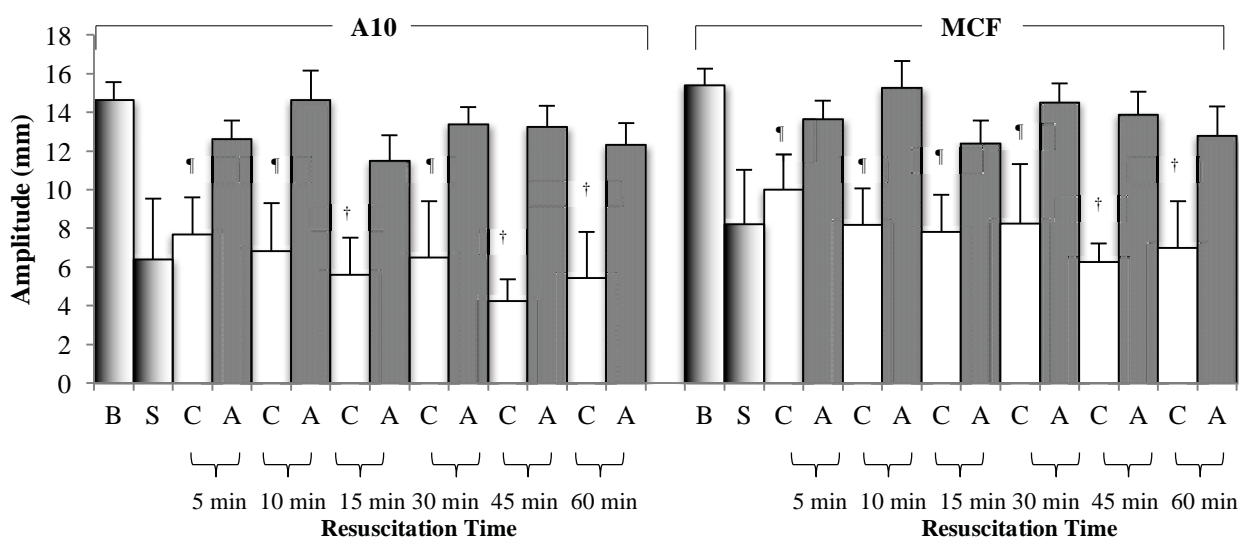
A) EXTEM



B) INTEM

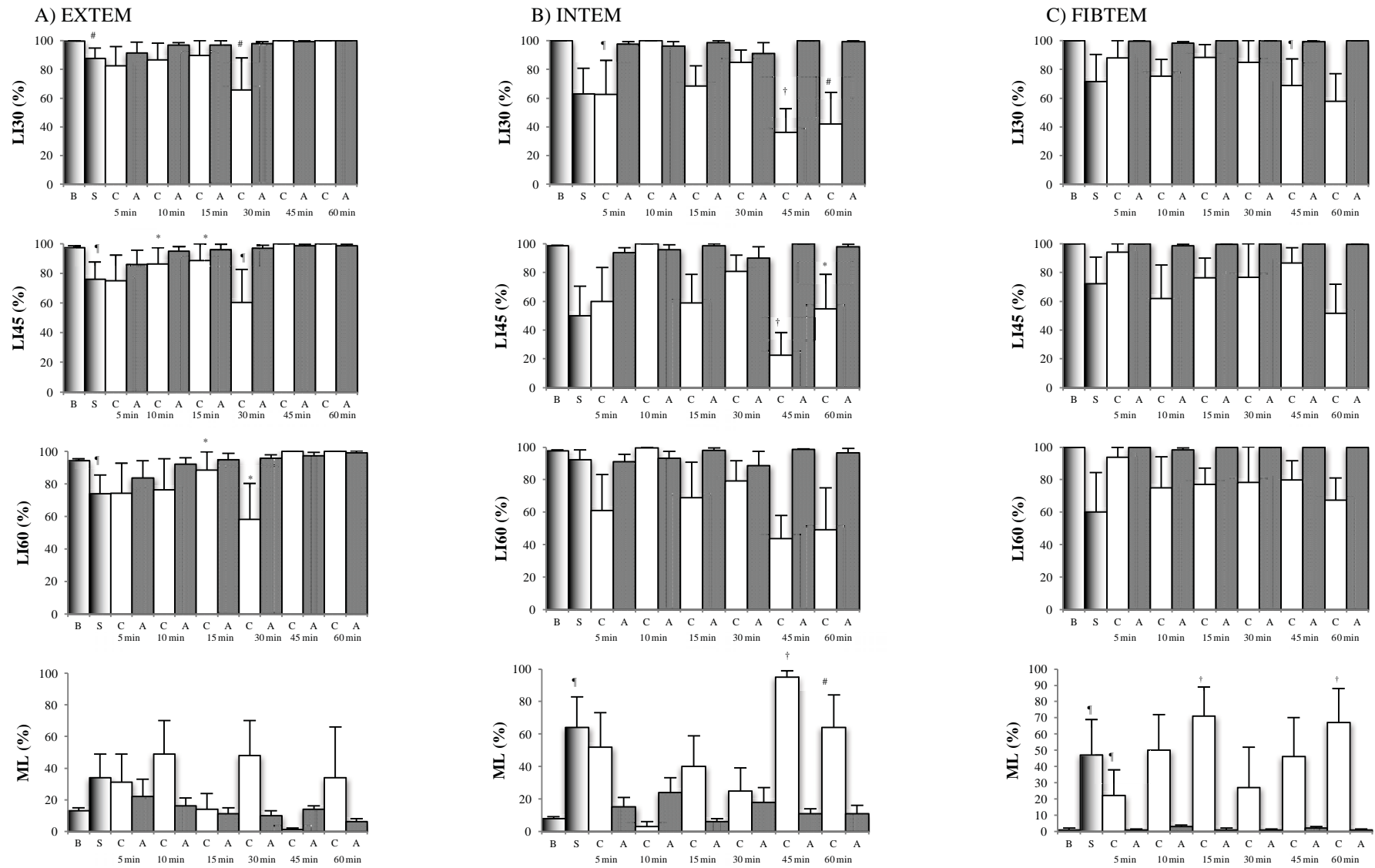


C) FIBTEM



**Figure 2.** Clot amplitudes at 10 min (A10) and maximum clot firmness (MCF) for EXTEM (A), INTEM (B) and FIBTEM (C) tests at baseline (B), following 60 min shock (S), and after 5, 10, 15, 30, 45 and 60 min resuscitation with 7.5% NaCl (C) and 7.5% NaCl with ALM (A). Values represent the mean  $\pm$  SEM. n=8 with the following exceptions due to non-physiological clot formation: EXTEM (A), n=5 for shock, 7.5% NaCl 5 min and 10 min groups; n=4 for 7.5% NaCl 30 min, 45 min, and 60 min groups. INTEM (B), n=7 for ALM 5 min, 10 min, and 15 min groups; n=6 for 7.5% NaCl 45 min group; n=5 for shock, 7.5% NaCl 5 min and 60 min groups, ALM 30 min and 45 min groups; n=4 for 7.5% NaCl 30 min group; n=2 for 7.5% NaCl 10 min group. FIBTEM (C), n=6 for 7.5% NaCl 5 min and 10 min groups; n=5 for shock, 7.5% NaCl 15 min and 60 min groups; n=4 for 7.5% NaCl 30 min and 45 min groups. \* $p$ <0.05 compared to baseline; # $p$ <0.05 compared to baseline, ALM 5-30 min groups; ¶ $p$ <0.05 compared to baseline and ALM at same resuscitation time; † $p$ <0.05 compared to baseline and all ALM groups; § $p$ <0.05 compared to baseline and 7.5% NaCl 45-60 min groups.

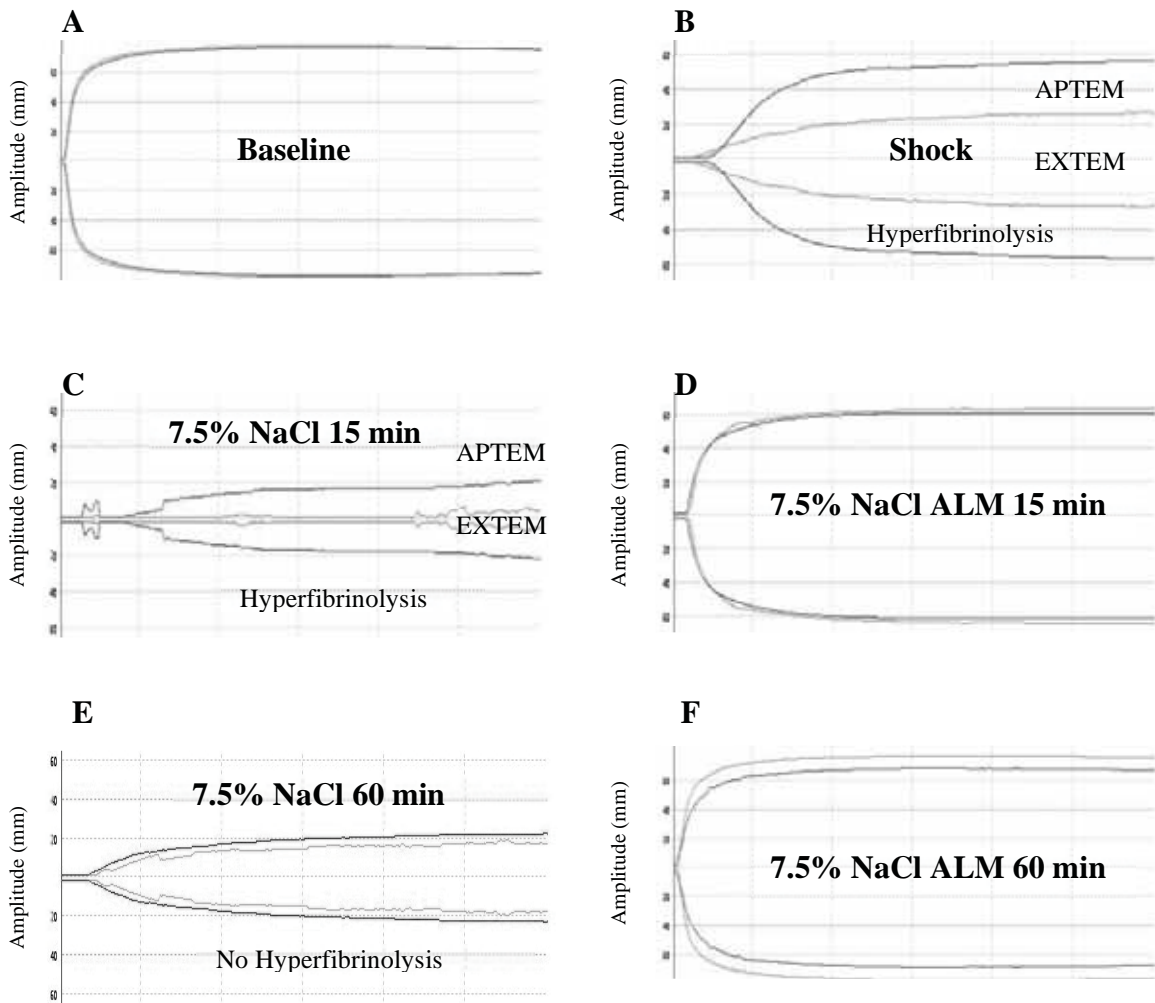
Figure 3





**Figure 3.** ROTEM lysis indices at 30 min (LI30%), 45 min (LI45%), and 60 min (LI60%), and maximum lysis (ML%) at baseline (B), after 60 min shock (S), and following 5, 10, 15, 30, 45 and 60 min resuscitation with 7.5% NaCl (C) and 7.5% NaCl with ALM (A). Values represent the mean  $\pm$  SEM. n=8 with the following exceptions due to non-physiological clot formation: EXTEM (A) n=5 for shock, 7.5% NaCl 5 min and 10 min groups; n=4 for 7.5% NaCl 30 min and 45 min groups, and n=3 for 7.5% NaCl 60 min group. INTEM (B) n=7 for 7.5% ALM 5 min, 15 min, and 60 min groups; n=6 for 7.5% NaCl 45 min and ALM 10 min groups; n=5 for shock, 7.5% NaCl 15 min, and ALM 45 min groups; n=4 for 7.5% NaCl 5 min, 30 min, and 60 min groups; and n=2 for 7.5% NaCl 10 min group. FIBTEM (C) n=6 for 7.5% NaCl 5 min, 10 min, and 60 min groups; n=5 for shock and 7.5% NaCl 15 min groups; and n=4 for 7.5% NaCl 30 min and 45 min groups. \* $p$ <0.05 compared to baseline; # $p$ <0.05 compared to baseline; † $p$ <0.05 compared to baseline and ALM 60 min; ‡ $p$ <0.05 compared to ALM 60 min group; † $p$ <0.05 compared to baseline and all ALM groups.

**Figure 4.** Representative ROTEM temograms of EXTEM and APTEM for baseline (A), shock (B), 15 min 7.5% NaCl resuscitation (C) and 60 min 7.5% NaCl resuscitation (E), and 15 min (D) and 60 min (F) 7.5% NaCl ALM resuscitation. The complete data for all animals can be found in Tables 2 and 3, and Figures 2 and 3.



## **ROTEM Parameters During Small-Volume 7.5% NaCl ± ALM Resuscitation after 60 min Shock**

**EXTEM:** CT for 7.5% saline controls at 5 and 10 min resuscitation increased two-fold over shock values to  $253\pm114$  and  $241\pm149$  sec, with significantly prolonged CFTs compared to baseline, indicating a worsening of hypocoagulopathy (Table 2). Similar to the haemodynamic profile, coagulopathy progressively worsened over 60 min 7.5% NaCl resuscitation highlighted by the percentage of animals able to generate a physiological clot (amplitude >20 mm) decreasing from 63% after 5 min resuscitation to 25% at 45 min and 38% at 60 min resuscitation (Table 2; Fig 2). Saline controls also had significantly reduced alpha angles and MCEs. In direct contrast, 7.5% NaCl ALM treatment led to a near full correction of clotting times with CT values of  $47\pm7$  sec at 5 min and  $53\pm11$  sec at 60 min. CFT, alpha angle, and CFR did not significantly differ to baseline during 60 min resuscitation (Table 2, Fig. 4).

EXTEM A10 values for 7.5% NaCl controls were 47, 37, 39, 54, 21 and 48 mm at 5, 10, 15, 30, 45 and 60 min resuscitation respectively, compared to 70 mm at baseline and 35 mm at shock (Fig. 2). The addition of ALM to 7.5% NaCl led to a near full correction of A10 and MCF (Fig. 2). EXTEM ML (%) was  $\geq 31\%$  after 5, 10, 30, and 60 min resuscitation with 7.5% NaCl alone indicating significant fibrinolysis (Fig. 3). EXTEM lysis indices in saline controls at 5 min and 30 min resuscitation were 83% and 66% (LI30), 75% and 61% (LI45), and 74% and 58% (LI60), supporting a fibrinolytic phenotype (Fig. 3). Conversely, LI(30-60)% for ALM-treated animals were comparable to baseline, as was ML (%) (Fig. 3).

**INTEM:** Clotting times (CTs) in animals treated with 7.5% NaCl bolus were 1728 sec at 5 min, 547 sec at 10 min, and increased to 2689 sec ( $p<0.05$ ) after 60 min resuscitation. CFTs were also prolonged and alpha angles reduced, with significantly lower clot elasticities, which dropped to 2 to 3% baseline at 45 and 60 min (Table 2). Similar to EXTEM, INTEM hypocoagulopathy was progressive in 7.5% NaCl-resuscitated rats and after 45 min resuscitation no saline-treated animals propagated physiological clots larger than 20 mm (Table 2; Fig. 2). Clot amplitude (A10) and MCF in saline controls were variable (<5mm to 56 mm) but remained lower than baseline

( $p < 0.05$ ) and ALM-treated rats during 60 min resuscitation (Fig. 2). INTEM clot times and kinetics for ALM-resuscitated animals did not differ from baseline, except at 30 min resuscitation with significantly prolonged CFT, and reduced alpha angle and CFR (Table 2). This corresponded with the minimum haemodynamics in the ALM group prior to increases in MAP, SP, DP, and PP (Table 1).

Clot amplitudes (A10) in 7.5% NaCl-treated animals were 20 mm, 18 mm, 2 mm, and 4 mm after 5 min, 15 min, 45 min, and 60 min resuscitation respectively, which were significantly lower than baseline (69 mm) and ALM-treated animals (66 mm, 65 mm, 69 mm, and 58 mm) (Fig. 2). INTEM lysis indices and ML showed significant clot breakdown (25-90%) for 7.5% NaCl at 5, 15, 30, 45 and 60 min resuscitation (Fig. 3). In contrast, for 7.5% NaCl ALM groups, the lysis index ranged from 89.8% to 100% indicating little or no clot breakdown (Fig. 3).

**FIBTEM:** CT for 7.5% NaCl-treated rats ranged from 304 to 1454 sec, and were 3 to 5 times higher than their corresponding EXTEM CT values at the same time points during 60 min resuscitation (Table 2). In direct contrast, FIBTEM CT for 7.5% NaCl ALM rats were 49, 41, 49, 47, 36 and 57 sec respectively, and did not differ from EXTEM CT values or baseline values, indicating the ALM correction of CT was in part due to preservation and/or restoration of platelets during resuscitation. FIBTEM clot amplitudes (A10) for saline controls were similar to shock (6 mm), and ranged from 4 to 8 mm over 60 min of resuscitation ( $p < 0.05$  compared to baseline) (Fig. 2). In contrast, FIBTEM (A10) values in the ALM group were similar to the baseline amplitude of 15 mm (12 to 15 mm) (Fig. 2). The FIBTEM/EXTEM A(10) ratio for 7.5% saline controls at 5, 10, 15, 30, 45 and 60 min were 16%, 19%, 15%, 12%, 20%, and 11% respectively, compared to baseline ratio of 21%. In contrast, 7.5% NaCl ALM-resuscitated rats preserved baseline FIBTEM/EXTEM A(10) ratios (18-22% over 60 min resuscitation). These differences between 7.5% NaCl controls and ALM-treated animals were also reflected in maximum clot firmness (Fig. 2).

The FIBTEM LI30(%) for 7.5% NaCl controls was 88%, 75%, 88%, 85%, 69% and 58% at 5, 10, 15, 30, 45, and 60 min respectively, compared to 98-100% for 7.5% NaCl ALM rats over the 60 min resuscitation period (Fig. 3). Lysis indices 45-60(%) for 7.5% NaCl ALM rats were also 98-100%, compared to 94% at 5 min, 62-75% at 10

min, 76-78% at 15 and 30 min, 80-87% at 45 min, and 52-67% at 60 min for saline controls (Fig. 4). The FIBTEM lysis index data and  $ML \geq 22\%$  throughout 60 min resuscitation suggest that controls were fibrinolytic, but not the ALM-treated animals.

**APTEM:** Lysis indices and clot amplitudes from APTEM and EXTEM assays run simultaneously were compared to confirm hyperfibrinolysis in shock and after 15 min and 60 min 7.5% NaCl resuscitation (Table 3). These groups had  $LI(30-60) \leq 85\%$  or  $ML \geq 15\%$  on EXTEM, INTEM, and FIBTEM assays indicating hyperfibrinolysis (Fig. 3). APTEM  $LI(30-60)\%$  for shock group were corrected from 74-88% (EXTEM) to 98-99%, and ML was corrected from 34% to 7%, confirming hyperfibrinolysis after 60 min shock. Shock clot amplitudes were also increased by 12-18 mm after addition of aprotinin in APTEM assay, further supporting a hyperfibrinolytic state after 60 min haemorrhagic shock (Table 3; Fig. 4).

APTEM  $LI(30-60)\%$  for 7.5% NaCl controls at 15 min resuscitation were corrected to 97-98% (8-9% higher than EXTEM  $LI(30-60)\%$ ), and ML was corrected to 8%, suggesting hyperfibrinolysis (Table 3; Fig. 4). However, in contrast to shock group, inhibiting fibrinolysis with aprotinin did not correct clot amplitudes after 15 min saline resuscitation (Table 3). At 60 min resuscitation EXTEM and APTEM  $ML(\%)$  were the same (34%), and clot amplitudes were comparable, indicating the profound hypocoagulopathy after 60 min 7.5% NaCl resuscitation was not associated with hyperfibrinolysis (Tables 2-3; Figs. 2-4).

TABLE 3: Comparison of EXTEM and APTEM (EXTEM + aprotinin) clot lysis and clot amplitude parameters for detection of hyperfibrinolysis in shock and 7.5% NaCl resuscitation groups

Parameter	Shock (n=5)*		7.5% NaCl 15 min Resuscitation (n=8)		7.5% NaCl 60 min Resuscitation (n=3)**	
	EXTEM	APTEM	EXTEM	APTEM	EXTEM	APTEM
LI30%	<b>88±7</b>	<b>99±1</b>	<b>88±12</b>	<b>97±2</b>	100±0	100±0
LI45%	<b>76±11</b>	<b>99±1</b>	<b>89±11</b>	<b>98±2</b>	100±0	100±0
LI60%	<b>74±12</b>	<b>98±1</b>	<b>89±11</b>	<b>97±2</b>	100±0	100±0
ML%	<b>34±15</b>	<b>7±3</b>	<b>14±10</b>	<b>8±4</b>	34±32	34±31
A5 (mm)	29±10	41±11	37±9	33±9	37±18	34±15
A10 (mm)	35±10	49±8	39±10	39±9	43±19	39±18
A15 (mm)	38±10	52±7	41±10	41±8	47±17	42±18
A20 (mm)	39±11	54±7	43±9	42±8	50±18	43±19
A25 (mm)	39±10	54±7	45±9	43±8	51±17	43±19
A30 (mm)	37±10	55±7	46±9	43±8	51±17	44±20
MCF (mm)	41±9	57±7	48±10	47±8	49±20	46±19

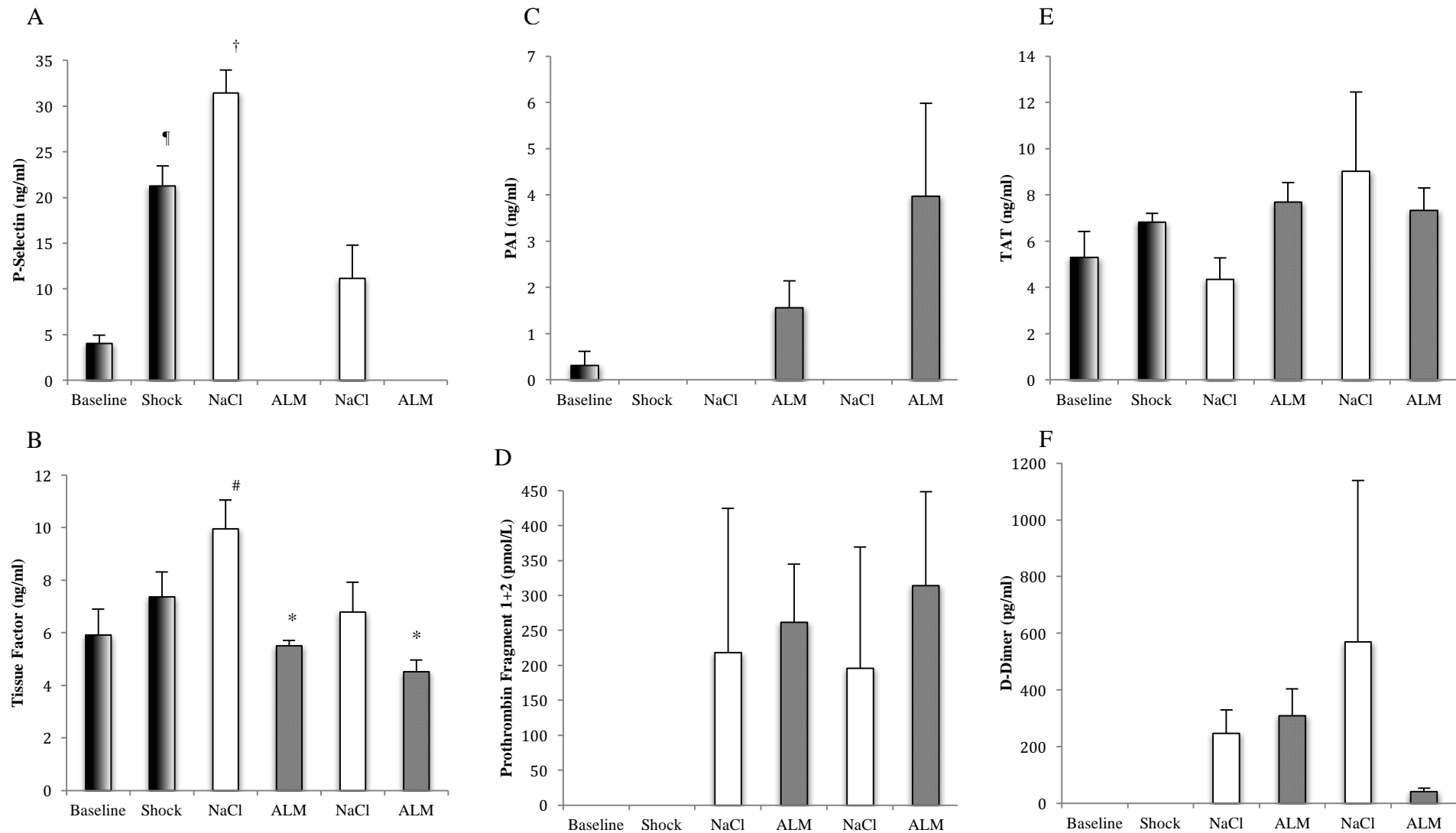
Values represent mean ± SEM. LI = lysis index, %; ML = maximum lysis, %; A = clot amplitude, mm; MCF = maximum clot firmness, mm. n values represent number of ROTEM calculated parameters dependent on minimum clot amplitude of 2 mm being reached. \*Three animals from shock group and \*\*five animals from 7.5% NaCl 60 min resuscitation group could not initiate tissue factor-activated clot formation (flat-line ROTEM assay). **Hyperfibrinolysis (bold values)** confirmed by normalization of lysis index in APTEM (EXTEM-activated assay with aprotinin for *in vitro* inhibition of fibrinolysis). Baseline and 7.5% NaCl ALM 15 min and 60 min resuscitation groups not shown because LI≥85% indicating no fibrinolysis (Fig. 3).

## **Plasma Levels of Soluble P-Selectin, Tissue Factor, PAI, tPA, TAT, F<sub>1+2</sub>, and D-Dimers**

P-Selectin levels significantly increased over 5-fold after 60 min haemorrhagic shock compared to baseline (Fig. 5). Values were similar to our previous study<sup>419</sup>. In 7.5% NaCl controls, P-Selectin values continued to rise after 15 min resuscitation (7.8-fold higher than baseline;  $p < 0.05$ ) and remained significantly elevated at 60 min (2.8 times higher than baseline). In contrast, P-Selectin was not detected in ALM-treated rats at 15 or 60 min resuscitation. Baseline tissue factor (TF) was 5.9 ng/ml and increased ~25% to 7.4 ng/ml after shock (Fig. 5). TF in ALM-treated rats was not significantly different from baseline at 5.5 and 4.5 ng/ml after 15 and 60 min resuscitation. Conversely, after 15 min 7.5% NaCl resuscitation TF levels were significantly higher than baseline and both ALM resuscitation groups, before decreasing to 6.8 ng/ml after 60 min.

Plasminogen activator inhibitor (PAI-1) was not detected after 60 min shock, supporting our results from a previous study<sup>419</sup>, and was also undetectable in saline controls (Fig. 5). In contrast, ALM-treated animals had 5-fold higher levels of PAI-1 than baseline after 15 min, which increased a further 2.5 times by 60 min resuscitation. Tissue-type plasminogen activator (tPA) was not detected at baseline or after 60 min shock, or in any resuscitation group. Thrombin-antithrombin (TAT) complex concentration was 5.29 ng/ml at baseline and increased 28% after shock but these values were not significantly different (Fig. 5). 7.5% NaCl controls at 15 and 60 min were 82% and 1.7 times baseline, and TAT complexes in 7.5% NaCl ALM-treated rats were ~1.4 times baseline after 15 and 60 min resuscitation. Prothrombin Fragment 1+2 (F<sub>1+2</sub>) could not be detected at baseline or after 60 min haemorrhagic shock (Fig. 5). F<sub>1+2</sub> values were highly variable at around ~200 pmol/L in saline controls, and 261 and 314 pmol/L in ALM-treated animals at 15 and 60 min resuscitation. D-Dimers could not be detected at baseline or shock. At 15 min resuscitation, D-Dimer levels were similar in saline controls and ALM rats (246 vs. 308 pg/ml) (Fig. 5). However, at 60 min resuscitation, control values rose to 570 pg/ml but levels fell dramatically in ALM rats (42 pg/ml).

Figure 5





**Figure 5.** Plasma concentrations of (A) P-selectin (ng/ml), (B) tissue factor (TF; ng/ml), (C) plasminogen activator inhibitor-1 (PAI-1; ng/ml), (D) prothrombin fragment 1+2 (F<sub>1+2</sub>; pmol/l), (E) thrombin-antithrombin complex (TAT; ng/ml), and (F) D-Dimers (pg/ml) at baseline, after 60 min shock, and following 15 min and 60 min resuscitation with 7.5% NaCl alone and 7.5% NaCl with ALM. Tissue-type plasminogen activator (tPA; pg/ml) was undetectable in all groups at assay sensitivity of 1.0 pg/ml. ¶*p*<0.05 compared to baseline; †*p*<0.05 compared to baseline, shock, and 7.5% NaCl 60 min group; #*p*<0.05 compared to baseline, and ALM 15 min and 60 min groups; \**p*<0.05 compared to shock group.

## **Discussion**

TIC is a dynamic haemostatic disorder that is associated with significant bleeding, transfusion requirements, morbidity and mortality<sup>440</sup>. The underlying mechanisms remain controversial and are believed to involve trauma, widespread tissue hypoperfusion, endothelial injury, inflammation, platelet dysfunction and hyperfibrinolysis<sup>421,441,442</sup>. Hyperfibrinolysis is recognized as the most lethal phenotype of TIC with reported mortality of >60%<sup>443</sup>. Based on ROTEM analysis, we report at the end of 60 min haemorrhagic shock, a single bolus of 7.5% NaCl exacerbated coagulopathy and fibrinolysis over 60 min resuscitation. In contrast, 7.5% NaCl ALM hypotensively resuscitated the animals and returned clot times and clot propagation kinetics to baseline with preservation of clot stability, and a full correction of hyperfibrinolysis at 15 min. ALM's antifibrinolytic activity was supported by EXTEM, FIBTEM and APTTEM comparisons, and was associated with higher plasma PAI-1, lower D-dimers and lower P-Selectin levels indicating reduced platelet and endothelial activation.

### **Hypotensive Resuscitation**

Small-volume bolus of 7.5% NaCl (0.3 ml) was not sufficient to resuscitate after 42% blood volume loss and 60 min severe haemorrhagic shock. The mean arterial pressure (MAP) increased only transiently and then returned to shock values after 60 min resuscitation (Table 1). Hypertonic saline resuscitation for traumatic haemorrhage is controversial, particularly after the failure of three major trauma trials from the Resuscitation Outcomes Consortium in the US and Canada<sup>131,444</sup>. Despite these outcomes, pre-hospital use of hypertonic fluids has been shown to partially restore normal activity and the apoptotic behaviour of polymorphonuclear neutrophils (PMNs) in trauma patients with severe traumatic brain injury<sup>445</sup>. Further trials are required to assess the safety and efficacy of hypertonic saline in trauma patients.

In contrast, small-volume 7.5% NaCl ALM raised MAP to 62 mmHg at 5 min and 68 mmHg at 60 min. This steady rise in MAP was associated with significant increases in systolic and diastolic pressure and pulse pressure, and significantly higher body temperature compared to 7.5% NaCl controls at 60 min (Table 1). This supports our

earlier work in both rats and pigs<sup>432-434,446</sup>. That ALM treatment reduces the fall in body temperature (34°C vs. 31.8°C) most likely arises from improved cardiac and haemodynamic coupling during resuscitation despite severe hypovolaemia, although improved control within the ‘Thermostat Centre’ of the hypothalamus cannot be ruled out.

### **Haemorrhagic Shock leads to Coagulopathy and Hyperfibrinolysis**

After 60 min haemorrhagic shock, the present study supports our earlier study indicating hyperfibrinolysis was associated with acute hypocoagulopathy (Table 2, Figs. 2-4)<sup>435</sup>. Increased fibrinolysis was further supported by the difference in clot amplitude decrease between APTEM A10 (22% fall) compared to EXTEM (50% fall) after 60 min shock (Table 3) and non-detectable plasminogen activator inhibitor (PAI-1) (Fig. 5)<sup>435</sup>. Low or non-detectable PAI-1 have been reported in severely injured trauma patients<sup>447-449</sup>, however the mechanism of PAI-1 inhibition remains unclear and may include activated protein C (aPC), tissue factor (TF) release, and tissue plasminogen activator (tPA) production<sup>413,450,451</sup>. The ability of aPC to deplete PAI-1 levels enough to promote fibrinolysis is questionable given PAI-1 circulates at ~10 times aPC<sup>451</sup>. TF increased ~25% after shock, however tPA was not detectable (Fig. 5). It is possible tPA peaked earlier than was measured in this study, as Wu and colleagues found elevated levels 30 min after injury which then decreased below baseline<sup>452,453</sup>. Despite other indicators of hyperfibrinolysis plasma D-dimers were not detected after 60 min shock (Fig. 5). This is consistent with findings from rat models of polytrauma and haemorrhage demonstrating an initial fall in D-dimers and no significant increase until at least two hours after injury<sup>452-454</sup>.

### **7.5% NaCl ALM Corrected Hyperfibrinolysis during Resuscitation at 15 min**

Our study further showed that small-volume 7.5% NaCl IV bolus treatment worsened shock-induced coagulopathy during 60 min resuscitation. EXTEM and INTEM CT and CFT were significantly prolonged with lower alpha angles and clot formation rates compared to baseline (Table 2). The hypocoagulopathy became progressively worse over the 60 min resuscitation period as demonstrated by INTEM clot amplitudes, which were significantly lower at 45 min and 60 min resuscitation compared with 30 min

resuscitation. FIBTEM CT values were 3 to 6 times longer than the corresponding EXTEM CTs during the first 30 min of saline resuscitation, suggesting platelet function and aggregation was sufficient and did not contribute to this hypocoagulopathy. FIBTEM clot amplitudes (A10) for saline controls remained similar to shock values and ranged from 4 to 7 mm compared to 15 mm for baseline (Fig. 2). The lack of rebound in clot amplitude after platelet inhibition with cytochalasin D in the FIBTEM test further supports minimal platelet contribution to the loss of clot strength in 7.5% NaCl controls and points to a possible fibrin(ogen) defect.

Hyperfibrinolysis was confirmed using APTEM LI(30-60)% and ML with a full reversal of EXTEM LI(30-60)% and ML at 15 min 7.5% NaCl resuscitation (Table 3). In contrast to the shock state, addition of aprotinin in the APTEM test did not correct clot amplitudes after 7.5% NaCl 15 min resuscitation. However, hyperfibrinolysis in saline controls was further supported by undetectable levels of plasma PAI-1, and increasing plasma levels of D-Dimers (Fig. 5). After 60 min 7.5% NaCl resuscitation, EXTEM, INTEM, and FIBTEM ML indicated fibrinolysis (Fig. 3), however comparison of EXTEM and APTEM assays showed no hyperfibrinolysis (Table 3).

Plasma levels of thrombin-antithrombin complex (TAT) were comparable across all groups, indicating thrombin generation was not a factor in the differences in coagulopathy between groups (Fig. 5). The significant 6-fold increase in P-selectin at 15 min and 2-fold increase at 60 min, as well as significantly higher tissue factor concentration at 15 min, indicates prolonged endothelium activation and inflammation with 7.5% NaCl resuscitation (Fig. 5). Together these ROTEM parameters and plasma markers reflect a dynamic worsening coagulopathy in 7.5% NaCl controls with early hyperfibrinolysis, and possible systemic inflammation, associated with worsening haemodynamics (Table 1).

In contrast to small-volume 7.5% NaCl alone, the presence of ALM rapidly corrected the shock-induced hypocoagulopathy and reversed hyperfibrinolysis early during resuscitation. This finding was based on the following results from clot initiation, propagation, strength, and lysis profiles:

- A near full correction of clotting times from EXTEM, INTEM and FIBTEM CT, CFT, alpha angle, CFR, and MCE (Table 2).

- A near full correction of A10 and MCF on all tests (Fig. 2).
- LI(30-60) and ML percentages near or close to baseline over 60 min resuscitation indicating no fibrinolysis (Fig. 3).

One of the interesting findings of this study was that small-volume 7.5% NaCl ALM bolus treatment corrected shock-induced hypocoagulopathy and hyperfibrinolysis despite mild hypothermia (34°C), which can contribute to trauma-induced coagulopathy and promote fibrinolysis (Table 1)<sup>158,240,241</sup>.

Our previous work also reported correction of coagulopathy at 5 min<sup>434</sup>, however, in that study we only speculated on the presence of hyperfibrinolysis after pressure-controlled bleeding and 60 min haemorrhagic shock, as we did not have access to the APTEM assay to directly inhibit fibrinolysis in EXTEM. Despite numerous studies confirming the validity of the APTEM test for diagnosis of hyperfibrinolysis<sup>430,438,439</sup>, its sensitivity for detection of profibrinolytic activation has been questioned<sup>194</sup>. Furthermore, Abuelkasem and colleagues have recently suggested that FIBTEM is more sensitive than EXTEM in identifying hyperfibrinolysis<sup>455</sup>. In this study after 60 min 7.5% NaCl resuscitation, FIBTEM LI(30-60)% were 57.8%, 51.6%, and 67.4%, strongly suggesting fibrinolysis, however addition of aprotinin in the APTEM assay did not change EXTEM lysis (34% vs. 34%), therefore it was concluded that the coagulopathy was not associated with hyperfibrinolysis (Fig. 3; Table 3). Full correction of APTEM lysis index at 15 min 7.5% NaCl resuscitation confirmed hyperfibrinolysis, however inhibition of fibrinolysis by aprotinin did not correct clot amplitude in this group (Table 3). Further studies are required to elucidate the correct test and parameter for diagnosis of hyperfibrinolysis since other studies have used a change in A15, A20 and MCF between EXTEM and APTEM<sup>438</sup>.

The increased plasma levels of PAI-1, the key inhibitor of fibrinolysis<sup>456,457</sup>, following ALM resuscitation provide further support for an antifibrinolytic effect contributing to ALM's correction of trauma-induced coagulopathy (Fig. 5). Platelets contain and release large amounts of active PAI-1<sup>455</sup>, suggesting ALM resuscitation may preserve platelet function. Further research is required on the duration of the antifibrinolytic effect of ALM therapy because fibrinolytic shutdown can also be pathologic and associated with poor late outcomes<sup>458</sup>. ALM-treated animals had significantly lower TF

levels and lower P-selectin levels (Fig. 5), which may be of significance since P-selectin has been linked with shock-induced cardiac and pulmonary injury<sup>459,460</sup>.

### **FIBTEM/EXTEM Ratio in Clot Strength and Possible Significance**

As mentioned in our previous study using ROTEM, we reported a differential contribution of platelets and fibrinogen to early coagulopathy in the rat model, with a platelet defect occurring after 20 min controlled haemorrhage, and a fibrinogen defect after 60 min of untreated shock<sup>435</sup>. We further showed that the fibrinogen to platelet ratio in clot amplitude (FIBTEM/EXTEM A10 and MCF) remained unchanged at 1:5 from baseline despite different contributions of platelets and fibrinogen to the early coagulopathy<sup>435</sup>. This ratio may be of clinical significance in determining the treatment success of early coagulopathy. In the present study, this ratio was not maintained in saline controls and fell to 1:10 after 60 min resuscitation (Fig. 3). The functional significance of this ratio deviating from 1:5 on the underlying mechanical properties of the platelet-fibrin(ogen) clot are not currently known but it may make the coagulopathy harder to correct with blood products such as fibrinogen concentrate, platelets or plasma. In contrast, ALM-treated animals showed no such variability and maintained the fibrinogen to platelet ratio of 1:5 over 60 min hypotensive resuscitation. Further studies are required to investigate the importance of the fibrinogen to platelet ratio to various coagulopathy treatment strategies.

### **Possible Clinical Implications**

The ability to detect and treat coagulopathy early is a key strategy to haemorrhage control in the trauma patient<sup>420,422-425</sup>. ROTEM and other viscoelastic methods enable rapid detection of hyperfibrinolysis to guide treatment in severely injured trauma patients<sup>430</sup>. Antifibrinolytics such as tranexamic acid (TXA), aminocaproic acid and aprotinin inhibit the activation of plasminogen to plasmin, and prevent the break-up of fibrin and maintain clot stability. The CRASH-2 trial led to adoption of TXA for trauma patients<sup>461</sup>, however, Harvin and colleagues did not confirm a survival benefit in trauma patients with hyperfibrinolysis on hospital admission<sup>462</sup>, and a retrospective analysis using propensity score matching found increased mortality in severely injured patients administered TXA<sup>463</sup>. The ALM antifibrinolytic effect combined with its

small-volume resuscitation capabilities may offer an alternative therapeutic intervention in traumatic haemorrhagic shock.

### **Limitations of the Study**

An ongoing problem in resuscitation research is selecting the appropriate haemorrhagic shock model with clinical significance. A potential limitation of pressure-controlled haemorrhage and shock is that it may not mimic the clinical scenario of traumatic blood loss<sup>464</sup>. Clinically relevant uncontrolled blood loss may elicit a different stress response influencing the underlying coagulopathy. The choice of animal species may also impact on translation. Further *in vivo* and *in vitro* studies are required to unravel the mechanisms underlying ALM's coagulation restorative and antifibrinolytic properties including a possible thrombomodulin-thrombin 'switch' mechanism, and improved cross-talk between cardiac-endothelium perfusion, inflammation and coagulation. Cardiac function, regional blood flows and tissue oxygenation of vital organs and whole body oxygen consumption also need to be quantified during hypotensive resuscitation.

### **Conclusions**

In the rat model of severe haemorrhagic shock, small-volume 7.5% NaCl failed to resuscitate and exacerbated fibrinolysis and coagulopathy. In contrast, 7.5% NaCl ALM resuscitated animals and rapidly corrected hyperfibrinolysis at 15 min with possibly improved platelet and endothelial function.

## CHAPTER 7

### MECHANISMS OF EARLY TRAUMA-INDUCED COAGULOPATHY: THE CLOT THICKENS OR NOT?

Since Brohi and colleagues first demonstrated the presence of a clinically important coagulopathy in trauma patients that was not directly related to fluid administration<sup>147</sup>, acute traumatic coagulopathy (ATC) has become a key focus in the field of trauma resuscitation research. However, despite a large body of literature about this endogenous impairment of haemostasis, there is still little consensus on the pathophysiology and management of ATC or what constitutes a clear definition of ATC that everyone can agree on. Early diagnosis and treatment is clinically important since it is associated with higher transfusion requirements, prolonged intensive care unit stays, increased morbidity, and a four-fold increased risk of mortality in both adult and paediatric trauma patients<sup>153,154,465-467</sup>.

The review presented in Chapter 7 aims to summarise the various names and acronyms that have been introduced in the literature and four major mechanistic hypotheses including: 1) the DIC-fibrinolysis hypothesis, 2) the activated protein C hypothesis, 3) the glycocalyx hypothesis, and 4) the “fibrinogen-centric” hypothesis. It is concluded that no single hypothesis adequately explains the various manifestations of trauma-induced coagulopathy, and that the phenomenon is rather a dynamic entity that evolves over time. A more thorough understanding of the pathophysiology of ATC is required to elucidate the exact mechanisms of correction of trauma-induced hypocoagulopathy by adenosine, lidocaine, and magnesium (ALM) demonstrated in Chapters 2, 4 and 6. A key point that came out of our analysis was that too many researchers and clinicians are quick to label a bleeding phenotype as a form of DIC without evidence of fibrin deposits in the microvasculature.



## Mechanisms of early trauma-induced coagulopathy: The clot thickens or not?

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**ABSTRACT:** Traumatic-induced coagulopathy (TIC) is a hemostatic disorder that is associated with significant bleeding, transfusion requirements, morbidity and mortality. A disorder similar or analogous to TIC was reported around 70 years ago in patients with shock, hemorrhage, burns, cardiac arrest or undergoing major surgery, and the condition was referred to as a "severe bleeding tendency," "defibrination syndrome," "consumptive disorder," and later by surgeons treating US Vietnam combat casualties as a "diffuse oozing coagulopathy." In 1982, Moore's group termed it the "bloody vicious cycle," others "the lethal triad," and in 2003 Brohi and colleagues introduced "acute traumatic coagulopathy" (ATC). Since that time, early TIC has been cloaked in many names and acronyms, including a "fibrinolytic form of disseminated intravascular coagulopathy (DIC)." A global consensus on naming is urgently required to avoid confusion. In our view, TIC is a dynamic entity that evolves over time and no single hypothesis adequately explains the different manifestations of the coagulopathy. However, early TIC is not DIC because an increased thrombin-generating potential in vitro does not imply a clinically relevant thrombotic state in vivo as early TIC is characterized by excessive bleeding, not thrombosis. DIC with its diffuse anatomopathologic fibrin deposition appears to be a latter phase progression of TIC associated with unchecked inflammation and multiple organ dysfunction. (*J Trauma Acute Care Surg.* 2015;79: 301–309. Copyright © 2015 Wolters Kluwer Health, Inc. All rights reserved.)

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## CHAPTER 8

### **ADENOSINE, LIDOCAINE, AND Mg<sup>2+</sup> (ALM): FROM CARDIAC SURGERY TO COMBAT CASUALTY CARE – TEACHING OLD DRUGS NEW TRICKS**

Chapters 2, 3, 4 and 6 presented results of studies investigating small-volume adenosine, lidocaine, and magnesium (ALM) resuscitation fluid in rat and pig models of severe haemorrhagic shock. The fluid bolus was shown to resuscitate mean arterial pressure (MAP) into the permissive hypotensive range, correct trauma-induced coagulopathy, correct hyperfibrinolysis, and afford superior cardiovascular, acid-base, metabolic and renal protection compared to hypertonic saline alone<sup>345,377,527</sup>. ALM bolus or infusion has also demonstrated broad-spectrum protection in small and large animal models of regional myocardial ischaemia<sup>326,327,528</sup>, cardiac arrest<sup>395,529</sup>, endotoxaemia<sup>530</sup>, and polymicrobial sepsis<sup>394,531</sup>, including cardiac rescue and stabilisation, restoration of acid-base balance, correction of coagulopathy, attenuation of inflammation, and prevention of multiple organ dysfunction and failure.

Chapter 8 presents a historical review of the development and translation of ALM therapy from a “polarizing” cardioplegia to a small-volume intravenous bolus and drip treatment for haemorrhagic shock, cardiac arrest, polymicrobial infection, and sepsis. It further focuses on the studies presented in this thesis, and discusses future applications in major surgery, as well as our current working hypothesis to explain the multi-factorial benefits of ALM. This hypothesis will be broken down and discussed in detail in the next chapter.

# Adenosine, lidocaine, and Mg<sup>2+</sup> (ALM): From cardiac surgery to combat casualty care—Teaching old drugs new tricks

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**ABSTRACT:** New frontline drugs and therapies are urgently required to protect the body from primary and secondary injuries. We review more than 10 years of work on adenosine, lidocaine, and magnesium (ALM) and its possible significance to civilian and military medicine. Adenosine is an endogenous nucleoside involved in nucleotide production, adenosine triphosphate turnover, and restoration of supply and demand imbalances. Lidocaine is a local anesthetic and Class 1B antiarrhythmic, and magnesium is essential for ionic regulation and cellular bioenergetics. Individually, each plays important roles in metabolism, immunomodulation, inflammation, and coagulation. The original idea to combine all three was as a “polarizing” cardioplegia, an idea borrowed from natural hibernators. Two recent prospective, randomized human trials have demonstrated its safety and superiority in myocardial protection over high-potassium “depolarizing” solutions. The next idea came from witnessing how the human heart spontaneously reanimated after complex operations with little inotropic support. At high doses, ALM arrests the heart, and at lower doses, it resuscitates the heart. In rat and pig models, we have shown that ALM intravenous bolus and infusion “drip” protects against acute regional myocardial ischemia, lethal arrhythmias, cardiac arrest, compressible and noncompressible blood loss and shock, endotoxemia, and sepsis. Individually, adenosine, lidocaine, or magnesium fails to protect. Protection is afforded in part by reducing inflammation, correcting coagulopathy, and lowering energy demand. We propose a unifying hypothesis involving improved central, cardiovascular and endothelium coupling to maintain sufficient tissue oxygenation and reduce primary and secondary “hit” complications. As with any new drug innovation, translation into humans is challenging. (*J Trauma Acute Care Surg.* 2016;80: 135–145. Copyright © 2016 Wolters Kluwer Health, Inc. All rights reserved.)

**KEY WORDS:** Hemorrhage; trauma; injury; shock; inflammation.

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## CHAPTER 9

### DISCUSSION

*“In 1966, a US National Academy of Science report called trauma an ‘unrecognised epidemic’. Today it is called: ‘a neglected public health emergency’”*

The Lancet Editors (Oct 2016) <sup>575</sup>

#### 9.1 TRAUMATIC INJURY: A GLOBAL HEALTH EPIDEMIC

Traumatic injury and haemorrhagic shock is a global epidemic and major cause of morbidity and mortality in civilians and on the battlefield <sup>2,3</sup>. With approximately half of all trauma deaths occurring at the scene of injury or during transport to definitive care, maximum survivability depends on optimal pre-hospital care from the point of injury <sup>4,576</sup>. It has been estimated that up to 20% of civilian and battlefield trauma deaths may be prevented with optimal pre-hospital trauma care, equating to at least 30,000 less fatalities in the United States, and more than 2,000 Australian lives saved, annually <sup>14-17</sup>. At the October 2016 Clinical Congress, the American College of Surgeons (ACS) announced that trauma was a neglected US public health emergency and their commitment is to achieve zero preventable deaths from trauma <sup>575</sup>. This will require significant research funding and there is a drive in the field to develop evidence-based and consistent national and International protocols for pre-hospital trauma care. This thesis helps to address this urgent problem.

#### 9.2 ALM: TOWARDS THE DEVELOPMENT OF A NEW RESUSCITATION FLUID

With no consensus on what constitutes an optimal pre-hospital fluid resuscitation strategy for traumatic haemorrhagic shock (see Chapter 1), <sup>32,35-41</sup>, the major objective of this thesis was the development of a small-volume fluid for resuscitation and stabilisation that may translate to military and civilian use. The results from studies presented in Chapters 2, 3, 4, and 6 of this thesis suggest that small-volume ALM fluid

administered as a bolus after severe haemorrhagic shock resuscitates into the permissive hypotensive range, rescues and stabilises the heart, corrects trauma-induced coagulopathy, protects against hypothermia, restores acid-base balance, and in pigs has also been shown to improve left ventricular-arterial coupling, reduce whole body oxygen consumption, and appears to repay oxygen debt<sup>345,377,527</sup>. Chapter 6 further suggested an anti-inflammatory and endothelial protective effect of ALM resuscitation, however further studies are required to investigate markers of endothelial dysfunction, as well as neuroprotective effects.

### 9.3 THE ALM ‘RESYNCHRONIZATION’ HYPOTHESIS

One of the interesting features of the ALM fluid demonstrated in Chapter 2<sup>345</sup>, and my previous studies in rat models of severe to catastrophic blood loss<sup>31,317</sup>, is that adenosine alone, lidocaine alone, or magnesium alone fail to resuscitate mean arterial pressure (MAP) after haemorrhagic shock. Furthermore, unlike ALM, the combination of adenosine and lidocaine (AL) without magnesium was unable to correct trauma-induced coagulopathy<sup>345</sup>. All three components of ALM in combination are required for the resuscitative and coagulation restorative effects following traumatic haemorrhage.

A synthesis of the data presented in this thesis led to the formulation of the “ALM Resynchronization Hypothesis”, which was introduced in Chapter 8<sup>316</sup>. The term ‘resynchronization’ was chosen to signify ALM’s ability to help shift the system back to a more balanced homeostatic state favouring a pro-survival phenotype. It was hypothesized that this was achieved by: 1) improving central nervous system (CNS)-cardiac-endothelial coupling, 2) reducing endotheliopathy, and 3) improving tissue oxygenation. More recently, ALM has broadened its therapeutic potential by bolstering the host's immune system to improve survival after polymicrobial sepsis in the rat. Thus the ALM fluid therapy has also been shown in rat and pig models not only to protect against haemorrhagic shock but to bolster the body’s response to different types of ‘stressors’ including cardiac arrest<sup>395,529</sup>, myocardial ischaemia<sup>326,327,528</sup>, endotoxaemia<sup>530</sup>, and polymicrobial sepsis<sup>394,531</sup>.

What follows is a summary of the results that led to the formulation of the ALM Resynchronization Hypothesis, and ongoing investigations searching for the underlying mechanism of ALM action including heart rate variability (HRV) analysis and the possible involvement of nitric oxide (NO). I will also briefly discuss my most recent work with the US Special Operations Command (USSOCOM) demonstrating that ALM protects against internal blood loss<sup>577</sup>, moderate traumatic brain injury (TBI)<sup>577</sup>, and the lethal combination of uncontrolled haemorrhage and TBI<sup>578</sup>. Lastly, I will end with the future translational studies required to undertake human safety trials in the trauma setting.

### 9.3.1 Permissive Hypotension: A Precarious Balancing Act

There is much controversy about what is the lowest optimal mean arterial pressure (MAP) that can be maintained during resuscitation to achieve adequate tissue oxygenation without causing re-bleeding after haemorrhagic shock. Permissive hypotensive resuscitation with a MAP of around 60 mmHg has demonstrated a survival benefit in preclinical studies and clinical trials<sup>52,140-145</sup>. While my thesis did not address the optimal MAP for the rat or pig after severe haemorrhagic shock, it was found that small-volume 7.5% NaCl ALM fluid bolus resuscitated MAP to 64-69 mmHg in a permissive hypotensive range in rat (Chapters 2, 4, and 6) and around 50 mmHg in the pig (Chapter 3). I will now discuss the main findings.

#### *9.3.1.1 Haemodynamic resuscitation into the permissive range*

Rats resuscitated with ~0.7-1.0 ml/kg bolus 7.5% NaCl ALM following 20 min pressure-controlled bleeding and 60 min hypovolaemic shock had MAPs of 64 mmHg, 69 mmHg, and 68 mmHg after 60 min resuscitation in Chapter 2<sup>345</sup>, Chapter 4<sup>377</sup>, and Chapter 6, respectively. ALM also resuscitated into the permissive range in the pig model (Chapter 3) with a MAP of 48 mmHg and systolic arterial pressure of 79 mmHg at 60 min resuscitation<sup>527</sup>, which is within the target systolic pressure of 70-80 mmHg initially recommended by Cannon in 1918<sup>134</sup>. Interestingly, an infusion of ALM has also induced a reversible permissive hypotensive state in rat<sup>394</sup> and pig models<sup>530</sup> of polymicrobial sepsis and endotoxaemia.



### 9.3.1.2 Small-volume hypotensive fluid = low cube weight

Small-volume permissive hypotensive resuscitation also affords a logistical advantage in that it is low cube weight, which is of particular importance in combat casualty care and rural and remote environments where resources are limited<sup>16,117</sup>. In the rat model of severe haemorrhagic shock from Chapters 2, 4, and 6, the resuscitation bolus was 0.3 ml (~0.7-1.0 ml/kg), which represented ~3% of the shed blood volume<sup>345,377</sup>. Similarly, in the porcine model of pressure-controlled haemorrhagic shock (~75% blood loss), pigs received 4 ml/kg resuscitation bolus (140-160 ml for 35-40 kg pigs) which represented ~8% of the shed blood volume of ~2 L<sup>527</sup>. In contrast, traditional large-volume crystalloid resuscitation is given at three times blood loss volume<sup>102</sup>, which would have equated to ~35 ml fluid in the rat or ~6 L fluid in the pig.

### 9.3.1.3 Cardiac rescue and stabilisation

Crucial to the ALM resynchronization hypothesis is early rescue and stabilisation of optimal heart function and maintenance of ventricular-arterial coupling. Rescue of arterial pressure (MAP) does not guarantee rescue of cardiac output, which is also affected by systemic vascular resistance<sup>579</sup>. Cardiac dysfunction contributes to the high mortality of traumatic haemorrhagic shock<sup>105</sup>, and cardiac ‘uncoupling’ has been shown to be an independent cause of death in a retrospective analysis of 2088 trauma patients<sup>580</sup>.

Chapter 3 showed in the pig that a single 4 ml/kg bolus of 7.5% NaCl ALM led to a significant 1.6-fold increase in cardiac output (CO: 3.1±0.4 L/min vs. 2.0±0.2 L/min;  $p<0.05$ ), and a 1.9-fold increase in stroke volume (SV: 19±2 ml/beat vs. 10±1 ml/beat;  $p<0.01$ ) after severe haemorrhagic shock<sup>527</sup>. A 1.9-fold increase in SV following a 140-160 ml bolus being added to only ~25% of the animal’s normal circulating blood volume would suggest improved ventricular-arterial coupling between the heart and the arterial supply<sup>384</sup>. ALM fluid was also associated with a significantly higher ventricular-arterial coupling efficiency in the previously mentioned porcine endotoxaemia study<sup>530</sup>.

Although cardiac function was not specifically analysed in the rat studies in Chapters 2, 4, and 6, a ~0.7 ml/kg bolus of 7.5% NaCl ALM following ~40% blood loss significantly increased pulse pressure after 60 min resuscitation (42 mmHg vs. 34 mmHg;  $p < 0.05$ ), which is an index of stroke volume<sup>581</sup>. Cardiac stability was also evident in electrocardiograms of rats treated with ALM with absence of ventricular arrhythmias<sup>31,317,345</sup>, supporting previous findings in the *in vivo* rat model of myocardial ischaemia-reperfusion injury<sup>326,327</sup>. Furthermore, ALM has demonstrated cardiac rescue and stabilisation properties after asphyxial hypoxia-induced cardiac arrest<sup>395,529</sup>, and ventricular fibrillation-induced cardiac arrest and prolonged extracorporeal life support (ECLS) in the rat<sup>548</sup>. Further studies using echocardiography and measurement of pressure-volume loops are required to fully elucidate changes in cardiac function associated with haemodynamic rescue after ALM resuscitation.

### 9.3.2 Endothelial Protection

A second key feature of the ALM resynchronization hypothesis is protection against endothelial injury and restoration of endothelial integrity. The endothelium is an ideal target for pharmacological fluid therapies because of its involvement in multiple physiological functions including modulation of vasomotor tone, blood fluidity and permeability, haemostatic balance, platelet interactions, inflammatory responses, immune activity, and angiogenesis<sup>325,582</sup>. Within seconds of delivery injected intravenous fluids are in contact with the large ~4000-7000 m<sup>2</sup> endothelial surface area with the capacity to rapidly affect endothelial integrity and function<sup>384</sup>.

In Chapter 6, plasma P-selectin levels increased over five-fold after 20 min pressure-controlled bleeding and 60 min haemorrhagic shock, indicating widespread endothelial activation and inflammation<sup>583</sup>. Treatment with small-volume ALM bolus led to a significant reduction in P-selectin, which may indicate preserved endothelial homeostasis. Further studies are required to quantitate other markers of endothelial injury including E-selectin, von Willebrand factor (vWF), intracellular adhesion molecule-1 (ICAM-1), and syndecan, to investigate whether ALM has an endothelial protective effect.

### 9.3.2.1 Correction of trauma-induced coagulopathy

Shedding of the endothelial glycocalyx contributes to trauma-induced coagulopathy (TIC) and hyperfibrinolysis<sup>186,193,194,584</sup>, and Chapters 2 and 4-7 outlined the importance of correcting TIC early after traumatic injury. This thesis provides strong evidence that small-volume 7.5% NaCl ALM can correct haemorrhagic shock-induced hypocoagulopathy and hyperfibrinolysis. Chapter 2 showed that ~0.7-1.0 ml/kg bolus 7.5% NaCl ALM could correct prolonged prothrombin times (PT) and activated partial thromboplastin times (aPTT) back to baseline after 20 min pressure-controlled bleeding and 60 min haemorrhagic shock<sup>345</sup>. Chapter 4 further showed that this correction of PT and aPTT occurred within 5 min of the resuscitation bolus being administered<sup>377</sup>. In Chapter 4 coagulation was also assessed with ROTEM EXTEM, INTEM, and FIBTEM tests which showed a full correction of EXTEM and FIBTEM clots, and near full correction of INTEM clot kinetics, propagation, and stability parameters, at 5 min resuscitation<sup>377</sup>. After 60 min resuscitation ROTEM parameters for ALM-treated animals were not significantly different from baseline, and clot lysis was significantly reduced in ALM animals, indicating protection against fibrinolysis<sup>377</sup>. After confirming hyperfibrinolysis in this rat model of bleeding and 60 min shock in Chapter 5<sup>419</sup>, Chapter 6 confirmed ALM fluid could correct hyperfibrinolysis within 15 min and this was associated with higher levels of the fibrinolysis inhibitor, plasminogen activator inhibitor-1 (PAI-1), and lower D-dimers after 60 min resuscitation compared to saline controls (42 pg/ml vs. 570 pg/ml).

Correction of coagulopathy with ALM fluid therapy has also been demonstrated after cardiac arrest<sup>395</sup> and polymicrobial sepsis<sup>394,531</sup>. Further studies are required to tease apart the mechanisms of ALM correction of coagulopathy, including detailed investigations of platelet numbers and function, clotting factors, PAI regulation pathways, inflammatory pathways, and endothelial function. Results from Chapter 2 suggest the coagulopathy reversal is not related to improved haemodynamics since 7.5% NaCl AL bolus (no Mg<sup>2+</sup>) significantly increased MAP into the permissive range, but failed to correct coagulopathy in direct contrast to 7.5% NaCl ALM<sup>345</sup>. The working hypothesis presented in Chapter 7 is that ALM acts like a switch at the endothelial thrombomodulin-thrombin complex by shifting its substrate specificity from the protein C pathway which promotes fibrinolysis, to the thrombin activatable

fibrinolysis inhibitor (TAFI) pathway which promotes coagulation activation and correction<sup>383</sup>. The increased PAI-1 seen in Chapter 6 may indicate preserved platelet function since platelets are the source of active PAI-1<sup>455</sup>.

#### 9.3.2.2 Attenuation of inflammation

Chapter 1 described blunting of the inflammatory cascade as an important property of the ‘ideal’ resuscitation fluid. An exaggerated inflammatory response is one of the secondary ‘hit’ complications that arise from the host’s pathophysiological response to traumatic haemorrhage, and can contribute to multiple organ dysfunction and late mortality<sup>26,175,176</sup>. Inflammatory cytokines and chemokines were not measured in the rat and pig studies of severe haemorrhagic shock presented in Chapters 2, 3, 4, and 6, however plasma levels of tissue factor (TF), a key player in the cross-talk between inflammation and coagulation<sup>585</sup>, were measured at 15 min and 60 min following small-volume ALM resuscitation in the rat. TF was significantly attenuated in ALM-treated animals compared to hypertonic saline-treated animals 15 min after resuscitation, which may indicate an anti-inflammatory effect (Chapter 6).

Further support for an ALM-associated anti-inflammatory effect comes from *in vitro* studies of isolated porcine polymorphonuclear neutrophils (PMNs)<sup>328</sup>; a significant reduction in peak levels of the pro-inflammatory cytokine tumour necrosis factor-alpha (TNF- $\alpha$ ; ALM: 7121 pg/ml vs. saline: 11596 pg/ml,  $p=0.02$ ) during LPS-induced porcine endotoxaemia; and significant attenuation of C-reactive protein (CRP) and pro-inflammatory interleukins IL-1 $\beta$  and IL-6 following ALM fluid infusion in the rat model of polymicrobial sepsis<sup>531</sup>.

#### 9.3.2.3 Multiple organ protection

Protection against endothelial dysfunction and restoration of endothelial integrity will prevent haemorrhagic shock-induced cellular dysfunction, and thereby prevent development of multiple organ failure and late-stage mortality<sup>586</sup>. Chapter 3 showed cardioprotective effects and superior renal protection with ALM resuscitation in the pig model of ~75% blood loss and 90 min haemorrhagic shock<sup>527</sup>. After 60 min permissive hypotensive resuscitation and 180 min shed blood resuscitation, pigs receiving 4 ml/kg

7.5% NaCl ALM had three-fold higher urinary output (2.13 ml/kg/hr vs. 0.66 ml/kg/hr,  $p=0.001$ ), significantly lower plasma creatinine levels, significantly lower urine N-acetyl- $\beta$ -D-glucosaminidase (NAG)/creatinine ratio, and significantly higher creatinine clearance ratio, compared to saline controls. Urine protein/creatinine ratio was also lower in ALM-treated animals but this result was not significant<sup>527</sup>. These results indicated ALM resuscitation afforded global kidney as well as proximal tubule protection. Renal function was also preserved in the pig study of Granfeldt and colleagues which showed a significant return of glomerular filtration rate (GFR) to 83% of baseline in animals treated with ALM and Ringer's acetate, compared to 54% in animals treated with Ringer's acetate alone<sup>346</sup>. Protection against lung injury has been demonstrated in the rat model of CLP and endotoxaemic porcine model with significantly reduced pulmonary oedema and significantly higher PaO<sub>2</sub>/FiO<sub>2</sub> ratio in ALM-treated animals compared to saline controls<sup>394,530,531</sup>.

#### *9.3.2.4 Restoration of acid-base balance*

Resuscitation success during permissive hypotensive resuscitation may be indicated by acid-base status. Small-volume 7.5% NaCl ALM resuscitation in the pig model of haemorrhagic shock led to superior acid-base recoveries compared to saline controls in the study presented in Chapter 3. Pigs treated with 4 ml/kg 7.5% NaCl ALM bolus had significantly higher arterial pH (7.28 vs. 7.21;  $p<0.05$ ), significantly lower blood lactate levels (7.1 mM vs. 11.3 mM;  $p<0.05$ ), significantly higher base excess (-5.9 mEq/L vs. -11.2 mEq/L;  $p<0.05$ ), higher bicarbonate (19 mM vs. 15 mM), and significantly lower plasma K<sup>+</sup> (4.4 mM vs. 5.6 mM;  $p<0.05$ ) at the end of 60 min permissive hypotensive resuscitation<sup>527</sup>.

#### *9.3.2.5 Repayment of oxygen debt*

One of the challenges of permissive hypotensive resuscitation is avoiding further oxygen debt accumulation at lower arterial pressures<sup>161</sup>. This challenge may be overcome with the ALM resynchronization hypothesis by increasing oxygen delivery to tissues (i.e. increasing DO<sub>2</sub>) to create a high-flow, low-pressure state. In Chapter 3, a 4 ml/kg bolus of 7.5% NaCl ALM led to 46% higher DO<sub>2</sub> than saline controls (7.6 ml/min/kg vs. 5.2 ml/min/kg) at hypotensive pressures<sup>527</sup>. This higher oxygen delivery

may reduce the level of oxygen debt in these animals at the time of blood resuscitation, and therefore contribute to the improved organ function seen in this porcine model of severe haemorrhagic shock <sup>527</sup>. However, further investigation at the level of the microcirculation is required to determine oxygen delivery to individual tissue beds during hypotensive periods.

Lowering oxygen demand would also reduce debt accumulation and lead to faster debt repayment, and thereby improved outcomes <sup>255,257</sup>. A 10 ml bolus of 0.9% NaCl AL administered with reinfusion of shed blood in the aforementioned pig haemorrhagic shock study resulted in a 15% reduction of whole body oxygen consumption ( $VO_2$ ) from 5.7 ml/min/kg to 4.9 ml/min/kg <sup>527</sup>. This lower  $VO_2$  may be due to lower demand since this treatment group exhibited higher pH and base excess, and lower lactates indicating a lower ischaemic insult <sup>527</sup>. A reduction in whole body oxygen consumption was also reported when AL was administered at return of shed blood following hypotensive resuscitation with 7.5% NaCl ALM and Ringers acetate <sup>346</sup>, and with ALM infusion in the porcine endotoxaemia study <sup>530</sup>. Further studies are required to determine the mechanism by which ALM can lower oxygen demand and therefore consumption, but it may result from attenuation of catecholamines, which are known to increase oxygen consumption and oxygen wasting <sup>587</sup>.

#### *9.3.2.6 Autonomic nervous system regulation: Additional Analysis*

A key principle of the ALM Resynchronization Hypothesis is the involvement of the central nervous system (CNS) as the central controller <sup>316</sup>. While this was not a study aim for this thesis investigation, the resuscitative, coagulation restorative, and multi-organ protective effects of ALM may involve higher centre controls and modulation of the sympathetic and parasympathetic outflows. These outflows are found in the nucleus tractus solitarius (NTS) located in the medulla oblongata and may be responsible for regulating whole body neural balance, and protect and restore the endothelium <sup>316</sup>.

For example, sympathoadrenal overactivation following traumatic injury and haemorrhagic shock leads to a catecholamine surge, which can damage the endothelium, and has been reported to be an independent predictor of trauma mortality <sup>588</sup>. Multiple preclinical studies have demonstrated a benefit of inhibiting sympathetic

activation and/or increasing parasympathetic stimulation leading to improved outcomes and survival <sup>589-591</sup>. In a rat model of trauma-induced coagulopathy, inhibition of sympathetic activation with chemical sympathectomy led to an antifibrinolytic state and reduction of endothelial glycocalyx shedding <sup>592</sup>. Given the coagulation-restoring, antifibrinolytic, and anti-inflammatory effects of small-volume 7.5% NaCl ALM fluid, it was hypothesised that ALM's mechanism of action may involve sympathetic nervous system inhibition and/or upregulation of parasympathetic activity.

Heart rate variability (HRV) analysis is another possible method for assessing and quantifying modulation of parasympathetic and sympathetic activity <sup>593,594</sup>. Power spectral analysis of HRV (LabChart Pro 8 HRV Module Rat Preset, ADInstruments, Bella Vista, Australia) during 60 min permissive hypotensive resuscitation in saline- and ALM-treated animals from Chapter 2 <sup>345</sup>, found a significant reduction in the low frequency (LF) energy in the ALM group compared to 7.5% NaCl controls ( $7.45 \pm 3.52 \text{ ms}^2$  vs.  $16.33 \pm 5.88 \text{ ms}^2$ ;  $p < 0.05$ ). This was associated with a lower LF/high frequency (LF/HF) ratio in ALM animals ( $1.69 \pm 0.82$  vs.  $2.52 \pm 1.04$ ). While the LF component of spectral power is influenced by both sympathetic and parasympathetic activity, HF is influenced by parasympathetic activity alone <sup>595</sup>, therefore the LF/HF ratio is an index of sympatho-parasympathetic balance <sup>596,597</sup>. The lower LF and LF/HF ratio during 60 min permissive hypotensive resuscitation after small-volume ALM therapy indicate attenuation of sympathetic nervous system activity, suggesting that the CNS may be involved in improved whole body protection. Further HRV analyses are ongoing in the rat and pig after haemorrhagic shock.

### 9.3.3 Protection Against Hypothermia

An unexpected finding from this thesis was that small-volume 7.5% NaCl ALM bolus led to improved thermoregulation (Chapters 4 and 6). Thermal support was not used in the rat studies presented in this thesis in order to mimic the clinical presentation of accidental hypothermia. Following 60 min resuscitation ALM-treated rats maintained significantly higher body temperatures than hypertonic saline controls ( $34^\circ\text{C}$  vs.  $32^\circ\text{C}$ ) (Chapters 4 & 6) which may have therapeutic benefits including reduction of oxygen demand, reduction of oedema, decreases in free radical production, modulation of

inflammation and leukotriene production, prevention of apoptosis, and a reduction in vascular permeability<sup>598-600</sup>. Mild hypothermia has previously demonstrated a survival advantage in animal studies of pressure-controlled and uncontrolled haemorrhagic shock<sup>600-603</sup>.

Further studies are required to elucidate the mechanism of protection against accidental hypothermia following traumatic haemorrhage, and whether it results from improved cardiac and haemodynamic coupling, or involves higher thermoregulatory control centres in the brain. The pig model of severe haemorrhagic shock presented in Chapter 3 did not induce hypothermia, however the core temperature was significantly lower in the ALM group at 60 min permissive hypotensive resuscitation compared with saline controls (39.3°C vs. 39.7°C;  $p < 0.05$ ), and closer to the baseline core temperature of 38.2°C), which may indicate improved temperature regulation.

#### 9.4 DOES NITRIC OXIDE PLAY A ROLE IN THE ALM RESYNCHRONIZATION HYPOTHESIS?

The surprising array of whole body protective effects of small-volume ALM resuscitation from the data presented in this thesis led to the following question: Does nitric oxide (NO) play a role in ALM's ability to restore homeostasis after traumatic haemorrhagic shock? While these studies are only preliminary the data support a potential role for NO. Nitric oxide is a prime candidate for a role in ALM's mechanism of action because it is a ubiquitous signaling molecule with important regulatory functions in most cells and tissues, and is a fundamental mediator in the physiology of normal homeostasis and the pathophysiology of critical illness<sup>186,604-606</sup>. NO is involved in the control of vascular tone, cardiac contractility, leukocyte adhesion, adhesion molecular expression, platelet aggregation, body temperature, and functions as a neurotransmitter in the central nervous system (CNS)<sup>186,605-609</sup>.

In light of nitric oxide's known involvement in cardioprotection<sup>610,611</sup>, and the studies of Cabrales and colleagues in Golden Syrian Hamsters which demonstrated administration of exogenous NO during the early phase of haemorrhagic shock prevented cardiovascular collapse and preserved cardiac function<sup>612,613</sup>, I did



preliminary experiments to test  $N^G$ -nitro-L-arginine methyl ester (L-NAME), and different antagonists of NO with ALM resuscitation in the rat model of pressure-controlled bleeding and 60 min haemorrhagic shock. L-NAME is a non-specific inhibitor of nitric oxide synthase (NOS) <sup>614,615</sup>, and its addition to ALM led to early cardiovascular collapse, extensive ventricular arrhythmias, and 100% mortality within 20 min of administration. Therefore, inhibiting NO production with the non-selective NOS inhibitor, L-NAME, prevented small-volume bolus 7.5% NaCl ALM from resuscitating after haemorrhagic shock, and also prevented ALM's protection against ventricular arrhythmias <sup>31,317,326,327</sup>.

To further examine the potential role of nitric oxide in ALM resuscitation, a selection of specific NO inhibitors were tested, including specific inhibitors of the inducible form of NOS (iNOS) and the neuronal NOS isoform (nNOS), as well as a NO scavenger. Scavenging available NO prevented ALM increasing MAP, suggesting a requirement for NO in ALM resuscitation. Inhibitors of nNOS or iNOS did not affect permissive hypotensive resuscitation with ALM, leading to the hypothesis that ALM resuscitation in part involves eNOS-dependent NO production. Whether ALM prevents eNOS uncoupling <sup>616,617</sup>; increases bioavailability of the substrate L-arginine<sup>618</sup> or cofactors such as tetrahydrobiopterin (BH4) <sup>619</sup>; or regulates eNOS activity through changes in intracellular calcium <sup>620</sup>, phosphorylation <sup>605,621</sup>, or interaction with other regulatory proteins <sup>608,622</sup>; requires more detailed investigation.

## 9.5 FURTHER SUPPORT FOR SMALL-VOLUME ALM RESUSCITATION

Further support for small-volume ALM fluid therapy comes from my most recent studies funded by US Special Operations Command (USSOCOM) in three new small animal models: 1) Rat model of non-compressible haemorrhage induced by liver resection <sup>623</sup>; 2) Rat model of moderate traumatic brain injury (TBI) induced by fluid percussion <sup>577</sup>; and 3) Two-hit rat model of mild TBI (mTBI) and uncontrolled haemorrhage <sup>578</sup>.

Small-volume ALM fluid significantly improved survival over six hours, significantly reduced internal blood loss, and resuscitated MAP into the permissive range after liver

resection and uncontrolled bleeding<sup>623</sup>. Transthoracic echocardiography (TTE) demonstrated improved cardiac function with significantly increased cardiac contractility, increased CO and SV, and maintained ejection fraction (EF) after ALM treatment compared with saline controls and Hextend®-treated animals. Small-volume ALM therapy also improved acid-base status with higher pH, significantly lower blood lactates, and lower plasma K<sup>+</sup> compared to saline controls following non-compressible haemorrhage<sup>623</sup>. ALM also defended body temperature against accidental hypothermia, and corrected coagulopathy with significant preservation of ADP- and collagen-induced platelet aggregation compared to saline controls and Hextend®-treated animals<sup>623</sup>.

This study in the rat model of liver resection and uncontrolled bleeding also provided further support for an anti-inflammatory and multi-organ protective effect of ALM fluid. Systemic inflammation was attenuated with significantly lower levels of pro-inflammatory cytokines and chemokines including IL-1 $\alpha$ , IL-1 $\beta$ , TNF- $\alpha$ , IL-6, Regulation on Activation, Normal T-Cell Expressed and Secreted (RANTES), and interferon-gamma (IFN- $\gamma$ ). Rats receiving ALM fluid had significantly reduced levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), suggesting protection against liver injury<sup>623</sup>. These animals also had significant increases in local gut pO<sub>2</sub> and blood flow, which may be clinically important since the gut and splanchnic circulation is known to be particularly vulnerable to hypovolaemia, and intestinal ischaemia has been associated with the development of multiple organ failure<sup>172</sup>.

A neuroprotective effect was evident following ALM treatment in the rat model of fluid percussion-induced moderate traumatic brain injury, indicated by significantly increased cerebral blood flow, reduced neuroinflammation, and significantly reduced levels of the brain injury marker neuron specific enolase (NSE)<sup>577</sup>. ALM fluid also resulted in upregulation of anti-inflammatory cytokines IL-10 and IL-4 in the brain following moderate TBI in the rat, providing further evidence for an ALM anti-inflammatory effect; and protected cardiac and pulmonary function<sup>577</sup>. Furthermore, ALM-treated animals showed a significant reduction in plasma levels of syndecan-1, suggesting reduced systemic endothelial injury, which may be of clinical benefit.

Survivability over four hours was improved with small-volume ALM fluid resuscitation in the lethal two-hit model of mTBI and non-compressible haemorrhage<sup>578</sup>. A bolus of 0.7 ml/kg hypertonic saline with ALM improved acid-base balance with reduced arterial lactate levels compared to untreated animals and saline controls ( $3.7\pm 0.5$  mM vs.  $6.6\pm 1.1$  mM and  $5.2\pm 1.5$  mM, respectively)<sup>578</sup>. ALM also reduced blood loss by 50%, corrected trauma-induced coagulopathy, and reduced inflammation<sup>578</sup>. New studies are now underway to investigate survival over 72 hours with small-volume ALM therapy following uncontrolled haemorrhage with and without traumatic brain injury.

## 9.6 FUTURE TRANSLATIONAL STUDIES FOR HUMAN USE

As highlighted in Chapters 2-6 and Chapter 8, an ongoing challenge in trauma resuscitation research is selecting the right animal model to test new therapies. For example, detailed genomic analysis of murine responses to inflammatory stress demonstrated a poor correlation with human responses<sup>624</sup>. A mouse model may not be optimal because under stress the mouse can enter torpor, and so it may not be directly applicable for severe trauma and hypoxic studies<sup>625,626</sup>. In contrast, the rat, guinea-pig, rabbit, dog, and pig cannot enter torpor during stress, and may be better animal models for translation<sup>626</sup>. The development of an animal model of traumatic coagulopathy that reflects the human condition is another challenge for researchers, particularly given the known species differences in coagulation profile<sup>627</sup>. In this thesis I presented a small animal model of haemorrhagic shock-induced trauma-induced coagulopathy with hyperfibrinolysis (Chapter 5)<sup>419</sup>, however a suitable large animal model has proven more difficult<sup>451,628</sup>.

The ongoing problem with translation from small to large animal models, and then to human trials is that few are clinically successful. In the United States alone, less than 10% of drugs entering clinical trials go on to become an FDA-approved therapy<sup>629</sup>. Why so many translational studies fail may relate to: 1) the heterogeneity of the human condition which is difficult to reproduce in animal models<sup>197,630,631</sup>, and 2) poor trial design.

ALM may be different. As a cardioplegia it has already shown safety and superiority in randomised controlled human trials <sup>314,632</sup>. Further preclinical studies are currently underway in rat and pig models of uncontrolled haemorrhage with and without traumatic brain injury with an extended monitoring time of 72 hr to assess longer-term survival outcomes. My primary supervisor, Professor Geoffrey Dobson, and I have also recently teamed up with trauma surgeons of Cooper University, Camden, New Jersey, as well as the Level 1 trauma team at the Department of Surgery at Denver Health Medical Center, Colorado, to further develop the ALM therapy with a view to move to human safety trials. The hope is that the multi-factorial benefits of ALM therapy shown in rat and pig models of haemorrhagic shock <sup>31,317,345,346,377,527,623</sup>, including traumatic brain injury protection <sup>577,578</sup>, will translate and provide a new therapeutic option to meet the urgent needs outlined by the American College of Surgeons at the October 2016 Clinical Congress <sup>575</sup>.

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