Novel Therapeutic Approaches for Experimental Trauma-Haemorrhage

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Thesis submitted for the degree of Doctor of Philosophy in Pharmacology

i.   Abstract

Haemorrhagic shock (HS) is commonly associated with trauma. Severe haemorrhage causes hypoperfusion of tissues resulting in a global ischaemic state, and resuscitation is performed to restore circulating volume. However, the return of oxygen to ischaemic tissues causes the induction of a systemic inflammatory response, which contributes to cell death leading to organ failure. In trauma patients, failure of more than four organs is linked to certain mortality, highlighting the need for interventions that may reduce or prevent the deterioration in organ function.

The aim of this thesis was to investigate the effect of therapeutic approaches on the organ injury and dysfunction induced by HS. Briefly, male Wistar rats were subjected to haemorrhage by withdrawal of blood to reduce the mean arterial pressure to 35 ± 5 mmHg for 90 min. Followed by resuscitation with 20 ml/kg Ringer's lactate for 10 min and 50% of the shed blood for 50 min. Organ function was determined 4 h after the onset of resuscitation. This model was used to investigate the effect of three different interventions on the organ injury and dysfunction induced.

In the first study, administration of bone marrow-derived mononuclear cells (BMMNCs) upon resuscitation resulted in (1) significant attenuation of the organ injury and dysfunction associated with HS, and (2) restoration of the activation of the Akt pro-survival pathway. It is possible that these beneficial effects are mediated by paracrine mediators secreted by BMMNCs, which modulate this pathway, however injection of large numbers of cells is not practical in the acute setting of trauma.

Therefore, in the next study erythropoietin (EPO) was used as a daily pre-treatment for three days prior to the induction of haemorrhage, as EPO is a known stimulus of endothelial progenitor cell (EPC) mobilisation. EPO pre-treatment resulted in (1) significant attenuation of the organ injury and dysfunction associated with HS, (2) mobilisation of EPCs ($CD34^+$/flk-1^+), and (3) activation of the Akt pro-survival pathway with enhanced activation of eNOS. However, when used clinically EPO is associated

with an increased risk of thrombotic events, therefore in the final study a non-erythropoietic analogue of EPO was investigated.

Treatment with pyroglutamate helix B surface peptide (pHBSP) resulted in (1) significant attenuation of the organ injury and dysfunction associated with HS, and (2) activation of the Akt pro-survival pathway with enhanced activation of both eNOS and STAT3. Additionally, late pHBSP treatment, up to 60 min after the onset of resuscitation, exerted the highest degree of protection.

The findings of this thesis support the view that modulation of the Akt pro-survival pathway is a potential therapeutic target in the treatment of the ischaemia-reperfusion injury associated with severe haemorrhage and resuscitation.

ii.   Acknowledgments

I would like to extend my sincerest gratitude to my primary supervisor, Professor Christoph Thiemermann. Thank you providing me with the means to produce this thesis and always ensuring the conferences we attended were in the sunniest of locations! My gratitude also extends to my secondary supervisor, Dr Nimesh Patel, who has put up with my endless stream of questions, queries and qualms. He has been of great support to me during the 5 years I have known him and has become a great friend, for which I will always be thankful. My thanks also extended to my fellow collaborators in Turin, Dr Massimo Collino; in Singapore, Professor Fred Wong and (now) in Japan, Dr Kunihiko Takahashi, for their contributions to the studies in this thesis. I am also thankful to the British Heart Foundation for funding my PhD research.

I have been fortunate enough to meet some amazing people during my time at The William Harvey Research Institute and they have become dear friends of mine (you know who you are). Lastly, I wholeheartedly thank my parents, sisters and friends without whom I doubt I would have made it through this PhD. Thank you for the support, putting up with the tears and the moments of madness. Oh and I cannot forget to show my gratitude to the lives of the rats and mice that have been sacrificed for the all of the *in vivo* work I have completed in the past few years.

"Now this is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning" - Winston Churchill

iii.   Publications

PAPERS

NANDRA KK, PATEL NSA, BRINES M, COLLINO M, WONG WSF, KAPOOR A, BENETTI E, GOH FY, FANTOZZI R, CERAMI A, THIEMERMANN C (2011) A non-erythropoietic peptide that mimics the 3D structure of erythropoietin reduces organ injury/dysfunction and inflammation in experimental hemorrhagic shock. *Mol Med* **17**(9-10):883-92

NANDRA KK, TAKAHASHI K, COLLINO M, BENETTI E, WONG WS, GOH FY, SUZUKI K, PATEL NSA, THIEMERMANN C. (2012) Acute treatment with bone marrow-derived mononuclear cells attenuates the organ injury/dysfunction induced by hemorrhagic shock in the rat. *Shock* **37**(6):592-8

PATEL NSA, **NANDRA KK**, THIEMERMANN C (2012) Erythropoietin and its derivatives as therapies in critical care (Review). *Crit Care* **16**(4):229

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ABSTRACTS

NANDRA KK, TAKAHASHI K, COLLINO M, BENETTI E, WONG WS, GOH FY, SUZUKI K, PATEL NSA, THIEMERMANN C. (2011) Bone marrow-derived mononuclear cells protect against multiple organ failure in hemorrhagic shock through an Akt dependent pathway. *Shock* **36**(Suppl 1): 26

NANDRA KK, PATEL NSA, COLLINO M, BENETTI E, HARWOOD S, YAQOOB MM, THIEMERMANN C. (2012) Three day pre-treatment with erythropoietin attenuates the organ injury and dysfunction induced by hemorrhagic shock in the rat by a mechanism independent of endothelial progenitor cell mobilization. *Shock* **37**(Suppl 1): 96

iv.   Contents

v. Abbreviations

Chemicals and Substances

ABG	Arterial blood gas
AKI	Acute kidney injury
Akt	Also known as protein kinase B
ALT	Alanine aminotransferase
APACHE	Acute physiology and chronic health evaluation
ARDS	Acute respiratory distress syndrome
AST	Aspartate aminotransferase
ATP	Adenine triphosphate
Bad	Bcl-2 associated death promoter
Bax	Bcl-2 associated X protein
BCA	Bicinchoninic acid
Bcl-2	B-cell lymphoma – 2
Bcl-xL	B-cell lymphoma-extra large
BDNF	Brain derived neurotropic factor
Blm	Bloom syndrome protein
BMMNC	Bone marrow-derived mononuclear cell
cBase	Base deficit
CK	Creatine kinase
CO_2	Carbon dioxide
COX	Cyclo-oxygenase
DAMP	Damage associated molecular pattern
DIC	Disseminated intravascular coagulation
DNA	Deoxyribose nucleic acid
ECL	Enhanced chemiluminescence
ELISA	Enzyme-linked immunosorbant assay
eNOS	Endothelial nitric oxide synthase
EPC	Endothelial progenitor cell

EPO	Erythropoietin
EPO-R	Erythropoietin receptor
ERK	Extracellular signal kinase
FFP	Fresh frozen plasma
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
Flk-1	Foetal liver kinase – 1
FOXO1	Forkhead box protein O1
GM-CSF	Granulocyte macrophage – colony stimulating factor
GPCR	G-protein couple receptor
GSK-3β	Glycogen synthase kinase – 3beta
H$^+$	Hydrogen ion
H$_2$O$_2$	Hydrogen peroxide
HB-EGF	Heparin binding epidermal growth factor
HBSS	Hank's balanced salt solution
HEMS	Helicopter emergency medical service
HIF	Hypoxia inducible factor
HMGB1	High-mobility group protein B - 1
HO-1	Haem oxygenase – 1
HS	Haemorrhagic shock
Hsp	Heat shock protein
ICAM	Intercellular adhesion molecule
IGF	Insulin growth factor
IL	Interleukin
IKKα	IkappaB kinase alpha
iNOS	Inducible nitric oxide synthase
IRAK	IL-1 receptor-associated kinase
ISS	Injury severity score
JAK	Janus Kinase
JNK	c-Jun N-terminal kinase
K$^+$	Potassium ion

LTB$_4$	Leukotrine B$_4$
MAPK	Mitogen activated protein kinase
MAPKK	Mitogen activated protein kinase kinase
MAPKKK	Mitogen activated protein kinase kinase kinase
MCP-1	Monocyte chemoattractant protein – 1
MI	Myocardial infarction
MODS	Multiple organ dysfunction syndrome
MOF	Multiple organ failure
MPO	Myeloperoxidase
mPTP	Mitochondrial permeability transition pore
MSC	Mesenchymal stem cell
mTOR	Mammalian target of rapamycin
MyD88	Myeloid differentiation primary response gene 88
Na$^+$	Sodium ion
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced form of nicotinamide adenine dinucleotide
NADPH	Reduced form of nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor-kappa B
NHS	National health service
NO	Nitric oxide
O$_2$	Oxygen
O$_2^{\cdot}$	Superoxide ion
OH$^-$	Hydroxyl ion
ONOO$^-$	Peroxynitrate
p53	Protein 53
PAF	Platelet aggregating factor
PAMP	Pathogen associated molecular pattern
PARP	Poly ADP ribose polymerase
PBMNC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline

P_{CO2}	Partial pressure of carbon dioxide
PDGF	Platelet derived growth factor
PDK	Phosphoinositide-dependent kinase
PE	Phycoerythrin
PGI_2	Prostacyclin
pH	-log of hydrogen ion concentration
pHBSP	Pyroglutamate helix B surface peptide
PHD	Prolyl hydroxylase domain
PI3K	Phosphatidylinositol-3-kinase
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PIP_3	Phosphatidylinositol-3,4,5-triphosphate
PKB	Protein kinase B
P_{O2}	Partial pressure of oxygen
pRBCs	Packed red blood cells
PVDF	Polyvinylidene fluoride
Resus	Resuscitation
rhEPO	Recombinant human erythropoietin
RNA	Ribose nucleic acid
ROS	Reactive oxygen species
sca-1	Stem cell antigen – 1
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	Serine
SFNSL	Small fibre neuropathy screening list
SH_2	Src homology 2 domain
SIRS	Systemic inflammatory response syndrome
SOD	Superoxide dismutase
STAT	Signal transducer and activator of transcription
TGF	Transforming growth factor
Thr	Threonine
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-alpha

tPA	Tissue plasminogen activator
TRAF	TNF Receptor Associated Factors
TXA$_2$	Thromboxane
Tyr	Tyrosine
UK	United Kingdom
USA	United States of America
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
XIAP	X-linked inhibitor of apoptosis protein
βcR	Beta common receptor

%	Percentage
°C	Degrees Celsius
dL	Decilitre
g	Grams
g	Gravity
h	Hour
IU	International units
IU/kg	International units per kilogram
IU/kg/day	International units per kilogram per day
IU/L	International units per litre
IU/ml	International units per millilitre
kg	Kilogram
kPa	Kilopascal
L	Litre
M	Molar
MAP	Mean arterial blood pressure
mg	Milligram
min	Minute
ml	Millilitre
ml/kg	Millilitre per kilogram
ml/kg/h	Millilitre per kilogram per hour
mM	Millimolar
mm	Millimetre
mm^3	Millimetre cubed
mmHg	Millimetres of mercury
mmol	Millimole
mmol/L	Millimole per litre
mU/g	Milliunit per gram
nm	Nanometre

nmol/kg	Nanomole per kilogram
r.p.m	Rotations per minute
w/v	Weight per volume
μg/kg	Microgram per kilogram
μl	Microlitre
μm	Micrometre
μmol	Micromole
μmol/L	Micromole per litre
μU/g	Microunits per gram

Miscellaneous Terms

ANOVA	Analysis of variance
e.g.	For example
I.D.	Inner diameter
i.e.	That is
i.p.	Intra-peritoneal
i.v.	Intravenous
n	Number of experiments or animals
O.D.	Optical density
P	Probability
SEM	Standard error of the mean
vs.	Versus

CHAPTER 1

General Introduction

1.1 Trauma and haemorrhagic shock

Trauma is a leading cause of death in individuals under the age of 45 (Heron, 2007) and the associated blood loss or haemorrhage is responsible for 40% of all trauma deaths (Gruen *et al.*, 2006; Kauvar *et al.*, 2006; Sauaia *et al.*, 1995; Stewart *et al.*, 2003). Worldwide, trauma is the cause of approximately six million deaths every year (WHO, 2009) and in the United Kingdom (UK) there are approximately 10,000 deaths per annum resulting from trauma (ONS, 2011). This adds to the financial burden of healthcare as the treatment of trauma patients equates to 7% (£1.6 billion/year) of the National Health Service's (NHS) budget (DOH, 1999). Injuries such as gunshot and stab wounds (Rooney *et al.*, 1999) and injuries sustained in road traffic accidents (Troop, 2005) can all result in trauma, which is often coupled with severe haemorrhage. Traumatic brain injury is a serious form of injury frequently observed in trauma patients and is associated with a poor prognosis (Rosenfeld *et al.*, 2012). The increasing prevalence of knife and gang crime, the threat of terrorist attacks and various military conflicts, highlights that trauma-haemorrhage is an issue which has great current relevance.

When the word haemorrhage is combined with shock to give the phrase "haemorrhagic shock" (HS), it refers to a condition where the degree of blood loss results in severe hypotension. Shock can be a psychological or physiological state; the symptoms include considerable hypotension, tachycardia, decreased temperature and tachypnea (Rushing *et al.*, 2008). Extreme blood loss causes the manifestation of a hypovolemic state which is detrimental as it causes poor tissue perfusion resulting in tissue hypoxia. This state is defined as a patient having a systolic pressure of less than 80 mmHg or a patient requiring more than 10 L of resuscitative fluids over a period of 6 h (Fry *et al.*, 1980).

Hypovolemic shock is managed by resuscitation of the patient with combinations of crystalloids, colloids and blood products (see current perspectives on treatment). The combination of the hypoxia that occurs during haemorrhage and the return of oxygen that occurs during resuscitation can ultimately result in the development of the so-

called "lethal triad" of hypothermia, acidosis and coagulopathy (Ferrara *et al.*, 1990; Moore, 1996). Moore described this triad as a "bloody vicious cycle" whereby cellular shock, tissue injury and resuscitation contribute to the development of metabolic acidosis and hypothermia which exacerbate the progressive coagulopathy that is occurring (Moore, 1996). This pathophysiological state can result in mortality either "immediately"; due to respiratory dysfunction or substantial haemorrhage, "early"; due to further haemorrhage or brain injury, or "late" deaths due to multiple organ failure (Trunkey *et al.*, 1974). The focus of this thesis is the outcome of multiple organ failure as a result of severe haemorrhage and resuscitation.

1.2   Multiple organ failure

Multiple organ failure is a key endpoint in many disease states; it occurs when two or more organs are unable to function and is often observed in intensive care, sepsis and trauma patients. Organ failure has been tightly coupled with mortality throughout history. In World War II the kidney was believed to be the "weakest link", meaning failure of the kidney would ultimately result in death whereas failure of other organs would be less severe (Baue, 1975). In the 1960's, it was believed that lung or pulmonary failure was the most fatal and the term acute respiratory distress syndrome (ARDS) was first described (Ashbaugh *et al.*, 1967). Later retrospective investigation into post-operative patient records (553 patients who underwent emergency surgery including traumatic and non-traumatic procedures) by Fry and colleagues demonstrated that lung failure was associated with 67% mortality, liver failure with 53% mortality and kidney failure with 72% mortality. A striking statistic from this study is that the failure of four organs is associated with certain mortality (Fry *et al.*, 1980). These early reports are important as they are still indicative of the present clinical situation as patients with four-system organ failure still have a mortality that is close to 100% (Durham *et al.*, 2003).

The classification or definition of multiple organ failure is somewhat obscure which can lead to confusion in the early diagnosis of patients (Baue, 2006). For example, the

definition of acute kidney injury (AKI) varies between studies so very different parameters are used; specifically serum creatinine values greater than 1.4 to 5 mg/dL are described as indicators of AKI (Bell *et al.*, 1983; Dorinsky *et al.*, 1990; Faist *et al.*, 1983; Marshall *et al.*, 1988) and the values for urine output range from less than 479 to 750 ml over 24 h period (Knaus *et al.*, 1985; Ruokonen *et al.*, 1993). The failure of individual organ systems has been described by Fry and colleagues, see below:

- Cardiovascular failure is defined by hypotension, decreased cardiac output and either tachycardia or bradycardia. The cause can be electrical, i.e. if the heart is in ventricular fibrillation or it can be pathological, for example atherosclerosis of coronary arteries causing poor perfusion and necrosis of the cardiac muscle.

- Renal failure can be defined by measuring serum concentrations of urea and creatinine, toxins which should be readily excreted by the kidneys. Parameters such as urine flow, glomerular filtration rate and renal blood flow can also be measured. Inability to maintain electrolytes is dangerous as changes in sodium (Na^+) and potassium (K^+) ions can affect cardiac muscle contraction resulting in arrhythmias.

- Pulmonary failure occurs when the lungs are incapable of maintaining normal partial pressures of CO_2 and O_2 as breathing is suppressed, this requires the use of artificial ventilation.

- Hepatic failure is defined by high serum concentrations of aspartate transaminase (AST) and alanine transaminase (ALT) as these enzymes are released when hepatocytes undergo cell death.

- Gastrointestinal failure can be due to gastrointestinal bleeding resulting in the inability of the gut to function and/or bacterial translocation. Failure can be defined by elevated amylase and lipase levels due to the proximity of the pancreas.

- Other organ systems that can be affected: the coagulation system can fail thereby is unable to prevent any blood loss, the immune system may be unable to clear septic infection, the central nervous system can be affected resulting in coma, failure of the musculoskeletal system resulting in muscle wasting and bone degradation and the neuroendocrine system can fail requiring hormonal replacement.

These definitions have remained an important method of classifying organ failure and have influenced the development of modern scoring systems including the Injury Severity Score (ISS), Acute Physiology and Chronic Health Evaluation (APACHE) I and III scores, lung injury score (ARDS score), Multiple Organ Dysfunction Syndrome (MODS) score and the Marshall score. This has led to the understanding that multiple organ failure occurs as a range of organ dysfunctions which can result in eventual failure (Durham *et al.*, 2003; Marshall *et al.*, 1995).

In a recent multi-centre cohort study, the Marshall score was used to define multiple organ failure in 900 severely injured patients and it was reported that approximately 30% of patients had multiple organ failure and that the onset was within two days of admission (Minei *et al.*, 2012). Another smaller study which used the ISS and Denver MOF scoring systems (Ciesla *et al.*, 2006a; Ciesla *et al.*, 2006b) reported that mortality in trauma patients with multiple organ failure was 64% compared to 3% in trauma patients that did not have multiple organ failure (Jastrow *et al.*, 2009). These studies re-iterate the findings by Durham and colleagues as there is still a high mortality associated with the development of organ failure (Durham *et al.*, 2003; Jastrow *et al.*, 2009). These studies are also useful in describing the risk factors associated with poor prognosis in trauma patients with multiple organ failure. These include male gender, age, severity of injury, obesity, co-morbidity, length of time in shock and the transfusion of more than 6 units of blood (Minei *et al.*, 2012). Understanding the incidence and possible pre-disposition to the development of multiple organ failure could lead to better management of trauma patients and a better prognosis for patients with severe injury and haemorrhage.

There is a strong link between organ failure and patient mortality as the cause of death is not always associated with the initial insult but with the deterioration in organ function that occurs post-insult. The findings of Fry's study in 1980 and the current incidence of organ failure and the associated mortality highlight the need for interventions that may reduce or prevent the deterioration in organ injury and function. In order to develop targeted interventions the pathophysiology of HS needs to be understood.

1.3   Ischaemia-reperfusion injury and inflammation in haemorrhagic shock

Ischaemia-reperfusion injury occurs in many clinical disease states such as AKI, myocardial infarction (MI) and stroke, and it also occurs on a whole-body scale in HS. Haemorrhage causes a global ischaemic state as cardiac output is drastically reduced and therefore, perfusion of all tissue beds in all organs is affected. Resuscitation is a form of reperfusion which restores the cardiac output and perfusion of tissues, however resuscitation initiates an inflammatory response which results in tissue injury and consequent deterioration in organ function.

Ischaemia results in hypoxia and hypoglycaemia, which have a dramatic impact on the metabolic aspects of the cell (McCord, 1985). The cell is in an anaerobic state and thus the cell compensates by partially metabolising the small amount of glucose present via the glycolysis pathway to form lactic acid but this only provides a minimal amount of ATP. This lactic acid dissociates when in solution (i.e. in plasma) resulting in an increase in hydrogen ions (H^+) which causes a fall in pH. This fall in pH is detected by chemically sensitive receptors in the respiratory centres located in the medulla and the pons. As falls in pH are normally associated with an increase in carbon dioxide, which also combines with water to produce H^+, the change in pH is interpreted as a rise in carbon dioxide. Therefore, the rate and depth of breathing is increased as the body in order to exhale this apparent excess in carbon dioxide and restore the basal H^+ concentration. This state is known as metabolic acidosis (Widmaier *et al.*, 2006).

Metabolic acidosis is different from respiratory acidosis. Respiratory acidosis occurs when the respiratory rate is decreased (i.e. hypoventilation) resulting in inefficient removal of carbon dioxide from the body. This is a common complication of HS as physical trauma, in particular to the chest, and severe blood loss can result in hypoventilation. This causes increases in the partial pressure of carbon dioxide (P_{CO2}) and consequently, a fall in the blood pH, the respiratory rate is then increased in order to accelerate the removal of carbon dioxide from the body and restore the blood pH. This is a compensatory mechanism, however in metabolic acidosis the P_{CO2} is at a normal level and an increase in respiration can cause this to fall resulting in metabolic

alkalosis as the pH increases (i.e. reduction of H^+). These changes in pH can affect a multitude of factors in the body, especially the regulation of enzymic pathways and protein structure (Widmaier *et al.*, 2006). In the case of HS, metabolic acidosis occurs as the cell and tissue ischaemia and injury persists, therefore is usually observed secondary to the respiratory acidosis caused by the initial trauma.

In clinical situations base deficit is used as an indirect indicator of acidosis. It is also indicative of multiple organ failure and mortality (Davis *et al.*, 1996) and is believed to correlate with oxygen utilisation (Kincaid *et al.*, 1998). Base deficit is defined as the concentration (mmol/L) of additional bicarbonate that must be added to a litre of blood to normalise the pH. Blood lactate levels (mmol/L) are also measured as they can be used to quantify the degree of anaerobic respiration and hypoperfusion. Both these parameters are used in combination to determine the degree of injury and they can be used to define treatment endpoints. However the relevance of these data is under question, some have found a lack of correlation between lactate and base deficit and have found lactate to be superior over base deficit when predicting mortality in trauma patients and patients receiving major surgery (Husain *et al.*, 2003).

Resuscitation is a beneficial and vital process following haemorrhage as it restores the circulating volume (i.e. cardiac output and blood pressure), however it is well known that the return of oxygen to ischaemic tissues causes extensive damage (Grace, 1994). The initial site of injury following resuscitation is the vascular endothelium as these cells are highly sensitive to hypoxic conditions. In HS, endothelial cells undergo cellular swelling and depolarisation of the membrane potential (Illner *et al.*, 1982; Illner *et al.*, 1981), this is due to the reduction in ATP resulting in dysfunction of the Na^+/K^+ pump (Cunningham *et al.*, 1970; Shires *et al.*, 1972). This results in endothelial dysfunction and loss of endothelial integrity which promotes the development of a pro-inflammatory and pro-coagulant state.

During ischaemia, tissues are hypoxic and the return of oxygen to these tissues by resuscitation induces the production and release of reactive oxygen species (ROS). Endothelial cells possess enzymes which synthesise ROS, such as xanthine

dehydrogenase which is activated during hypoxia and is converted into xanthine oxidase (McCord, 1985). Normally this enzyme is involved in nicotinamide adenine dinucleotide (NAD) reduction but when in the oxidase form it reduces oxygen. Therefore when oxygen is returned to the hypoxic area there is an oxidative burst of superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl free radicals (OH^-) due to the accumulation of xanthine oxidase in the cells (Rushing *et al.*, 2008). ROS are free radicals as they have one or more unpaired electron meaning they are highly reactive and cause damage to living cells. When free radicals react with another molecule (e.g. a protein or lipid) the unpaired electron is transferred, therefore the other species becomes a free radical. Superoxide is formed by an oxygen molecule accepting an unpaired electron, this species then undergoes dismutation catalysed by superoxide dismutase (SOD) to form H_2O_2. H_2O_2 then reacts with superoxide anions to form hydroxyl free radicals in a reaction known as the Haber-Weiss reaction. This is catalysed by the oxidation of a ferrous ion to a ferric ion and is also called the Fenton reaction (Carden *et al.*, 2000; Grace, 1994; Nath *et al.*, 2000).

Nitric oxide (NO) is a gaseous species produced by endothelial cells to promote relaxation of vascular smooth muscle cells and in basal conditions it scavenges superoxide. However, in pathophysiological states superoxide levels are increased and as superoxide is highly reactive it can combine with NO to form peroxynitrite ($ONOO^-$) which is highly oxidising (Carden *et al.*, 2000). $ONOO^-$ reacts with sulphur containing groups, such as tyrosine and cysteine, within proteins by nitrating them thus hindering their function. There are many examples of target proteins such as NADH hydrogenase in complex I of the electron transport chain and endothelial nitric oxide synthase (eNOS) (Pacher *et al.*, 2007).

ROS have detrimental effects on structural components of the cell as well as on membrane bound proteins and intracellular enzymes. This can cause structural damage, for example, peroxidation of lipids in the plasma membrane affecting its fluidity, and it can cause metabolic damage by oxidising functional domains of enzymes. As free radicals are lipophilic these species are able to enter the nucleus

freely and cause direct damage to DNA, altering gene expression or even inducing cell death (refer to Figure 1.1) (Nath *et al.*, 2000).

The damage that occurs to cells induces a pro-inflammatory response independent of an infection. The production and release of chemokines such as interleukin (IL) – 8, particularly by endothelial cells promotes the chemotaxis of leukocytes to sites of tissue damage. There is also release of pro-inflammatory cytokines such as tissue necrosis factor (TNF) - α, IL–1 and IL-6, these induce the expression of cell adhesion molecules on endothelial cells such as intercellular adhesion molecule (ICAM) – 1 and on leukocytes such as CD11b on neutrophils. As the endothelium has lost its integrity the combination of these effects results in leukocyte infiltration of damaged tissues and once activated these neutrophils further potentiate the inflammatory response (refer to Figure 1.1) (Carden *et al.*, 2000; Rushing *et al.*, 2008).

Damaged and dead cells also release entities known as alarmins or danger associated molecular patterns (DAMPs) which act in a similar way to their bacterial counterparts; pathogen associated molecular patterns (PAMPs). DAMPs are usually intracellular or nuclear components, examples include high mobility group box 1 (HMGB1), heat shock proteins, monosodium urate and nucleic acids (Hwang *et al.*, 2011; Manson *et al.*, 2012; Zhang *et al.*, 2010). DAMPs enhance the inflammatory response observed in HS by acting on toll-like receptors (TLRs) which usually mediate the innate inflammatory response to PAMPs such as endotoxin; both TLR-4 and TLR-9 have been implicated in pathogenesis of HS (see Section 1.5) (Gill *et al.*, 2011).

Neutrophils are the primary leukocyte of the innate immune system and the first leukocytes to be recruited to site of injury and inflammation as they are able to phagocytose bacteria and initiate an immune response (Friedewald *et al.*, 2004). Depleting neutrophils in models of ischaemia-reperfusion inhibits the development of tissue injury and inflammation (Paller, 1989; Thornton *et al.*, 1989), therefore suggesting that in some cases neutrophils can induce damage to host cells. Neutrophils infiltrate most tissues by extravasation through the post-capillary venules, in the lung however, extravasation occurs in the capillaries (Grommes *et al.*, 2011). This process

consists of neutrophil rolling which is mediated by selectin molecules expressed on the endothelium such as E-selectin and L-selectin on leukocytes. The cells are then tethered to the endothelium by interaction of β1 and β2 integrins with ICAM-1 and ICAM-2 (Ley *et al.*, 2007). Once neutrophils have infiltrated the inflamed tissue they can exert host damage by release of their granular contents. These include serine proteases such as neutrophil elastase which degrades the elastin present in the extracellular matrix of tissues to assist infiltration of tissues (Ginzberg *et al.*, 2001). Neutrophils also possess oxidative enzymes, in particular NADPH oxidase (Lambeth, 2004) and myeloperoxidase (El Kebir *et al.*, 2008) which are known to produce ROS to assist in the degradation of phagocytosed material. However, in ischaemia-reperfusion injury neutrophil infiltration is extensive and the ROS produced also damage host cells. Once activated neutrophils secrete a host of inflammatory cytokines which potentiate the inflammatory response by recruiting more leukocytes, therefore contributing to tissue injury (refer to Figure 1.1).
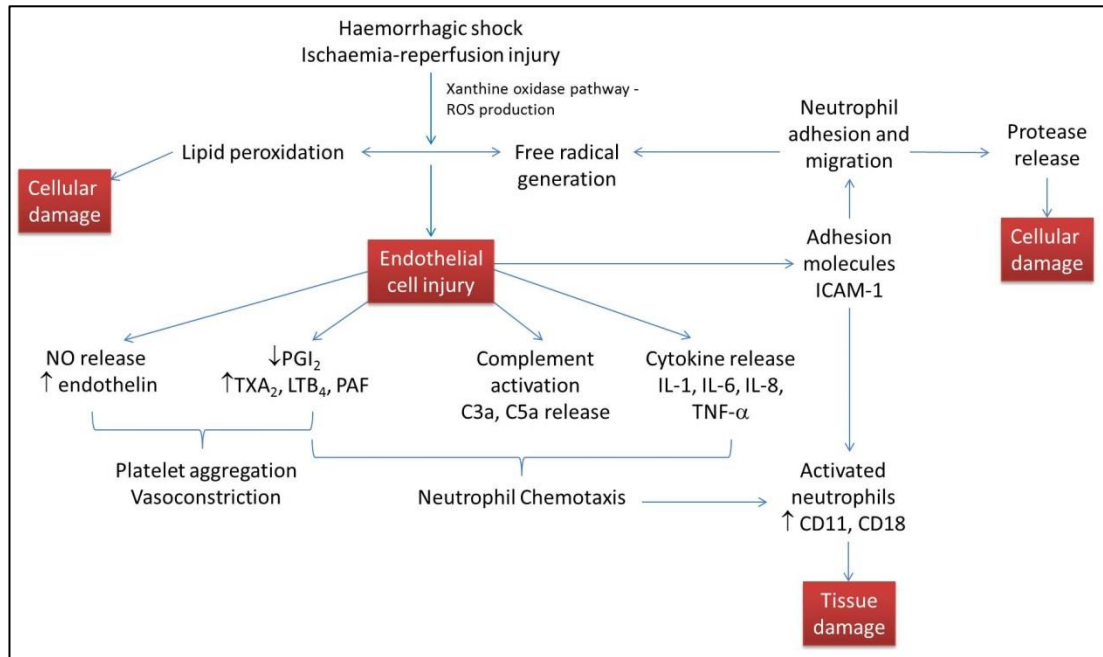
Figure 1.1

Figure 1.1: Overview of the inflammatory pathways involved in the ischaemia-reperfusion injury that occurs in HS. The production of ROS results in cellular damage, particularly endothelial cells. This causes release of inflammatory mediators such as C5a and IL-8 which attract and activate neutrophils. This combined with the increased expression of ICAM-1 on endothelial cells results in neutrophil infiltration of host tissues which contributes to the tissue damage [Adapted from (Grace, 1994)].

Ultimately, the endogenous responses to haemorrhage and resuscitation result in a massive inflammatory response known as the systemic inflammatory response syndrome (SIRS). This is defined clinically as having a heart rate greater than 90 beats per min, breathing rate greater than 20 breaths per min, P_{CO2} less than 32 mmHg, temperature either higher than 38°C or less than 36°C and blood leukocyte levels either higher than 12,000 cells per mm^3 or less than 4000 cells per mm^3 (Bone, 1992; Nathens *et al.*, 1996).

Another complication that occurs in trauma patients is disseminated intravascular coagulation (DIC), haemorrhage causes an increase in thrombin activity and a reduction in fibrinolysis in order to control the bleeding. This combined with the endothelial dysfunction that occurs, results in the formation of clots, particularly in capillaries (Gando *et al.*, 2002). This can contribute to a state of "no re-flow" following resuscitation where the tissue beds within organs are not sufficiently perfused (Carden *et al.*, 2000). However, excessive coagulation results in consumption of coagulation factors leading to coagulopathy as the patient is unable to control the loss of blood (Frith *et al.*, 2012).

The global ischaemia-reperfusion injury that occurs in HS can also cause cellular damage to the gastro-intestinal epithelium resulting in the disruption of the integrity of the gut wall. The consequent release of bacteria and bacterial components, in particular endotoxin, is thought to contribute to the inflammatory response observed in these patients (Deitch, 1994). However, this theory is not widely accepted as it has been shown that multiple organ failure can occur independent of a septic infection (Goris *et al.*, 1985).

1.4   Cell death in haemorrhagic shock

The cell damage and inflammation caused by the combination of haemorrhage and resuscitation results in cell death in many tissues which contributes to the deterioration in organ function.

Apoptosis is the process of programmed cell death which regulates cell proliferation; it is also triggered when the cell is under stress, in this case the cell is under metabolic stress due to hypoxia, hypoglycaemia and acidosis (Kroemer *et al.*, 2009). Apoptotic cells undergo nuclear shrinkage and fragmentation, chromatin condensation, cleavage of cytoskeletal proteins and formation of plasma membrane blebs before forming apoptotic bodies which are phagocytosed by leukocytes (Adams *et al.*, 2007; Marsden *et al.*, 2003). Apoptosis is initiated either by the death receptor pathway, in which

TNF-α binds a membrane-bound receptor that initiates the activation of caspase-8 (Hirsch *et al.*, 1997; Marsden *et al.*, 2003), or the mitochondrial pathway where ROS and cellular damage cause the opening of the mitochondrial permeability transition pore (mPTP) which results in the release of pro-apoptotic proteins, in particular cytochrome C which activates caspase-9 (Strasser, 2005). Caspases are serine proteases which mediate apoptotic signalling by promoting the degradation of DNA. Ischaemia induces apoptosis through the mitochondrial pathway and inhibition of mPTP opening is able to inhibit apoptosis of cardiomyocytes (Piot *et al.*, 2008). In HS, the ischaemia that occurs during haemorrhage induces apoptosis (Hsieh *et al.*, 2006) and the inhibition of caspase enzymes can limit the associated tissue injury (Abdelrahman *et al.*, 2004b).

Autophagy is another form of programmed cell death where cells digest their own organelles and macromolecules in order to generate energy and allow survival of the cell. It is an adaptive process to sublethal stress, however when the stress exceeds a tolerable level autophagy can lead to death of the cell (Klionsky, 2007; Kroemer *et al.*, 2005). It is unclear whether autophagy plays a role in cell death due to ischaemia, although autophagy has been shown to occur in cardiomyocytes subjected to hypoxia *in vitro* (Sybers *et al.*, 1976).

Necrosis results in swelling of the cell and organelles causing release of proteases which degrade cellular components, and the loss of cell membrane integrity results in bursting of the cell with release of the cell contents (Lemasters, 2005). During ischaemia, ATP levels are significantly reduced, there is release of ROS, increased intracellular calcium concentration, poly ADP ribose polymerase (PARP) and calpain activation, which are all triggers for necrosis (Conus *et al.*, 2008). There is a theory that necrosis may be partly a controlled form of cell death, as the release of DAMPs stimulates the innate immune system and this may be a trigger to manage the injury in an organised manner through apoptotic cell death rather than further necrosis (Lotze *et al.*, 2005). Necrosis occurs in response to ischaemia and can impair organ function, for example, HS can induce acute tubular necrosis in the kidney resulting in AKI (Mayeur *et al.*, 2011). Also inhibition of calpain, which is a protease activated by

elevated intracellular calcium levels, in HS reduced the generation of inducible nitric oxide synthase (iNOS) and cyclo-oxygenase-2 (COX-2) in the kidney which contributed to a reduction in tissue injury (McDonald *et al.*, 2001).

1.5   Key cell signalling pathways in the pathophysiology of haemorrhagic shock

The inflammatory response, tissue damage and cell death that occurs in HS is a result of the cell's response to ischaemia and reperfusion. This is regulated by intracellular signalling proteins which are activated by changes in both the intracellular and extracellular environments. The downstream effect is activation and/or translocation of transcription factors to the nucleus in order to influence gene expression. In the context of HS, this results in upregulation of pro-inflammatory cytokines and pro-apoptotic proteins which potentiate the inflammation and promote cell death.

Phosphatidylinositol-3-kinase (PI3K); particularly class I, is an important kinase involved in cell survival and growth. Following activation of tyrosine kinase receptors by growth factors and cytokines, PI3K mediates the phosphorylation of membrane-bound phosphatidylinositol-4, 5-biphosphate (PIP_2) to produce the intracellular signalling molecule phosphatidylinositol-3, 4, 5-triphosphate (PIP_3) (Whitman *et al.*, 1988). PIP_3 is then able to activate a host of kinases downstream of PI3K including phosphoinositide-dependent kinase 1 (PDK1) (Alessi *et al.*, 1997). This is illustrated in Figure 1.2. PDK1 has been shown to phosphorylate and activate protein kinase B (PKB; also known as Akt) at the Thr^{308} site, the phosphorylation of Akt at the hydrophobic Ser^{473} site allows molecular binding to occur which also contributes to its activation (Yang *et al.*, 2002). Activation of the Akt pro-survival pathway results in interaction with multiple signalling proteins and transcription factors to cause an anti-apoptotic effect, as can be seen in Figure 1.2. For example, once activated Akt is able to induce the phosphorylation of glycogen synthase kinase (GSK) – 3β. GSK-3β is a serine-threonine kinase that was originally recognised as a kinase that phosphorylates glycogen synthase. Unlike most other kinases, GSK-3β is active in a resting cell state and is inactivated by phosphorylation of Ser^9 by Akt (Cross *et al.*, 1995). GSK-3β has many downstream

targets, however it is known that it is able to interact with the transcription factor; nuclear factor (NF) - κB (Schwabe *et al.*, 2002). NF-κB plays an important role in regulating the transcription of a number of genes, especially those involved in producing mediators involved in local and systemic inflammation, such as cytokines, chemokines, cell adhesion molecules, apoptotic factors, and other mediators (Senftleben *et al.*, 2002). The structure of NF-κB consists of combinations of p65 or RelA, RelB and c-Rel subunits. The p65 subunit of NF-κB is thought to have four phosphorylation sites for the action of GSK-3β and specific inhibition of GSK-3β has been shown to directly inhibit NF-κB-dependent gene transcription (Schwabe *et al.*, 2002). Also, GSK-3β may inhibit the activation of NF-κB by phosphorylating IκBα resulting in its ubiquitination and degradation; IκBα in the resting cell is required to prevent NF-κB nuclear translocation and transcription of pro-inflammatory genes (Takada *et al.*, 2004).
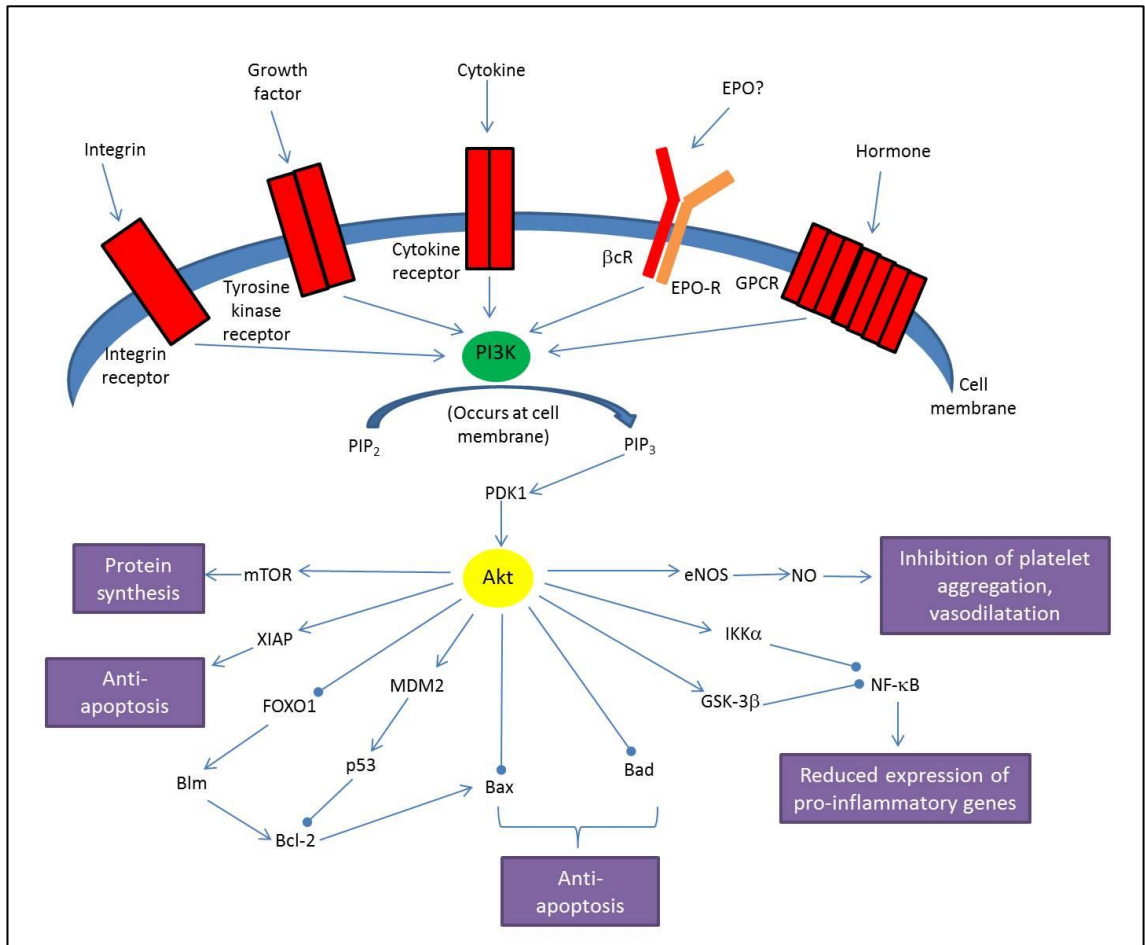
Figure 1.2

Figure 1.2: Summary of some of the intracellular proteins that Akt can interact with once activated by its upstream regulator PI3K. Interaction of a cell surface receptor with its ligand can result in recruitment and activation of PI3K, which activates Akt through a PDK-1-dependent pathway. Akt can phosphorylate and activate many proteins including eNOS, XIAP and mTOR, resulting in increased NO production, anti-apoptotic effects and increased protein synthesis. The inhibitory effects of Akt activation include inhibition of NF-κB and Bax/Bad to cause an anti-apoptotic effect. Akt is also able to interact with other intracellular proteins to influence the cell cycle and proliferation which have not been included in the figure. Pointed arrow heads indicate activation of protein, round arrow heads indicate inhibition of protein [Adapted from (Cell signaling technology, 2011)].

In states of cellular stress or injury the Akt pro-survival pathway is dysfunctional as there is a lack of activation of PI3K resulting in increased activation of transcription factors such as NF-κB leading to a pro-apoptotic and pro-inflammatory effect. NF-κB is a particularly important transcription factor in the pathophysiology of HS as it can be activated by multiple stimuli in addition to GSK-3β. For example, ischaemia can indirectly promote the translocation of NF-κB to the nucleus by causing the inhibition of prolylhydroxylase (PHD) enzymes. PHDs hydroxylate proline residues present in the hypoxia-inducible transcription factor (HIF) - 1α subunit in response to a reduction in P_{O2} which results in activation of HIF-1α which subsequently regulates the transcription of hypoxia-dependent genes such as erythropoietin. This pathway is also able to interact with NF-κB resulting in its activation in response to hypoxia (Eltzschig *et al.*, 2011). NF-κB activation is also effected by ROS produced by the mitochondria during cellular stress as ROS are able to induce the dissociation of IκBα from the active subunits of NF-κB (Cairns, 1998).

The Akt pro-survival pathway is disrupted in many tissues during HS resulting in inhibition of the pro-survival effect. The liver is particularly sensitive to ischaemia-reperfusion injury during HS; Liu and colleagues have demonstrated the dysfunction of the Akt pro-survival pathway that occurs in the liver following HS in the rat. This dysfunction has been linked to a corresponding reduction in the expression haem oxygenase (HO) – 1, which controls the expression of hypoxia-regulated genes such as erythropoietin (Liu *et al.*, 2011c).

Modulation of the Akt pro-survival pathway using interventions in animal models of HS has demonstrated a significant improvement in outcome. For example, 17β-estradiol attenuated the lung injury and inflammation induced by HS, as well as restoring the phosphorylation of Akt and increasing HO-1 expression (Hsu *et al.*, 2012). Angiopoietin-1 has been shown to increase vascular reactivity in the early stages of HS by activating its receptor, which interacts with Akt to cause activation of eNOS thus ensuring sufficient production of NO (Xu *et al.*, 2012). The anti-emetic, tropisetron, reduces the cardiac dysfunction associated with HS also by an Akt-dependent pathway

(Liu *et al.*, 2012). The administration of heparin binding epidermal growth factor (HB-EGF) in animals subjected to HS resulted in inhibition of adhesion molecule expression on endothelial cells by acting on Akt (Zhang *et al.*, 2012). Hypothermia has also been shown to increase the expression of Akt as well as increasing its co-localisation with heat shock proteins (Hsp), specifically Hsp27 (Li *et al.*, 2011). Histone deacetylase inhibitors such as valproic acid, also promote anti-apoptotic effects by preventing expression of pro-apoptotic proteins such as Bad and increasing the activation of Akt (Zacharias *et al.*, 2011). Akt has not been investigated clinically as a treatment target in the context of trauma or haemorrhage, however, modulation of Akt in cases of ischaemia does provide benefit in patients. Treatment of patients undergoing non-coronary artery cardiac surgery with simvastatin (20 mg before and after surgery), reduced the myocardial injury associated with cardiac surgery. It also increased the activation of Akt and Akt-dependent phosphorylation of eNOS, which is thought to be responsible for the tissue-protection observed (Almansob *et al.*, 2012).

As mentioned above, release of DAMPs from necrotic cells results in activation of TLRs; the release of HMGB1 in HS has been confirmed as early as 2 h after the ischaemic insult (Fan *et al.*, 2007). There are 13 homologues of this receptor and they are known to mediate a variety of PAMP signals (Medzhitov *et al.*, 1997), in addition DAMPs released during HS are also able to interact with these receptors as the expression of TLR – 2, 3, 4 and 6 have all been confirmed to increase in the lung following HS (Chen *et al.*, 2007; Tsan *et al.*, 2004). Once activated TLRs can activate an intracellular protein known as myeloid differentiation factor 88 (MyD88), which interacts with a series of other proteins including IL-1 receptor associated kinase (IRAK) – 1 and 4, and TNF receptor associated factor (TRAF) – 6. The combination of these cytokine mediated effects results in the activation of mitogen activated protein kinases (MAPKs), NF-κB and type I interferon pathways, therefore promoting the expression of pro-inflammatory genes (Mollen *et al.*, 2006).

MAPKs are a family of kinases that are also implicated in the regulation of cell proliferation and the development of inflammation. Members of this family include; p38 MAPK, c-Jun N-terminal kinase (JNK) 1/2 and extracellular signal-regulated kinase

(ERK) 1/2. These are serine-threonine kinases that are able to translocate to the nucleus and directly influence gene transcription by phosphorylating and activating transcription factors, for example p38 MAPK interacts with NF-κB causing its activation (Donnahoo *et al.*, 1999). This occurs in response to receptor activation by an extracellular stimulus which causes recruitment of MEK kinase (MAPKKK) to the membrane, once activated this phosphorylates MAPK kinase (MAPKK), which in turn phosphorylates and activates one of the above MAPKs so that it is able to enter the nucleus (Hancock, 2010). The phosphorylation of both ERK1/2 and p38 has been shown in animal models of HS and has been associated with both renal dysfunction and liver injury (Fukudome *et al.*, 2010; Sato *et al.*, 2011; Sato *et al.*, 2012). These studies suggest that the activation of these pathways by phosphorylation of MAPKs results in increased apoptosis and inflammation in multiple organs as a response to HS.

The complexity of these signalling pathways and their interaction with one another control and influence the survival state of the cell as well as the inflammatory response observed in the case of HS. Therefore, modulation of these pathways using pharmacological interventions may result in an altered cellular response which can result in a controlled inflammatory response and limited cell death therefore improving outcome.

1.6   Current perspectives on treatment

Current clinical views on the treatment of HS initially involve restoration of the circulating volume by resuscitation in order to improve cardiac output and perfusion of tissues, as well as preventing exsanguination. This treatment needs to be initiated soon after the ischaemic period as there is a "golden hour" in which treatments can be administered to prevent or limit tissue injury. In most situations it is either impossible or unpractical to be able to resuscitate with blood immediately, therefore resuscitative fluids are often used. There has been extensive debate over the last five decades as to which resuscitative methods are most favourable. A considerable amount of data regarding resuscitation fluids is from the military; during the time of the Vietnam War

it was established that large volumes of isotonic saline (3:1, i.e. three times the volume of blood lost) was the best volume replacement to improve survival (Dillon *et al.*, 1966; Shires *et al.*, 1973). However, pre-clinical studies of HS in animals showed that with such large volumes of saline there was increased metabolic acidosis as the saline was unable to neutralise the accumulation of acid from the ischaemic period (Traverso *et al.*, 1986). It was therefore proposed that a better resuscitative fluid is Ringer's lactate (Healey *et al.*, 1998).

Both saline and Ringer's lactate are crystalloid fluids which are isotonic with blood, therefore do not have any osmotic effects on cells but they do not possess oxygen carrying capacity. Ringer's lactate contains a high concentration of bicarbonate ions in the form of lactate which is useful as this can correct abnormal base deficits caused by hypoxia. Even though Ringer's lactate was considered a better resuscitative fluid, it can diffuse freely out of the vascular lumen so large volumes are required and the volumes used at that time were associated with the development of ARDS (Ashbaugh *et al.*, 1967; Demling, 1980). The development of ARDS could be associated with a pro-inflammatory response; Alam and colleagues demonstrated in a pig model of HS that the highest degree of neutrophil activation was associated with resuscitation of a large volume of Ringer's lactate infused at a fast rate (Alam *et al.*, 2004). Fresh frozen plasma (FFP) resuscitation in blunt force injury patients with HS has also been associated with an increased risk of ARDS (Watson *et al.*, 2009); even though pre-clinical studies have demonstrated its efficacy at maintaining endothelial integrity and reduce shedding of the endothelial glycocalyx layer (Haywood-Watson *et al.*, 2011).

To avoid the development of ARDS colloids such as albumin were thought to be a better option. When investigated experimentally colloids were shown to increase plasma oncotic pressure without causing pulmonary oedema (Haupt *et al.*, 1982), hence reducing the risk of ARDS. However, in patients the use of colloids has been associated with a rise in mortality of critically ill patients (Choi *et al.*, 1999; Velanovich, 1989). Therefore, crystalloids, in particular Ringer's lactate, are preferred as they are inexpensive and do not provoke adverse effects such as interference with blood group cross-matching.

In the UK, the guidelines for resuscitation of trauma patients state that initially intravenous fluid boluses (preferably Ringer's lactate) of 500 ml to 1 L should be administered followed by 40 ml/kg of cross-matched blood once it is possible i.e. once admitted into hospital (Draper *et al.*, 2011). There are frequently new advances with the management of trauma care, for example the Barts and The London helicopter emergency medical service (HEMS) is now able to transport blood in appropriate storage conditions, in the aptly named "golden box", to the scene of an accident where it can be administered. This should improve patient outcome as it allows blood resuscitation to occur within the "golden hour" (Bowdler, 2012). The combination of resuscitative fluids with blood or blood products such as packed red blood cells (pRBCs) appears to provide an efficient way of ensuring sufficient resuscitation along with restoration of the oxygen carrying capacity of the blood.

A "modern" view on the treatment of trauma patients is damage control resuscitation which combines both haemostatic resuscitation and permissive hypotension. Haemostatic resuscitation is the combination of pRBCs and FFP (Borgman *et al.*, 2007); whereas permissive hypotension is a restrictive form of resuscitation where either smaller volumes of fluid are administered or the fluids are administered over a longer period of time in order to maintain a lower systolic blood pressure (Jansen *et al.*, 2009). Haemostatic resuscitation has been used extensively by the military and retrospective analysis has shown that a ratio of pRBCs and FFP of 1:1 resulted in a significant reduction in mortality compared to patients that were resuscitated with higher ratios of pRBCs and FFP (Borgman *et al.*, 2007). Permissive hypotension has also been described to afford a beneficial effect in trauma patients by reducing the mortality associated with penetrating torso trauma (Bickell *et al.*, 1994), however there is also evidence that there is no difference in mortality of patients that receive restrictive or standard resuscitation (Dutton *et al.*, 2002). The outcome of using blood products early in the resuscitative phase and using a less aggressive approach allows clinicians to effectively tackle the "lethal triad" of hypothermia, acidosis and coagulopathy (Thomas *et al.*, 2010). However, over the past 50 years there has been much confusion and controversy with regards to resuscitation regimes for trauma and

HS patients and when reviewing the literature now it appears this confusion still remains (Curry *et al.*, 2011).

There are no specific pharmacological treatments used in the management of trauma patients, however pharmacological agents are used to control coagulation. In a large, multi-centre clinical trial of the anti-fibrinolytic; tranexamic acid, in trauma patients [Clinical Randomisation of Anti-fibrinolytics in Significant Haemorrhage (CRASH) – 2], mortality was significantly reduced and there was a reduction in the risk of re-bleeding (Roberts *et al.*, 2012). Fibrinogen has also been proposed as an agent that can benefit outcome in trauma patients by reducing the volume requirement of allogeneic blood products (Schochl *et al.*, 2011). However, it seems that treatment with clotting factors such as recombinant factor VIIa, actually serve no benefit in patients with severe haemorrhage and coagulopathy (Hauser *et al.*, 2010) leaving some confusion over the management of coagulopathy in trauma patients.

With the interest of multiple organ failure there are no treatments as such, therefore substitutive methods are normally used. Examples include respiratory ventilation, dialysis in renal failure and in some cases eventual organ transplantation. As a result a pharmacological treatment or an intervention which can help to reduce the severity of organ injury would be advantageous in reducing late mortality. However, the treatment of critically ill patients, particularly those with sepsis, with such interventions has been unsuccessful in the clinic. Recently, there has been an in-depth analysis of the genomic response to sepsis, burn injury and trauma in both mouse models and human patients, which demonstrated that there is an apparent difference in the degree and timing of change in gene expression between the species. Hence, despite many anti-inflammatory treatments demonstrating efficacy in animal models of disease they lack an effect in patients (Seok *et al.*, 2013). There are various pre-clinical studies in which interventions have been demonstrated to protect against the organ injury and inflammation induced by HS (Abdelrahman *et al.*, 2004a; Dugo *et al.*, 2006; McDonald *et al.*, 2001). However, these findings have not been translated to the clinic further emphasising the need for effective pharmacological agents that may be able to limit tissue injury. There is a possibility that these interventions may be

unsuccessful in patients as the pathophysiology of sepsis and HS is very similar, however the interventions studied in this thesis act on multiple protective pathways and this may be sufficient to reduce tissue injury.

1.7 Conclusions

In conclusion, the ischaemia-reperfusion injury that occurs following HS results in a systemic inflammatory response which can contribute to tissue injury and eventual organ failure. The development of pharmacological interventions that may modulate the cell signalling pathways behind this response could have a potential tissue-protective effect. This could have beneficial effects on the poor prognosis of trauma patients that develop multiple organ failure as a result of the combination of haemorrhage and resuscitation.

1.8 Aims and Objectives

The main aim of this thesis was to investigate potential therapeutic approaches for the treatment of the organ injury and dysfunction associated with trauma-haemorrhage. This was performed using a reliable and clinically relevant model of HS in the rat which was developed and characterised during my extramural placement year.

The first aim was to investigate the potential of stem cell therapy in the acute setting of HS. Specifically, using bone marrow-derived mononuclear cells (BMMNCs) as a treatment upon resuscitation and investigating the effect BMMNCs can exert on the damage caused by HS to the kidney, liver and lung. In particular, focusing on the ability of BMMNCs to interact with the Akt pro-survival pathway in the liver.

The second aim was to investigate the possibility of using a stem cell mobiliser to achieve similar tissue-protective effects observed with BMMNC administration. The hormone erythropoietin (EPO) was used as it is a known stimulus for endothelial progenitor cell (EPC) mobilisation when repetitively administered. EPO was also investigated in the context of its own tissue-protective effects as it can interact with multiple cell survival pathways.

The third aim was to investigate a non-erythropoietic analogue of EPO called pyroglutamate helix B surface peptide (pHBSP). The use of EPO clinically in trauma patients is associated with an increase in the incidence of thrombotic events, therefore delineating the tissue-protective and erythropoietic effects of EPO increases its therapeutic potential. As pHBSP and EPO act via similar mechanisms their interaction with the similar cell survival signalling pathways was also investigated.

CHAPTER 2

Acute treatment with bone marrow-derived mononuclear cells

attenuates the organ injury and dysfunction induced by

haemorrhagic shock

2.1 Introduction

Recent evidence suggests that stem cells, such as those present in bone marrow–derived mononuclear cells (BMMNCs), have both regenerative and paracrine properties. Regenerative medicine tends to focus on the use of induced pluripotent stem cells which behave in a similar manner to embryonic stem cells and can differentiate into multiple cell types under certain conditions (Liu *et al.*, 2011b; Takahashi *et al.*, 2007). However, stem cells also secrete anti-inflammatory proteins such as vascular endothelial growth factor (VEGF), IL-1β, β-fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF) - 1, and transforming growth factor (TGF) - β (Takahashi *et al.*, 2006), which can exert protective effects in ischaemic conditions and offer potential therapeutic benefit.

BMMNCs are a mixture of cells from the bone marrow which contain a proportion of stem or progenitor cells (Mao *et al.*, 2010; Tsai *et al.*, 2011). As they are relatively easy to isolate and manipulate, BMMNCs have become the focus of many pre-clinical studies using *in vivo* models of ischaemic disease. In a model of cerebral ischaemia, rats were subjected to permanent bilateral common carotid ligation followed by intravenous administration of 2×10^7 BMMNCs 24 h after the ischaemic insult. This resulted in significantly increased cell proliferation in the ischaemic subventricular zone and a significant increase in the expression of brain-derived neurotrophic factor (BDNF) (Gubert *et al.*, 2013). The intravenous administration of BMMNCs has also been investigated in the context of MI. Here, rats were subjected to left anterior descending coronary artery occlusion for 25 min followed by reperfusion for a period of 2 h, rats were treated with 1×10^7 BMMNCs upon reperfusion. The authors reported a 42% reduction in infarct size as well as a reduction in apoptosis and necrosis in the area at risk. An improvement in cardiac function was also observed in a longer term model where rats were allowed to recover over 7 days (Lovell *et al.*, 2010).

In the context of HS, BMMNCs play an important role in the host response to the ischaemic insult. Stem cells are known to be mobilised following trauma; in a rat model of unilateral lung contusion homing of bone marrow-derived stem cells to the site of

injury is observed. This effect is potentiated when the rats were subjected to unilateral lung contusion and HS, as homing of these cells was also observed in the non-injured lung (Badami *et al.*, 2007). It has also been reported, however, that bone marrow failure can occur following trauma and HS in patients within 4 days of the insult (Livingston *et al.*, 2003). Taken together, these findings suggest that exploiting the therapeutic potential of stem cells in HS could be beneficial.

This has already been investigated experimentally by using a proportion of stem cells present within the BMMNC fraction; bone marrow-derived mesenchymal cells (MSCs), as a treatment in a recovery model of mild HS. Rats were subjected to fixed-volume haemorrhage of 30% of the circulating blood volume followed 1 h later by resuscitation with Ringer's lactate. Rats were treated with 2×10^6 MSCs by intravenous injection, upon resuscitation and again 24 h after the onset of resuscitation. Treatment with MSCs resulted in a significant reduction in the release of inflammatory cytokines including TNF-α and monocyte chemoattractant protein (MCP) - 1, 96 h after the onset of resuscitation, as well as reducing the pulmonary oedema and increased endothelial permeability in the lung caused by HS (Pati *et al.*, 2011).

Although both experimental animal and clinical studies have utilised BMMNCs as a possible therapeutic approach, the mechanism(s) underlying any of the observed (or expected) beneficial effects of BMMNCs are largely unclear. In acute settings, it is highly unlikely that the beneficial effects observed are due to regeneration of tissue, but is much more likely to be secondary to either direct and/or paracrine effects on the host tissue. In the MI study, hearts from rats subjected to MI and treated with BMMNCs had increased activation of the Akt pro-survival pathway after 2 h of reperfusion (Lovell *et al.*, 2010). As discussed in Chapter 1, this pathway is of particular interest in HS as modulation of this pathway can result in modulation of a tissue's response to the ischaemia-reperfusion that occurs.

In order to study the effects of BMMNC treatment in HS, I developed model of HS during my extramural year placement. This model is an adaptation of the rat model previously used in Professor Thiemermann's research group which produces

reproducible organ injury and dysfunction (Abdelrahman *et al.*, 2004a; Abdelrahman *et al.*, 2004b; McDonald *et al.*, 2001).

One may argue that the model used previously had the disadvantage of utilising a resuscitation protocol that involves resuscitation with the entire volume of shed blood mixed with a very high concentration of heparin [shed blood was mixed with heparinised saline at a concentration of 7500 IU/ml (Dugo *et al.*, 2006)]. Therefore, the aim of developing a new model was to make the model more clinically relevant by using an appropriate resuscitation protocol involving a combination of a clinically used crystalloid fluid; Ringer's lactate, and shed blood mixed with a much lower concentration of heparin. The use of heparin was unavoidable as the re-injection of shed blood required sufficient anti-coagulation to minimise the risk of embolus in the animal. In this model, the amount of heparin each animal receives has been minimised to approximately 100 IU for the entire 6 h duration of the experiment. However, heparin is also well-known for its anti-inflammatory properties, which it can exert at doses lower than its anti-coagulant properties (Lever *et al.*, 2010). Therefore, it is possible that the dose of heparin used here is able to exert beneficial effects against the inflammation associated with HS. If heparin was strongly anti-inflammatory in the context of HS, its administration would attenuate the injury observed. This is not the case as a significant degree of injury is observed with the present and previous models, however the degree of inflammation may be muted to some degree. Contradicting data demonstrates that heparin can contribute to injury by causing release of lipases into the lymph which can cause endothelial damage (Qin *et al.*, 2011).

Many variables from the old model were mirrored in the new model: For example, young male rats were used to avoid interference of oestrogens which are thought to be protective in HS (Shimizu *et al.*, 2008) and as many trauma patients are young males this may be representative of a typical clinical situation. Also, the haemorrhage technique used here is a fixed-pressure haemorrhage protocol, in which the MAP is reduced from baseline (usually 120 mmHg) to 35 ± 5 mmHg for a period of 90 min (Dugo *et al.*, 2006). This controlled method of haemorrhage allows the degree of hypotension and so the ischaemic insult to be controlled, and this protocol has

previously been shown to induce significant organ injury and dysfunction (McDonald *et al.*, 2001). Additionally, arterial blood gas (ABG) analysis was used to characterise the progression of acidosis in the new model, which gave a useful insight into the development of injury but also the degree of hypoxia that could be induced by haemorrhage. These data are presented in Appendix 1 and demonstrates that a significant degree of acidosis was induced following haemorrhage (decreased pH and P_{CO2}, and increased P_{O2}, base deficit and blood lactate concentration) which was minimally recovered by the end of the experimental period. The modifications made to the old model of HS have improved the clinical relevance but has retained the model's ability to induce significant injury, therefore this model is sufficient to study the effects of novel interventions against the injury induced by haemorrhage and resuscitation.

Considering the previous studies performed with BMMNCs and the success of MSC treatment in HS. The aim of this study was to use a similar study design as that used by Lovell and colleagues in the rat model of MI, to investigate if BMMNCs could afford a similar degree of protection against the organ injury induced by HS. Also to investigate whether BMMNCs can activate the Akt pro-survival pathway in organs other than the heart, particularly focussing on the liver.

2.2 Methods

2.2.1 Surgical Procedure

This study was carried out on 37 male Wistar rats (Charles River Ltd, Margate, UK) weighing 295 ± 7 g receiving a standard diet and water *ad libtum*. All procedures were carried out in accordance with the Home Office Guidance in the Operation of the Animals (Scientific Procedures) Act 1986, published by Her Majesty's Stationery Office, London, UK. All data from rats that had died during the experiment were excluded from data analysis, hence the numbers (n) presented represent the 'survivors' of the entire experimental protocol.

All animals were anaesthetised with sodium thiopentone (Thiovet©, 120 mg/kg i.p.) and anaesthesia was maintained by supplementary injections (~10 mg/kg i.v.) of sodium thiopentone as and when required. A tracheotomy was performed to facilitate respiration; a small length of polyethylene tubing [Internal Diameter (ID) 1.67 mm, Portex, Kent, UK] was inserted into the lumen of the trachea. The left femoral artery was cannulated (ID 0.40 mm, Portex) and connected to a pressure transducer (SP844 blood pressure sensor, Memscap, Durham, USA) for the measurement of MAP which was displayed on a data acquisition system (Powerlab 8SP, Chart v5.5.3, AD Instruments, Hastings, UK) installed on Hewlett-Packard computer for the duration of the experiment. The right carotid artery was cannulated (ID 0.58 mm, Portex) for the withdrawal of blood into a heparinised syringe. The right jugular vein was cannulated (ID 0.40 mm, Portex) for the administration of Ringer's lactate, shed blood, vehicle or intervention. Body temperature was maintained at 37 ± 1°C by placing the anaesthetised rats onto a homeostatically controlled heating mat (Harvard Apparatus Ltd., Kent, UK) and temperatures were monitored by the means of a rectal probe thermometer. Upon completion of the surgical procedure, cardiovascular parameters were allowed to stabilise for a period of 15 min.

2.2.2 Haemorrhage and Resuscitation

After the stabilisation period, blood was withdrawn via the cannula inserted in the right carotid artery in order to achieve a fall in MAP to 35 ± 5mmHg within 10 min. From this point onwards, MAP was maintained at 35 ± 5mmHg for a period of 90 min either by further withdrawal of blood during the compensation phase (MAP rises following withdrawal due to sympathetic response) or administration of Ringer's lactate i.v. during the decompensation phase (animals are unable to increase and maintain high MAP). The average volume of blood withdrawn during haemorrhage was 9.80 ± 0.26 ml (n=23, across all haemorrhaged groups). At the end of the haemorrhage period, resuscitation was performed with 20 ml/kg Ringer's Lactate over a period of 10 min and then half the shed blood mixed with 100 IU/ml heparinised saline over a period of 50 min both administered i.v. Heparin was used as anti-coagulant and this dose was chosen following preliminary experiments where the concentration of heparinised saline was incrementally reduced from 7500 IU/ml to a concentration where the anti-coagulant effects were still preserved. MAP was maintained by 1.5 ml/kg/h i.v. bolus of Ringer's lactate for a period of 4 h after the onset of resuscitation when the experiment was terminated. Rats were sacrificed and the organs harvested; lung and liver samples were taken and stored at -80°C and additional lung samples were taken and stored in formalin for further analysis.

2.2.3 Isolation and characterisation of bone marrow–derived mononuclear cells

Bone marrow was freshly isolated from femurs and tibias of male Wistar rats from which BMMNCs were extracted using density gradient centrifugation at 400 *g* for 30 min at 4°C (Histopaque-1077©, Sigma-Aldrich, Dorset, UK). BMMNCs were re-suspended in 10 ml Hank's balanced salt solution (HBSS) and centrifuged at 250 *g* for 10 min at 4°C, the resultant supernatant was aspirated and the pellet re-suspended in the volume of PBS required for injection. BMMNCs from one donor rat were used to treat up to three rats that were either sham operated or subjected to HS, resulting in the use of six rats to donate BMMNCs.

BMMNCs were characterised using flow cytometry; 1 million cells were incubated with primary antibodies for c-kit (sc-5535; Santa Cruz Biotechnology, Heidelberg, Germany), CD34 (sc-9095; Santa Cruz Biotechnology), CD45 (554875; BD, Oxford, UK), and CD133 (sc-30219; Santa Cruz Biotechnology) in 80 µl of HBSS or with HBSS alone for 30 min at room temperature. Excess antibody was removed by washing with 1 ml HBSS and centrifuging at 330 g for 5 min at room temperature and the supernatant was aspirated. The cells were re-suspended in secondary antibody conjugated with FITC and incubated for a further 30 min at room temperature. The wash step was then repeated and cells were re-suspended in 500 µl of HBSS. Analysis was performed using Fortessa analyser (Beckman Coulter, High Wycombe, UK) and data analysed using FlowJo v7.6.5 (Ashland, Oregon, USA).

2.2.4 Experimental groups

Rats were randomly allocated into the following groups:

1. Sham + PBS   (n = 10)
2. Sham + BMMNC   (n = 4)
3. HS + PBS   (n = 10)
4. HS + BMMNC   (n = 13)

Rats were treated with either 1 ml/kg vehicle (PBS) or 1×10^7 BMMNCs suspended in 1 ml/kg PBS upon resuscitation; this treatment protocol is based on that used by Lovell and colleagues when investigating the effect of BMMNC treatment in MI (Lovell *et al.*, 2010). Sham-operated rats underwent identical surgical procedures but without haemorrhage or resuscitation.

2.2.5 Quantification of organ function and injury

At the end of the experiment (4 h after the onset of resuscitation), 1.1 ml of blood was withdrawn from the cannula inserted in the right carotid artery into serum gel tubes

(Idexx Laboratories, Sussex, UK) and centrifuged at 9900 r.p.m. for 3 min at room temperature. All serum samples were analysed within 24 h by a contract laboratory for veterinary clinical chemistry (Idexx Laboratories). The following parameters were measured in the serum as biochemical indicators of multiple organ injury and dysfunction as defined by Fry and colleagues (Fry *et al.*, 1980):

1. Renal dysfunction was determined by measuring serum levels of urea (mmol/L) and creatinine (µmol/L). Urea and creatinine are by-products of metabolism of protein and muscle, respectively. Serum levels of both can be used as indirect indicators of glomerular filtration rate and hence renal function.

2. Hepatic injury was determined by measuring serum levels of aspartate aminotransferase; AST, (IU/L) and alanine aminotransferase; ALT, (IU/L). Both AST and ALT are common clinical markers for liver injury and are released following hepatocyte cell death. However AST is also present in the heart, muscle, kidneys and brain, therefore is not specific for liver injury, whereas ALT is only found in liver parenchymal cells.

3. Skeletal muscle injury was determined by measuring serum creatine kinase; CK (IU/L) levels. CK is a surrogate marker for muscle injury as it is mainly present in the skeletal muscle and released following damage. However, as CK is also involved in energy metabolism in many cells it is not highly specific.

2.2.6 Histological Evaluation

At the end of the experiment, lung samples were excised and fixed in 10 % (w/v) formalin, buffered with PBS (0.01 mol/L, pH 7.4) for 1 week. Samples were then dehydrated using graded ethanol, embedded in paraffin wax and cut into sections using a Leica rotary microtome (thickness 5 µm). Sections were deparaffinised with xylene, stained with Gills haematoxylin and washed. Sections were then subsequently counterstained with 1% eosin, dehydrated with ethanol and cleared with Neo-Clear (Darmstadt, Germany) before mounting using HistoMount (Atlanta, Georgia, USA).

Sections were analysed using a Leica DM2000 upright microscope (Wetzlar, Germany). To determine lung injury, the sections were examined blind using a grading scale (0: none, 1: rare, 2: mild, 3: moderate, and 4: severe) as described by Akinci and colleagues with minor modifications (Akinci *et al.*, 2005). Features examined were inflammatory cell infiltration, thickening of the alveolar septa and pulmonary congestion. A total of 10 fields were evaluated randomly for each sample. The score for each group was the average score for all samples in the group.

2.2.7 Determination of Myeloperoxidase (MPO) Activity

Lung samples were homogenised in a solution containing 0.5% (w/v) hexadecyltrimethyl-ammonium bromide dissolved in 10 mmol/L potassium phosphate buffer (pH 7) and centrifuged for 30 min at 20,000 g at 4°C. An aliquot of the supernatant was then allowed to react with a solution of 1.6 mM tetramethylbenzidine and 0.1 mmol/L H_2O_2. The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 µmol of peroxide per min at 37°C and was expressed in µU/g of wet tissue.

2.2.8 Western Blot Analysis

Three separate experiments of western blot analysis were performed for each marker and tissues were analysed separately for each western blot experiment. Liver and lung samples were homogenised and centrifuged at 4,000 g for 5 min at 4°C. Supernatants were removed and centrifuged at 15,000 g at 4°C for 40 min to obtain the cytosolic fraction. The pelleted nuclei were re-suspended in extraction buffer. The suspensions were centrifuged at 15,000 g for 20 min at 4°C. The resulting supernatants containing nuclear proteins were carefully removed, and protein content was determined using a bicinchoninic acid (BCA) protein assay following the manufacturer's directions (Fisher Scientific, Loughborough, UK). Proteins were separated by 8% sodium dodecyl

sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinyldenedifluoride (PVDF) membrane (Fisher Scientific), which was then incubated with a primary antibody (lung samples were incubated with rabbit anti-ICAM-1 dilution 1:1000, and liver samples incubated with rabbit anti-total Akt dilution 1:1000; mouse anti-phospho Akt Ser[473] dilution 1:1000; rabbit anti-total GSK-3β, dilution 1:200; goat anti-phospho GSK-3β Ser[9] dilution 1:200; rabbit anti-NF-κB p65 dilution 1:400). Blots were then incubated with a secondary antibody conjugated with horseradish peroxidase (dilution 1:10000) and developed using the enhanced chemiluminescence (ECL) detection system (Fisher Scientific).

The immunoreactive bands were visualised by autoradiography. The membranes were stripped and incubated with β-actin monoclonal antibody (dilution 1:5000) and subsequently with an anti-mouse antibody (dilution 1:10000) to assess gel-loading homogeneity. Densitometric analysis of the bands was performed using Gel Pro®Analyzer 4.5, 2000 software (Media Cybernetics, Silver Spring, Maryland, USA) and optical density analysis was expressed as fold-increase versus the sham group. In the sham group, the immunoreactive bands of the gel were respectively measured and normalised against the first immunoreactive band (standard sham sample) and the results of all the bands belonging to the same group were expressed as mean ± standard error of the mean (SEM). This provides SEM for the sham group where a value of 1 is relative to the first immunoreactive band.

2.2.9   Materials

Unless otherwise stated, all compounds used in this study were purchased from Sigma-Aldrich Company Ltd (Poole, Dorset, UK). All stock solutions were prepared using non-pyrogenic saline (0.9 % [w/v] NaCl; Baxter Healthcare Ltd, Thetford, Norfolk, UK). Ringer's lactate was purchased from Baxter Healthcare Ltd. Antibodies for Western blot analyses were purchased from Santa Cruz Biotechnology (Heidelberg, Germany).

2.2.10 Statistical analysis

All values described in the text and figures are expressed as mean ± SEM for n observations. Each data point represents biochemical measurements obtained from up to 13 separate animals. Statistical analysis was carried out using GraphPad Prism 5.03 (GraphPad Software, San Diego, California, USA). Data without repeated measurements were assessed by one-way analysis of variance followed by Dunnett's post-hoc test. Data with repeated measurements were assessed by two-way analysis of variance followed by Bonferroni post-hoc test. A P value of less than 0.05 was considered to be significant.

2.3 Results

2.3.1 Characterisation of BMMNCs

Bone marrow–derived mononuclear cells were characterised to be c-kit^{+} (7 ± 1 %, n = 10), CD34^{+} (7 ± 1 %, n = 10), CD45^{+} (54 ± 6 %, n = 10), and CD133^{+} (15 ± 1 %, n = 10).

2.3.2 Effect of BMMNC administration on the circulatory failure induced by haemorrhagic shock

Baseline MAP values were similar amongst all four groups. When compared with sham-operated rats, haemorrhage caused a significant decline in MAP which was improved by resuscitation; however MAP was still significantly different from that of sham-operated rats ($P<0.05$, Figure 2.1). The administration of BMMNCs was sufficient to increase MAP during resuscitation so that it was similar to that of sham-operated rats ($P<0.05$; Figure 2.1), although this declined towards the end of the experiment. Administration of BMMNCs in sham-operated rats had no significant effect on MAP ($P>0.05$; Figure 2.1).

Figure 2.1
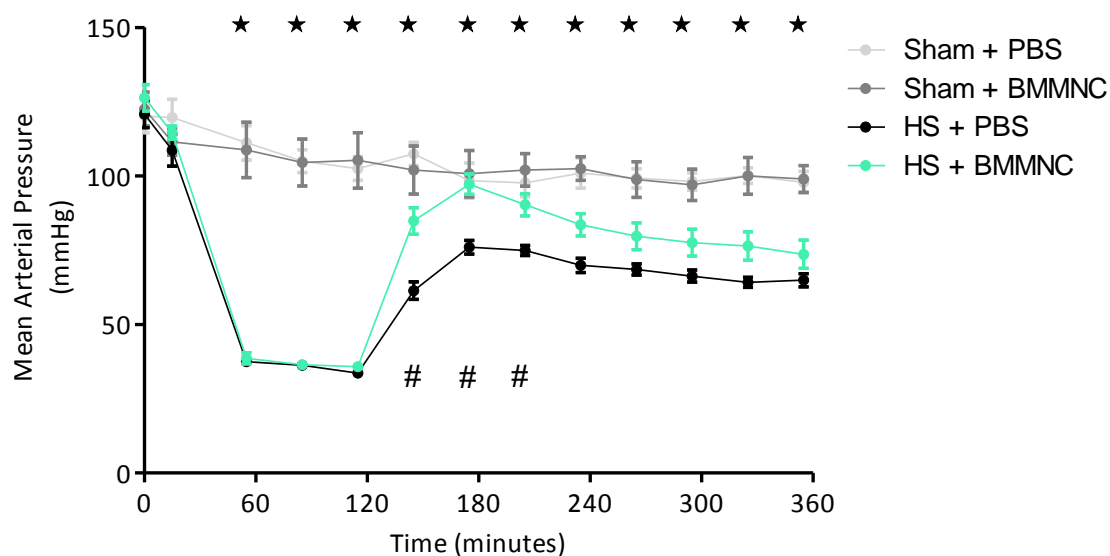
Figure 2.1: Alterations in MAP; in rats subjected to sham-operation or HS and treated with either PBS (Sham + PBS, n=10; HS + PBS, n=10), or BMMNCs (Sham + BMMNC, n=4; HS + BMMNC, n=13). Data are expressed as mean ± SEM for n observations, *P<0.05 Sham vs. HS + PBS, #P<0.05, HS + PBS vs. HS + BMMNC.

2.3.3 Effect of BMMNC administration on the renal dysfunction induced by haemorrhagic shock

When compared with sham-operated rats, HS-rats treated with vehicle developed significant increases in serum urea (P<0.05; Figure 2.2A) and creatinine (P<0.05; Figure 2.2B), indicating the development of renal dysfunction. Administration of BMMNCs in HS-rats significantly attenuated the rise in serum urea (P<0.05; Figure 2.2A) and creatinine (P<0.05; Figure 2.2B). Administration of BMMNCs in sham-operated rats had no effect on either serum urea or creatinine levels (P>0.05; Figure 2.2).
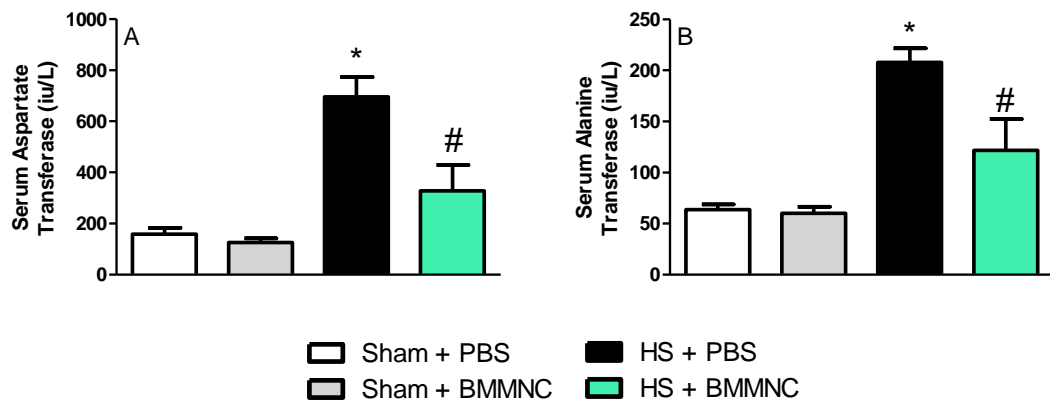
Figure 2.2
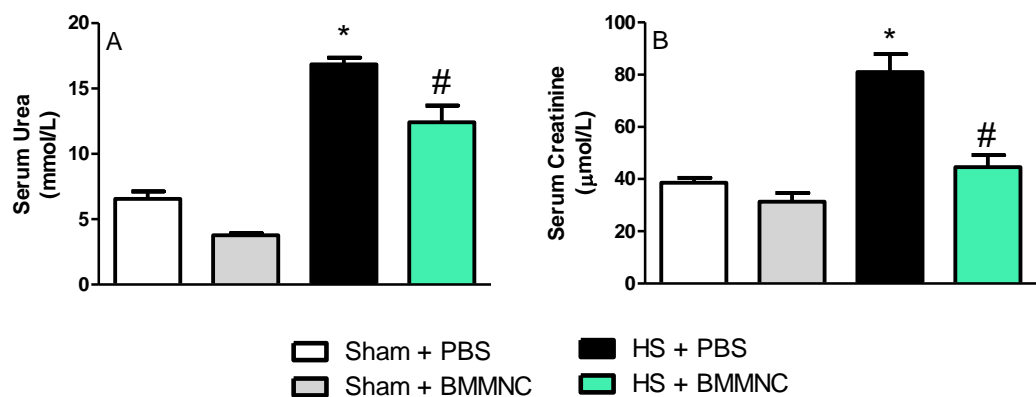
Figure 2.2: Alterations in serum levels of (A) urea and (B) creatinine; in rats subjected to sham-operation or HS and treated with either PBS (Sham + PBS, n=10; HS + PBS, n=10), or BMMNCs (Sham + BMMNC, n=4; HS + BMMNC, n=13). Data are expressed as mean ± SEM for n observations, *P<0.05 Sham vs. HS + PBS, #P<0.05, HS + PBS vs. HS + BMMNC.

2.3.4 Effect of BMMNC administration on the hepatic injury induced by haemorrhagic shock

When compared with sham-operated rats, HS-rats treated with vehicle developed significant increases in serum AST ($P<0.05$; Figure 2.3A) and ALT ($P<0.05$; Figure 2.3B), indicating the development of hepatic injury. Administration of BMMNCs in HS-rats significantly attenuated the rise in serum AST ($P<0.05$; Figure 2.3A) and ALT ($P<0.05$; Figure 2.3B). Administration of BMMNCs in sham-operated rats had no effect on either serum AST or ALT levels ($P>0.05$; Figure 2.3).

Figure 2.3

Figure 2.3: Alterations in serum levels of (A) AST and (B) ALT; in rats subjected to sham-operation or HS and treated with either PBS (Sham + PBS, n=10; HS + PBS, n=10), or BMMNCs (Sham + BMMNC, n=4; HS + BMMNC, n=13). Data are expressed as mean ± SEM for n observations, *$P<0.05$ Sham vs. HS + PBS, #$P<0.05$, HS + PBS vs. HS + BMMNC.

2.3.5 Effect of BMMNC administration on the skeletal muscle injury induced by haemorrhagic shock

When compared with sham-operated rats, HS-rats treated with vehicle developed a significant increase in serum CK ($P < 0.05$; Figure 2.4), which may indicate the development of skeletal muscle injury. Administration of BMMNCs in HS-rats significantly attenuated the rise in serum CK ($P < 0.05$; Figure 2.4). Administration of BMMNCs in sham-operated rats had no effect on serum CK levels ($P > 0.05$; Figure 2.4).
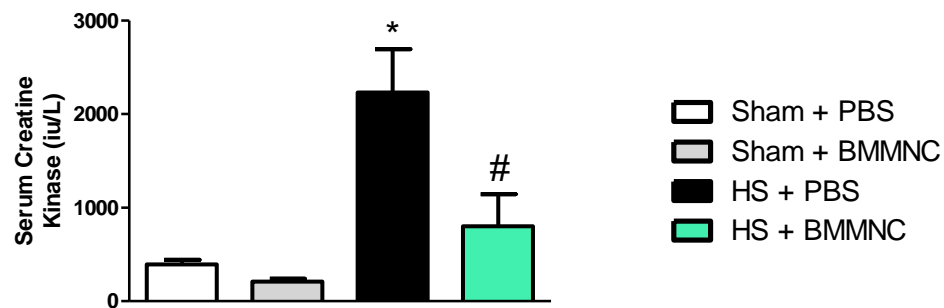
Figure 2.4

Figure 2.4: Alterations in serum levels of CK; in rats subjected to sham-operation or HS and treated with either PBS (Sham + PBS, n=10; HS + PBS, n=10), or BMMNCs (Sham + BMMNC, n=4; HS + BMMNC, n=13). Data are expressed as mean ± SEM for n observations, *$P < 0.05$ Sham vs. HS + PBS, #$P < 0.05$, HS + PBS vs. HS + BMMNC.

2.3.6. Effect of BMMNC administration on the pulmonary injury and inflammation induced by haemorrhagic shock

When compared with sham-operated rats (Figure 2.5A), the lung histology of HS-rats treated with vehicle confirmed inflammatory cell infiltration ($P<0.05$; Figure 2.5B and D) and alveolar septal thickening ($P<0.05$; Figure 2.5B and E), suggesting development of lung injury and inflammation. Administration of BMMNCs in HS-rats attenuated the degree of inflammatory cell infiltration ($P<0.05$; Figure 2.5C and D) but not the alveolar septal thickening ($P>0.05$; Figure 2.5C and E).
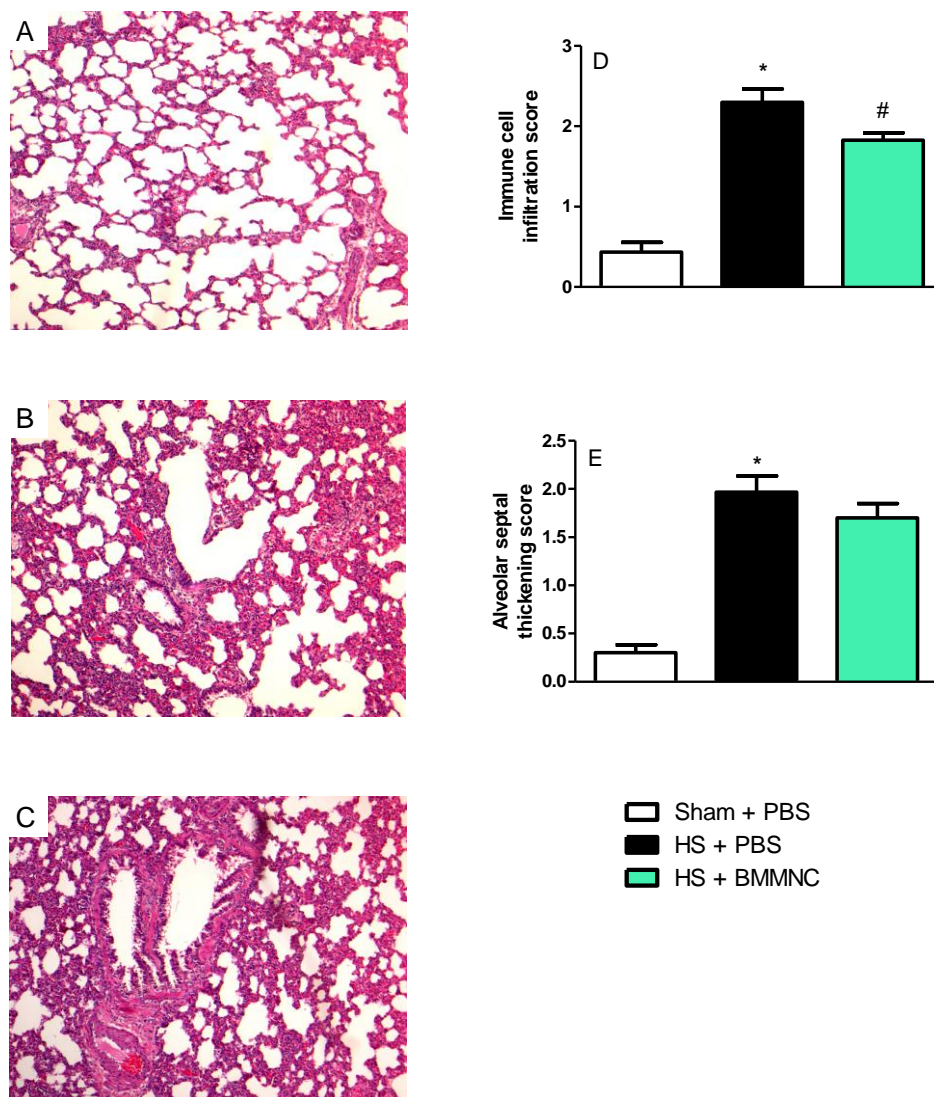
Figure 2.5

Figure 2.5: Lung histological sections taken from (A) sham-operated rat with inflammatory cell infiltration grade 1 and no observable alveolar septal thickening, (B) HS-rat treated with vehicle with inflammatory cell infiltration grade 3 and widespread alveolar septal thickening and (C) HS-rat treated with BMMNCs with inflammatory cell infiltration grade 2 and alveolar septal thickening. Haematoxylin-eosin stain, magnification ×100; figures are representative of at least three experiments performed on different days. Histological scoring for (D) inflammatory cell infiltration and (E) alveolar septal thickening, in rats subjected to sham-operation or HS and treated with either PBS (Sham + PBS, n=4, HS + PBS, n=4) or BMMNCs (HS + BMMNC, n=4). Data are expressed as mean ± SEM for n observations, *P<0.05 Sham vs. HS + PBS, #P<0.05 HS + PBS vs. HS + BMMNC.

2.3.7 Effect of BMMNC administration on the pulmonary congestion induced by haemorrhagic shock

When compared with sham-operated rats (Figure 2.6A), the lung histology of HS-rats treated with vehicle confirmed pulmonary congestion ($P<0.05$; Figure 2.6B and D), suggesting development of lung injury and inflammation. Administration of BMMNCs in HS-rats attenuated the degree of pulmonary congestion ($P<0.05$; Figure 2.6C and D). However, this was not to the same level as that in sham-operated rats.
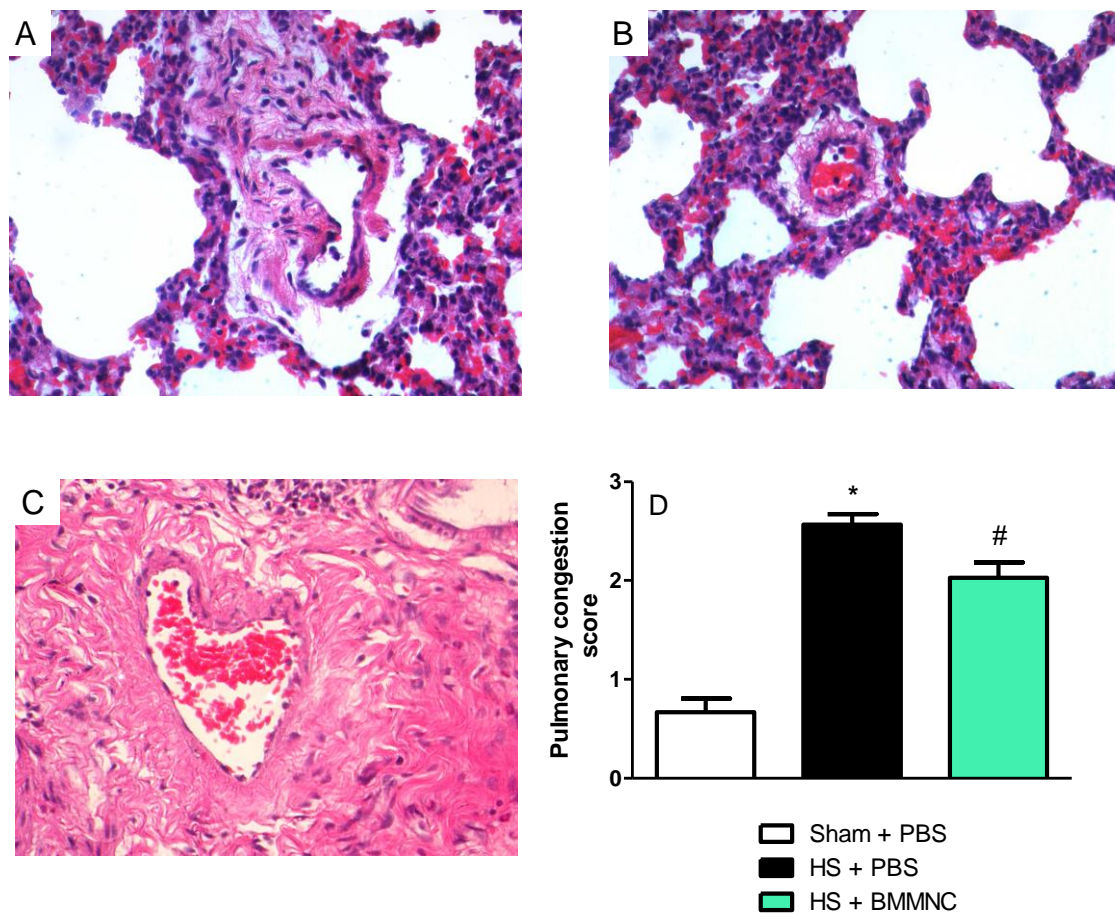
Figure 2.6

Figure 2.6: Lung histological sections taken from (A) sham-operated rat with pulmonary congestion grade 0, (B) HS-rat treated with vehicle with pulmonary congestion grade 3 and (C) HS-rat treated with BMMNCs with pulmonary congestion grade 2. Haematoxylin-eosin stain, magnification ×400; figures are representative of at least three experiments performed on different days. Histological scoring for (D) pulmonary congestion, in rats subjected to sham-operation or HS and treated with either PBS (Sham + PBS, n=3, HS + PBS, n=3) or BMMNCs (HS + BMMNC, n=4). Data are expressed as mean ± SEM for n observations, *P<0.05 Sham vs. HS + PBS, #P<0.05 HS + PBS vs. HS + BMMNC.

2.3.8 Effect of BMMNC administration on the MPO activity in the lungs from rats that underwent haemorrhage and resuscitation

MPO activity was determined to estimate the degree of neutrophil infiltration in the lung. When compared with sham-operated rats, HS-rats treated with vehicle had significantly higher MPO activity in the lung (P<0.05; Figure 2.7), suggesting the presence of neutrophils in the lung. Administration of BMMNCs in HS-rats significantly reduced the rise in MPO activity caused by haemorrhage and resuscitation in the lung (P<0.05; Figure 2.7).
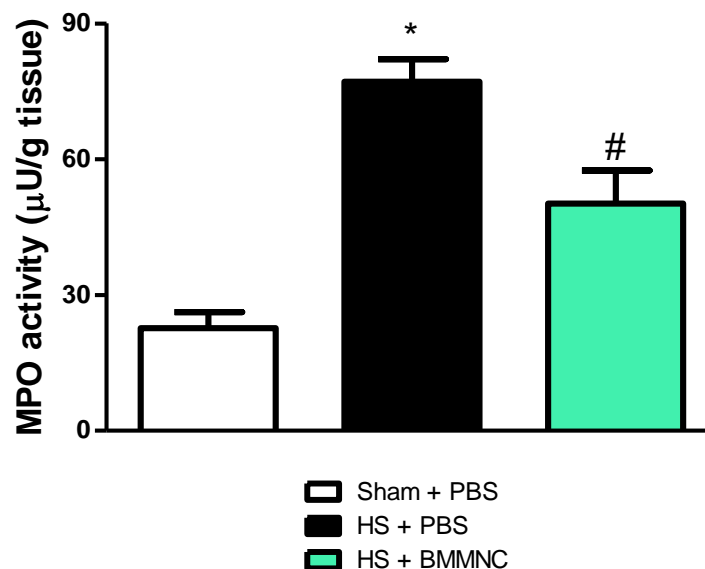
Figure 2.7

Figure 2.7: Activation of MPO in the lung in rats subjected to sham-operation or HS and treated with either PBS (Sham + PBS, n=3, HS + PBS, n=3) or BMMNCs (HS + BMMNC, n=3). Data are expressed as mean ± SEM for n observations, *P<0.05 Sham vs. HS + PBS, #P<0.05 HS + PBS vs. HS + BMMNC.

2.3.9 Effect of BMMNC administration on the expression of ICAM-1 in the lungs from rats that underwent haemorrhage and resuscitation

The expression of ICAM-1 was measured as an indication of the inflammation occurring in the lung. When compared with sham-operated rats, HS-rats treated with vehicle developed a significant increase in the expression of ICAM-1 in the lung (P<0.05; Figure 2.8). Administration of BMMNCs in HS-rats attenuated the increase in ICAM-1 expression caused by haemorrhage and resuscitation in the lung (P<0.05; Figure 2.8).
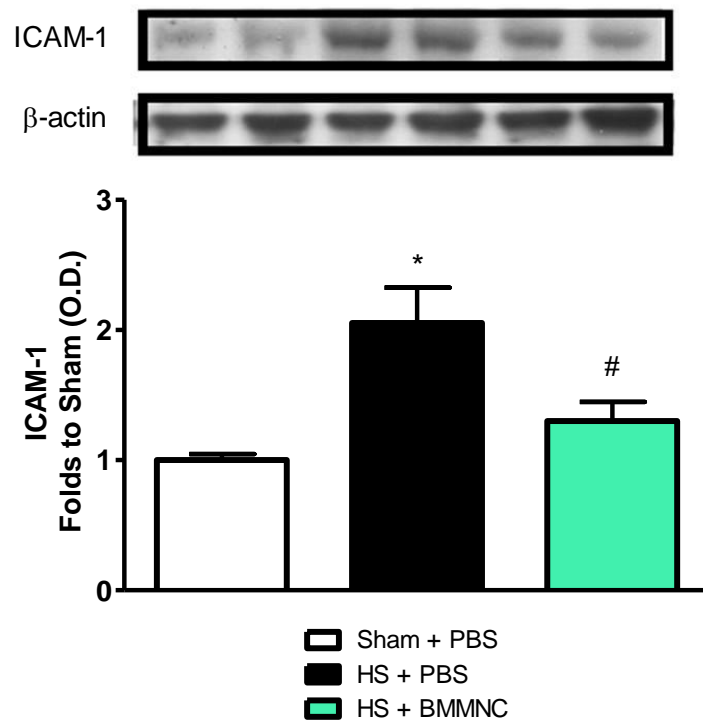
Figure 2.8

Figure 2.8: Expression of ICAM-1 in the lung; in rats subjected to sham-operation or HS and treated with either PBS (Sham + PBS, n=3, HS + PBS, n=3) or BMMNCs (HS + BMMNC, n=3). Data are expressed as mean ± SEM for n observations, *P<0.05 Sham vs. HS + PBS, #P<0.05 HS + PBS vs. HS + BMMNC.

2.3.10 Effect of BMMNC administration on the phosphorylation of Akt in the livers from rats that underwent haemorrhage and resuscitation

To gain a better insight into the potential mechanism(s) underlying the observed beneficial effects of BMMNCs, the effects of BMMNC administration on cell signalling pathways known to confer tissue protection or to inhibit inflammation were investigated in the liver. When compared with sham-operated rats, HS-rats treated with vehicle developed a significant decrease in the phosphorylation of Akt on Ser^{473} in the liver ($P<0.05$; Figure 2.9). Administration of BMMNCs in HS-rats attenuated the decline in the phosphorylation of Akt caused by haemorrhage and resuscitation in the liver ($P<0.05$; Figure 2.9).
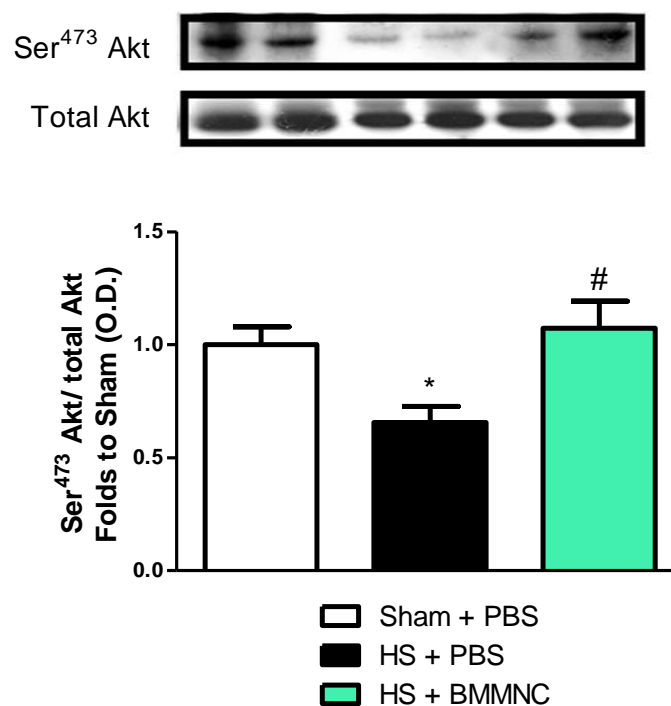
Figure 2.9

Figure 2.9: Phosphorylation of Akt on Ser^{473} in the liver; in rats subjected to sham-operation or HS and treated with either PBS (Sham + PBS, n=3, HS + PBS, n=3) or BMMNCs (HS + BMMNC, n=3). Data are expressed as mean ± SEM for n observations, *$P<0.05$ Sham vs. HS + PBS, #$P<0.05$ HS + PBS vs. HS + BMMNC.

2.3.11 Effect of BMMNC administration on the phosphorylation of GSK-3β in the livers from rats that underwent haemorrhage and resuscitation

When compared with sham-operated rats, HS-rats treated with vehicle developed a significant decrease in the phosphorylation of GSK-3β on Ser^9 in the liver (P<0.05; Figure 2.10). Administration of BMMNCs in HS-rats attenuated the decline in the phosphorylation of GSK-3β caused by haemorrhage and resuscitation in the liver (P<0.05; Figure 2.10).
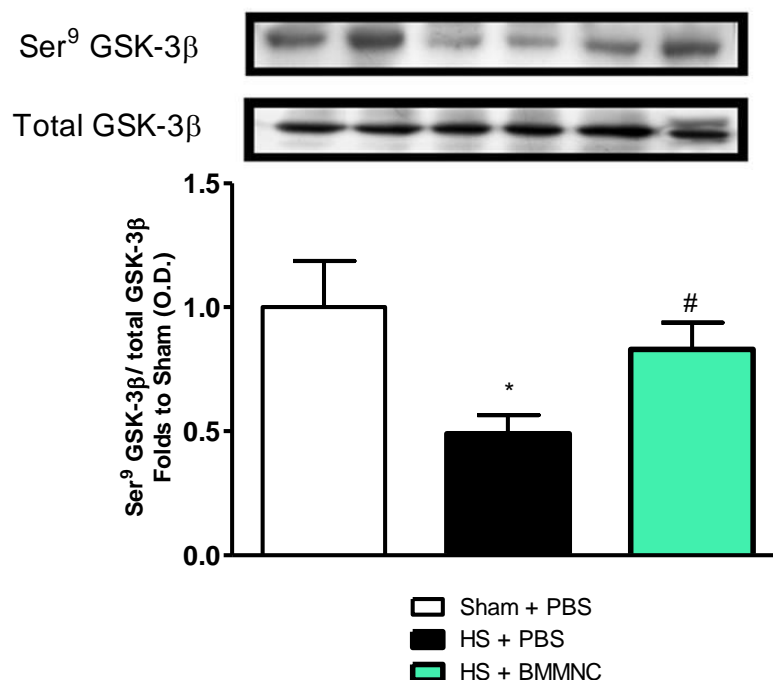
Figure 2.10

Figure 2.10: Phosphorylation of GSK-3β on Ser^9 in the liver; in rats subjected to sham-operation or HS and treated with either PBS (Sham + PBS, n=3, HS + PBS, n=3) or BMMNCs (HS + BMMNC, n=3). Data are expressed as mean ± SEM for n observations, *P<0.05 Sham vs. HS + PBS, #P<0.05 HS + PBS vs. HS + BMMNC.

2.3.12 Effect of BMMNC administration on the nuclear translocation of the p65 subunit of NF-κB in the livers from rats that underwent haemorrhage and resuscitation

When compared with sham-operated rats, HS-rats treated with vehicle developed significant increases in the nuclear translocation of the p65 NF-κB subunit in the liver, indicating the activation of NF-κB (P<0.05; Figure 2.11). Administration of BMMNCs in HS-rats resulted in a significant reduction in nuclear translocation of p65 and, hence, the activation of NF-κB in the liver (P<0.05; Figure 2.11).
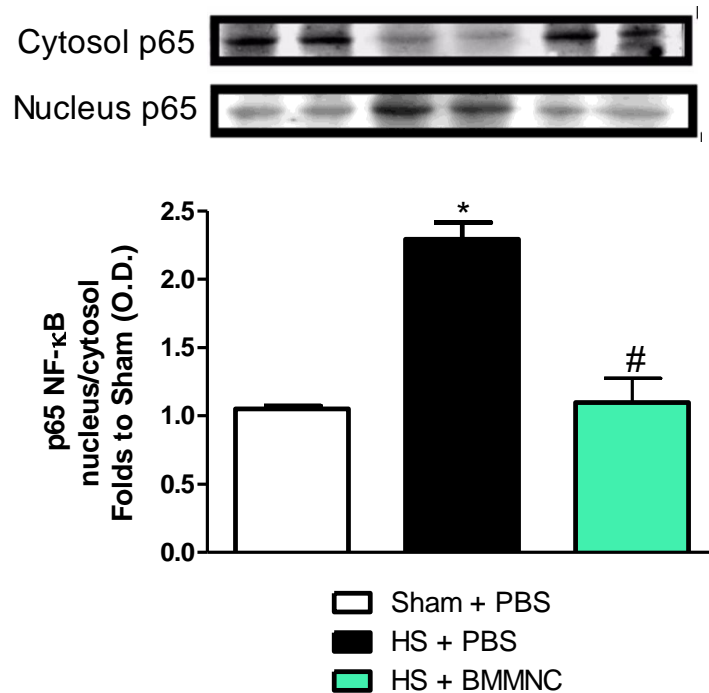
Figure 2.11

Figure 2.11: Nuclear translocation of the p65 subunit of NF-κB in the liver; in rats subjected to sham-operation or HS and treated with either PBS (Sham + PBS, n=3, HS + PBS, n=3) or BMMNCs (HS + BMMNC, n=3). Data are expressed as mean ± SEM for n observations, *P<0.05 Sham vs. HS + PBS, #P<0.05 HS + PBS vs. HS + BMMNC.

2.4 Discussion

This study was designed to investigate whether administration of BMMNCs upon resuscitation could limit the organ injury and dysfunction associated with severe haemorrhage and resuscitation. Acute treatment with BMMNCs [1 x 10^7 cells: c-kit$^+$ (7 ± 1%, n=10), CD34$^+$ (7 ± 1%, n=10), CD45$^+$ (54 ± 6%, n=10) and CD133$^+$ (15 ± 1%, n=10)] attenuated the renal dysfunction, hepatic injury, skeletal muscle injury and pulmonary injury and inflammation, caused by HS.

The Akt pro-survival pathway and its role in the pathophysiology of HS have been discussed in detail in Chapter 1. In line with other studies (Liu *et al.*, 2011a), this model of HS resulted in significant reduction in the phosphorylation of Akt in the liver. Reduction in the activation of this pathway would suggest that organs would become more susceptible to injury and inflammation (Hanlon *et al.*, 2005). This decline in phosphorylation was restored by the administration of BMMNCs to the level seen in sham-operated rats. A downstream phosphorylation target of Akt is GSK-3β; consistent with the decline in the phosphorylation of Akt, HS also caused a significant decline in the phosphorylation of GSK-3β. As GSK-3β is normally phosphorylated at rest in an inactive state, this excessive activation would drive both inflammation (Dugo *et al.*, 2005) and tissue injury (Nishihara *et al.*, 2006). Similar to the effects on Akt phosphorylation, administration of BMMNCs resulted in restoration of GSK-3β phosphorylation to the level seen in sham-operated rats. The transcription factor; NF-κB is known to be modulated by GSK-3β and consistent with the activation of GSK-3β, HS resulted in a significant increase in the activation of NF-κB (measured here as nuclear translocation of p65). This was also attenuated with the administration of BMMNCs. All of the above findings support the view that BMMNC treatment restores the activation of Akt resulting in inhibition of the activation of GSK-3β and NF-κB. This effect is similar to that already reported in hearts subjected to ischaemia-reperfusion (Lovell *et al.*, 2010) and suggests that BMMNCs may, in part, exert their beneficial effects via this pathway.

How the BMMNCs activate these pathways is still unclear. It is possible that the stem cells themselves interact with host cells that they come into contact with through the many markers expressed on their cell surfaces. Pati and colleagues demonstrated that when bone marrow-derived MSCs were co-cultured with pulmonary endothelial cells, there was a reduction in the endothelial permeability induced by VEGF-A treatment and in the endothelial-leukocyte aggregation in response to TNF-α treatment. This data suggests a direct interaction between cells. However, when pulmonary endothelial cells were treated with conditioned medium from cultured MSCs a similar effect was observed (Pati *et al.*, 2011). These findings support the view that stem cells release anti-inflammatory factors which act in a paracrine manner on nearby cells. The injection of conditioned medium in an animal model of limb ischaemia had a beneficial angiogenic effect indicating this as a possible, if not better treatment regimen (Di Santo *et al.*, 2009).

The present study reports a beneficial effect associated with BMMNC treatment, however, the practicality of such a treatment in the clinic may not be as easy. To date, there have been no clinical studies that investigate the efficacy of stem cells as a treatment for trauma. One study has been completed to assess the safety; children (5 – 14 years old) with traumatic brain injury were subjected to bone marrow harvest and were injected intravenously with 6 x 10^6 bone marrow-progenitor cells between 24 and 36 h post-injury (ClinicalTrials.gov.ID: NCT00254722). The results are still to be published and a secondary adult study is currently recruiting (ClinicalTrials.gov ID: NCT01575470) suggesting that the use of stem cells in an acute, emergency setting may be viable.

The main limitation of stem cell therapy is the need for huge numbers of cells to ensure that a sufficient number of viable cells reach the area of injury. This is not advantageous as it can result in accumulation of cells in various tissues. This was observed in the lungs of the BMMNC-treated rats in this study as there was a visible cell infiltrate in the lung; as a result the scoring for the immune cell infiltration, alveolar septal thickening and pulmonary congestion was higher than expected. However, when MPO activity and ICAM-1 expression was measured in these lungs it

appeared that BMMNC treatment actually reduces the inflammation in the lung despite causing a degree of pulmonary congestion. The accumulation of BMMNCs in the lung may not be deleterious as Badami and colleagues demonstrated that HS caused mobilisation of endogenous bone marrow cells to the lungs as the lungs are a major site of HS-induced injury (Badami *et al.*, 2007). Therefore, the presence of these cells in the lungs during the first 4 h following haemorrhage may be beneficial and over time these cells would be cleared from the lungs, however as this is an acute model this could not be investigated.

Another limitation is that BMMNCs consist of a mixture of different cell types including MSCs (Tsai *et al.*, 2011) and endothelial progenitor cells (EPCs) (Mao *et al.*, 2010), both of which have demonstrated efficacy in animal models of ischaemia-reperfusion injuries, therefore the attenuation in injury observed with BMMNC treatment may or may not be attributable to a particular cell type. Characterisation of the BMMNCs used here demonstrated only a small proportion of the cells as progenitors (approximately 7%) and the markers used are predominantly expressed on EPCs. Therefore, the mixture of mature cells present within BMMNCs may also be attributable to the protective effects observed here. Although, treatment with cell types that lack progenitors, for example fibroblasts, have not demonstrated any efficacy in a model of MI when compared to the cell types that do consist of progenitors such as BMMNCs (van der Bogt *et al.*, 2008).

The model of HS used here induced a significant degree of organ injury and dysfunction which was comparable to data previously published with the original model (Abdelrahman *et al.*, 2004a; McDonald *et al.*, 2001). There are some weaknesses with this model as it is a controlled form of haemorrhage and does not have an element of trauma, therefore it is not entirely clinically relevant (Hauser, 2005; Tsukamoto *et al.*, 2009). However, this model allows the direct effects of ischaemia and resuscitation on organ function to be investigated and the effect of interventions against this type of injury rather than the direct mechanical injury associated with trauma. Another factor is that anaesthesia has to be maintained for the duration of the experiment due to Home Office legislation, however this would only occur clinically if a

patient suffered haemorrhage during surgery. In the USA, anaesthesia is only used for the surgical procedure and haemorrhage and resuscitation is induced in conscious animals (Shimizu *et al.*, 2008; Shimizu *et al.*, 2006; Wang *et al.*, 1992); this would provide a much more realistic sympathetic response. Home Office legislation also states that this model cannot be used to study mortality; such studies would be ideal as the effect of interventions on survival is important but as a result this model is not sufficient to study the recovery processes that organs may undergo following an ischaemic insult. Unfortunately no model used to study HS is entirely perfect therefore it is more realistic to use the current model whereby there is a compromise between the clinical relevance and the scientific reproducibility (Calzia *et al.*, 2012; Hauser, 2005; Tsukamoto *et al.*, 2009).

In conclusion, the acute administration of BMMNCs upon resuscitation attenuated the multiple organ injury and dysfunction in rats subjected to severe haemorrhage and resuscitation. BMMNC treatment resulted in activation of the Akt pro-survival pathway resulting in inhibition of GSK-3β and NF-κB, which was similar to the effects observed with BMMNC treatment in a rat model of MI. The signalling events initiated by BMMNC treatment contributed to the beneficial effects observed in HS and may be a result of the action of paracrine mediators secreted by the BMMNCs. In light of the limitations of this study, it may be more therapeutically viable to induce mobilisation of endogenous bone marrow-derived stem cells using pharmaceutical agents.

CHAPTER 3

Pharmacological pre-conditioning with erythropoietin attenuates the organ injury and dysfunction induced by haemorrhagic shock

3.1   Introduction

Erythropoietin (EPO) is a 34-kilodalton glycoprotein secreted by the kidneys which controls erythropoiesis by regulating the proliferation of erythroid progenitor cells (EPCs) in the bone marrow. It acts via an anti-apoptotic mechanism to prevent death of erythroid progenitors allowing them to differentiate into circulating mature erythrocytes. EPO secretion is increased when oxygen delivery to the kidneys is low in order to increase the number of circulating erythrocytes, hence the oxygen carrying capacity. The endogenous mechanism by which EPO is able to stimulate erythroid progenitor cell differentiation has been exploited and recombinant human EPO (rhEPO) is used as a treatment in some cases of anaemia, for example in patients that require dialysis or cancer patients.

The expression of EPO is under the control of an oxygen sensitive transcription factor; HIF-1, this is an $\alpha\beta$ heterodimer and each domain has a typical helix-loop-helix motif. This is the area within the protein that interacts with a specific site in the DNA. Both the α and β subunits are synthesised constitutively, however the α-subunit is normally degraded in oxygenated cells, hence the levels of the α-subunit in normoxia are usually undetectable. The α-subunit is degraded by poly-ubiquitation which is an oxygen-dependent process, therefore when the cell is hypoxic, degradation is blocked and HIF-1α rapidly accumulates. The HIF-1α subunit interacts with DNA at a specific transcription factor binding site and increases expression of the genes encoding EPO. This factor can also influence expression of other genes such as VEGF for angiogenesis, tyrosine hydroxylase, iNOS to cause vasodilatation, glycolytic enzymes and glucose transporters to increase availability of glucose (Elliott *et al.*, 2005; Huang *et al.*, 1998).

EPO mediates its anti-apoptotic effects on erythroid progenitor cells by binding to its receptor (EPO-R) on the cell surface and activating a downstream signalling pathway to influence gene expression. EPO-R exists as a monomer on the cell membrane and belongs to the cytokine family of receptors; when bound by EPO the receptors dimerise to form a homodimer. Activation of this receptor initiates the stimulation of

the JAK/STAT (Janus Kinase/Signal Transducer and Activator of Transcription) pathway. These receptors do not possess any tyrosine kinase properties however each monomer is associated with a JAK molecule on the cytoplasmic domain, when the receptor is activated JAKs phosphorylate tyrosine residues on the receptor. This allows recruitment of STAT proteins from the cytoplasm, these STAT proteins bind to the phosphorylated residues via their SH2 domain and are consequently phosphorylated by the activated JAKs. The phosphorylated STAT proteins then leave the receptor and form dimers so that they are able to enter the nucleus and bind to specific sites on DNA as a transcription factor. An example is the STAT-5 homodimer which specifically increases the expression of Bcl-XL which blocks apoptosis by preventing the action of caspase-9 (Elliott *et al.*, 2005).

EPO receptors were previously believed to be expressed on haematopoietic cells only but EPO-R has now been found in many other tissues including the endothelium (Anagnostou *et al.*, 1990), the brain (Tan *et al.*, 1992), the kidneys (Westenfelder *et al.*, 1999) and the heart (Depping *et al.*, 2005). Sakanaka and colleagues demonstrated that apoptosis induced by brief periods of cerebral ischaemia in gerbils was exacerbated with administration of a soluble EPO-R (Sakanaka *et al.*, 1998). With this in mind EPO's potential as a tissue-protective agent has been investigated in many animal models of ischaemia-reperfusion type injury; examples include AKI (Sharples *et al.*, 2004), stroke (Siren *et al.*, 2001), HS (Abdelrahman *et al.*, 2004b) and MI (Brunner *et al.*, 2009).

In the majority of studies EPO is administered either on or prior to reperfusion, however there are also studies which have investigated the protective effects exerted by EPO pre-treatment in similar contexts. For example, daily pre-treatment with EPO for a period of three days has been demonstrated to protect against the injury induced by ischaemia-reperfusion injury in the hind-limb (Heeschen *et al.*, 2003) and kidney (Patel *et al.*, 2004).

Repetitive dosing with EPO could potentially activate numerous different targets. In particular, EPO is a known stimulus of EPC mobilisation; EPCs are mobilised in response

to hypoxia in order to promote angiogenesis but are also able to secrete paracrine proteins, which include anti-inflammatory cytokines such as IL-10 and growth factors including VEGF (Heeschen *et al.*, 2003). EPCs express numerous cell surface markers, which are used to characterise and identify these cells, examples include CD34, CD45, CD133, stem cell antigen-1 (sca-1) and vascular endothelial growth factor receptor-2 (VEGFR2). VEGFR2 is a type III tyrosine kinase receptor and its activation results in increased proliferation, migration, survival and permeability of vascular endothelial cells and EPCs. These actions are beneficial in hypoxia as they promote improved perfusion of ischaemic tissues [reviewed in (Holmes *et al.*, 2007)]. Additionally, the autologous transplantation of EPCs (1 x 10^7 cells/kg i.v. 24 h after the injection of endotoxin) in a pig model of combined HS and endotoxaemia resulted in improved capillary density in organs including the heart, liver, kidney, intestine and lung seven days after treatment. This was correlated with a decreased incidence of multiple organ failure in the EPC-treated pigs when compared to the vehicle treated pigs (Tianhang *et al.*, 2013). Taken together this suggests that EPO-induced mobilisation of EPCs could result in tissue-protective effects either indirectly through the secretion of paracrine proteins and/or by direct interaction of EPCs with host cells.

EPO can induce anti-inflammatory and anti-apoptotic effects in many different cell types either by interaction with its classical receptor; EPO-R, or the proposed molecular target responsible for EPO's tissue-protective effects; the β common receptor (βcR) (Brines *et al.*, 2004). The βcR is discussed in Chapter 4. There are multiple cell signalling pathways known to be activated by EPO, in particular the JAK/STAT, Akt and MAPK [reviewed in (Chateauvieux *et al.*, 2011)]. As discussed in Chapter 1 these pathways play a pivotal role in the pathophysiology of HS and the data reported in Chapter 2 demonstrates that modulation of the Akt pro-survival pathway can result in reduced tissue injury. Therefore, EPO could have the potential to be used as a therapy, which is administered prior to the onset of ischaemia.

However, many ischaemic insults occur spontaneously and cannot be predicted, for example trauma due to road traffic accident, and the preferred treatment time point is at the location of the accident or upon hospital admission, i.e. when resuscitation is

performed. Alternatively, in situations where the ischaemic insult is foreseeable such as prior to major surgery or in military conflicts in which blood loss is likely, EPO pre-treatment may be a viable therapeutic option. EPO is already clinically used as treatment prior to surgery in order to reduce the risk of post-operative anaemia and to reduce the requirement of peri-operative blood transfusions (Naran *et al.*, 2012; Yoo *et al.*, 2011). It is possible that in addition to the erythropoietic effects that EPO exerts in these situations EPO could protect tissues against the ischaemic damage associated with blood loss.

Taking into account the beneficial effects observed with EPO pre-treatment in the context of hind-limb ischaemia (Heeschen *et al.*, 2003) and AKI (Patel *et al.*, 2004), and the potential of EPO pre-treatment as a therapeutic option in cases of foreseeable haemorrhage. The aim of this study was to investigate the tissue-protective effect of EPO as a pharmacological pre-conditioning agent over a three day period prior to the induction of haemorrhage and resuscitation. Also to investigate the mechanism(s) by which EPO exerts this beneficial effect, by quantifying the degree of EPC mobilisation and the activation of the Akt pro-survival pathway.

3.2 Methods

This study was carried out on 32 male Wistar rats (Charles River Ltd, Margate, UK) weighing 283 ± 6 g. An additional six rats were used for the isolation of peripheral blood mononuclear cells. For details on surgical procedure, haemorrhage and resuscitation and quantification of organ injury and function, refer to sections 2.2.1, 2.2.2 and 2.2.5, respectively. The average volume of blood withdrawn during the haemorrhage period was 10.45 ± 0.19 ml (n=24, across all haemorrhaged groups).

3.2.1 Experimental groups

Rats were randomly allocated into the following groups:

1. Sham + PBS 3 day pre-treatment (n=4)
2. Sham + EPO 3 day pre-treatment (n=4)
3. HS + PBS 3 day pre-treatment (n=12)
4. HS + EPO 3 day pre-treatment (n=12)

Rats were treated with either 1 ml/kg PBS or 1000 IU/kg recombinant human EPO i.p. daily for 3 days and on day 4 were subjected to sham-operation (surgical procedure only) or HS. This dose of EPO and length of treatment was selected on the basis of the pre-treatment protocol used by Patel and colleagues (Patel *et al.*, 2004).

3.2.2 Measurement of haematocrit

The haematocrit was measured prior to haemorrhage in rats treated with either PBS or EPO for 3 days, approximately 100 μl blood was withdrawn 24 h after the final dose from the cannula inserted in the right carotid artery and analysed using ABL77 v1.41 analyser (Radiometer, Brønshøj, Denmark).

3.2.3 Measurement of the serum concentration of recombinant human EPO concentration

The serum concentration of injected rhEPO was measured prior to haemorrhage in rats treated with either PBS or EPO for 3 days. Approximately 500 µl blood was withdrawn 24 h after the final dose from the cannula inserted in the right carotid artery from which serum was obtained and analysed as per manufacturer's instructions using a Human EPO Immunoassay (R&D Systems Europe Ltd., Abingdon, UK).

3.2.4 Flow cytometry

The proportion of circulating EPCs was measured in rats treated with either PBS or EPO for 3 days. Approximately 3 ml blood was withdrawn 24 h after the final dose from the cannula inserted in the right carotid artery from which peripheral blood mononuclear cells (PBMNCs) were extracted using density gradient centrifugation at 400 g for 30 min at 4°C (Histopaque-1077©, Sigma-Aldrich, Dorset, UK). PBMNCs were re-suspended in 10 ml Hank's balanced salt solution (HBSS) and centrifuged at 250 g for 10 min at 4°C, the resultant supernatant was aspirated and the pellet re-suspended in 100 µl HBSS. Three million cells were incubated with 21 µl of mouse anti-CD34 antibody conjugated with fluorescein (FITC) and 24 µl of mouse anti-flk-1 (foetal liver kinase-1; the murine analogue of VEGFR2) antibody in 80 µl of HBSS or with HBSS alone for 30 min at room temperature. Excess antibody was removed by washing with 1 ml HBSS and centrifuging at 330 g for 5 min at room temperature and the supernatant was aspirated. The cells were re-suspended in 5 µl of goat anti-mouse IgG-phycoerythrin (PE) and incubated for a further 30 min at room temperature. The wash step was then repeated and cells were re-suspended in 500 µl of HBSS. Analysis was performed using Fortessa analyser (Beckman Coulter, High Wycombe, UK) and data analysed using FlowJo v7.6.5 (Ashland, Oregon, USA).

3.2.5 Western blot analysis

For details refer to section 2.2.8, in addition to the measurement of Akt and GSK-β phosphorylation and p65 nuclear translocation, eNOS phosphorylation was also measured using following primary antibodies; rabbit anti-total eNOS dilution 1:200 and goat anti-phospho eNOS Ser[1177] dilution 1:200.

3.2.6 Materials

For details refer to section 2.2.9, additionally antibodies for flow cytometry were purchased from Insight Biotechnology Ltd. (Middlesex, UK). NeoRecormin (rhEPO) was manufactured by Roche (Welwyn Garden City, U.K).

3.2.7 Statistical Analysis

For details refer to section 2.2.10, additionally data with comparison of only 2 groups was assessed by unpaired, two-tailed t-test.

3.3   Results

3.3.1   Effect of EPO pre-treatment on the circulatory failure caused by haemorrhagic shock

Baseline MAP values were similar amongst all four groups. When compared with sham-operated rats, haemorrhage caused a significant decline in MAP, which was improved by resuscitation, however MAP was still significantly different from that of sham-operated rats. Pre-treatment with EPO had no significant effect on the decline in MAP; although there was a slight increase in MAP during the resuscitation phase, when compared with PBS pre-treated rats (P>0.05, Figure 3.1). EPO pre-treatment also had no significant effect on the MAP of sham-operated rats (P>0.05, Figure 3.1).
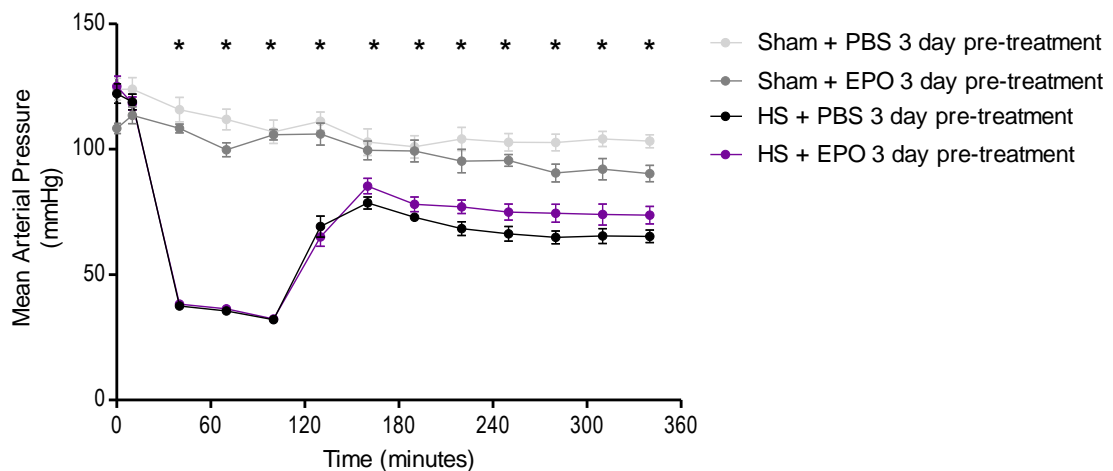
Figure 3.1

Figure 3.1: Alterations in MAP; in rats pre-treated daily over three days and subjected to sham-operation on the fourth day (Sham + PBS 3 day pre-treatment, n=4, Sham + EPO 3 day pre-treatment, n=4) or HS on the fourth day (HS + PBS 3 day pre-treatment, n=12; HS + EPO 3 day pre-treatment, n=12). Data are expressed as mean ± SEM for n observations, *P<0.05 Sham vs. HS + PBS 3 day pre-treatment.

3.3.2 Effect of EPO pre-treatment on the renal dysfunction induced by haemorrhagic shock

When compared with sham-operated rats pre-treated with PBS, HS-rats pre-treated with PBS developed significant increases in serum urea (P<0.05; Figure 3.2A) and creatinine (P<0.05; Figure 3.2B), indicating the development of renal dysfunction. Pre-treatment of HS-rats with EPO significantly attenuated the rise in serum creatinine (P<0.05; Figure 3.2B) but had no effect on the rise in serum urea (P>0.05, Figure 3.2A). EPO pre-treatment in sham-operated rats had no effect on either serum urea or creatinine levels (P>0.05; Figure 3.2).
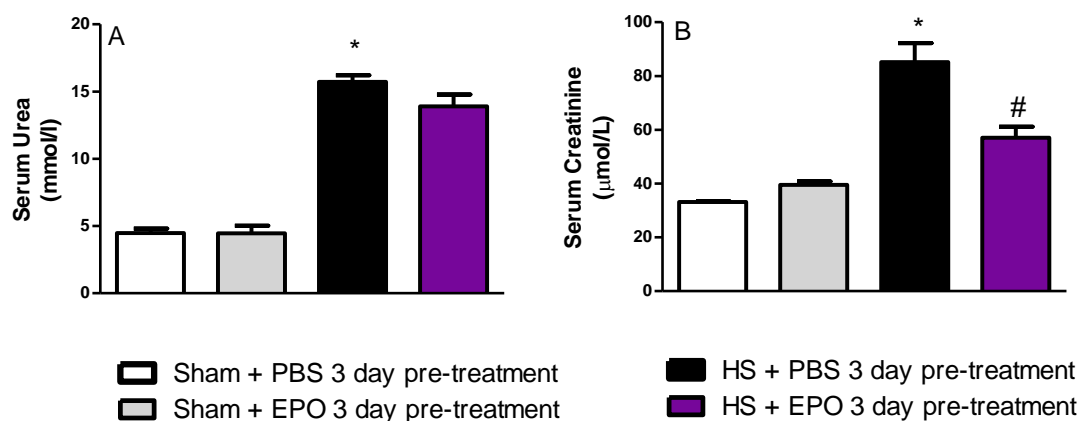
Figure 3.2

Figure 3.2: Alterations in serum levels of (A) urea and (B) creatinine; in rats pre-treated daily over three days and subjected to sham-operation on the fourth day (Sham + PBS 3 day pre-treatment, n=4, Sham + EPO 3 day pre-treatment, n=4) or HS on the fourth day (HS + PBS 3 day pre-treatment, n=12; HS + EPO 3 day pre-treatment, n=12). Data are expressed as mean ± SEM for n observations, *P<0.05 Sham vs. HS + PBS 3 day pre-treatment, #P<0.05, HS + PBS 3 day pre-treatment vs. HS + EPO 3 day pre-treatment.

3.3.3 Effect of EPO pre-treatment on the hepatic injury induced by haemorrhagic shock

When compared with sham-operated rats pre-treated with PBS, HS-rats pre-treated with PBS developed significant increases in serum AST (P<0.05; Figure 3.3A) and ALT (P<0.05; Figure 3.3B), indicating the development of hepatic injury. Pre-treatment of HS-rats with EPO significantly attenuated the rises in serum AST (P<0.05; Figure 3.3A) and ALT (P<0.05; Figure 3.3B). EPO pre-treatment in sham-operated rats had no effect on either serum AST or ALT levels (P>0.05; Figure 3.3).

Figure 3.3

Figure 3.3: Alterations in serum levels of (A) AST and (B) ALT; in rats pre-treated daily over three days and subjected to sham-operation on the fourth day (Sham + PBS 3 day pre-treatment, n=4, Sham + EPO 3 day pre-treatment, n=4) or HS on the fourth day (HS + PBS 3 day pre-treatment, n=12; HS + EPO 3 day pre-treatment, n=12). Data are expressed as mean ± SEM for n observations, *P<0.05 Sham vs. HS + PBS 3 day pre-treatment, #P<0.05, HS + PBS 3 day pre-treatment vs. HS + EPO 3 day pre-treatment.

3.3.4 Effect of EPO pre-treatment on the skeletal muscle injury induced by haemorrhagic shock

When compared with sham-operated rats pre-treated with PBS, HS-rats pre-treated with PBS developed a significant increase in serum CK ($P<0.05$; Figure 3.4), which may indicate the development of skeletal muscle injury. Pre-treatment of HS-rats with EPO significantly attenuated the rise in serum CK ($P<0.05$; Figure 3.4). EPO pre-treatment in sham-operated rats had no effect on serum CK levels ($P>0.05$; Figure 3.4).

Figure 3.4

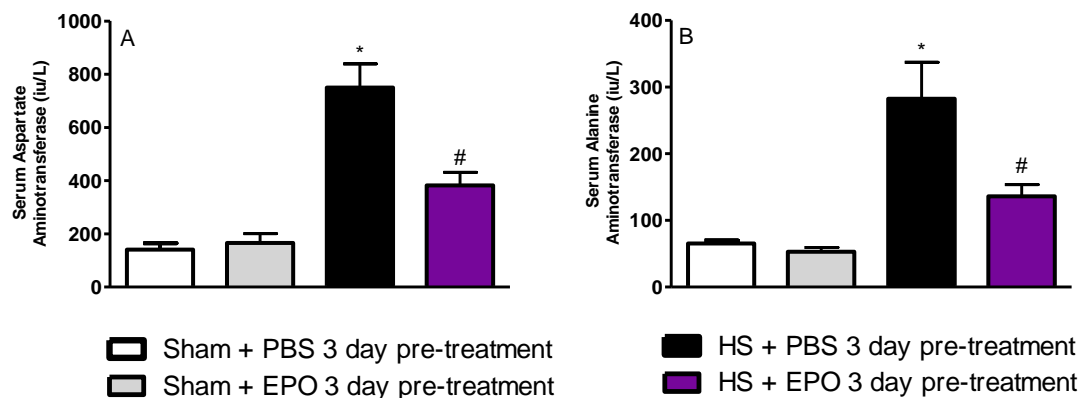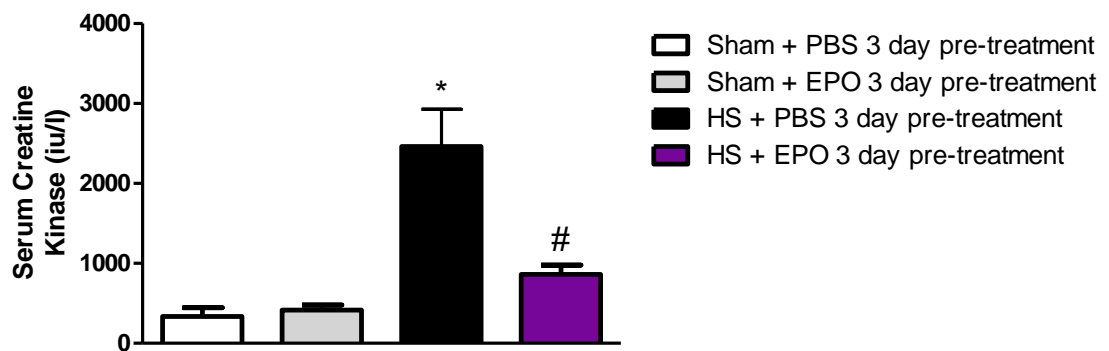**Figure 3.4:** Alterations in serum levels of CK; in rats pre-treated daily over three days and subjected to sham-operation on the fourth day (Sham + PBS 3 day pre-treatment, n=4, Sham + EPO 3 day pre-treatment, n=4) or HS on the fourth day (HS + PBS 3 day pre-treatment, n=12; HS + EPO 3 day pre-treatment, n=12). Data are expressed as mean ± SEM for n observations, *$P<0.05$ Sham vs. HS + PBS 3 day pre-treatment, #$P<0.05$, HS + PBS 3 day pre-treatment vs. HS + EPO 3 day pre-treatment.

3.3.5   Effect of EPO pre-treatment on the haematocrit and serum EPO concentration

When compared with PBS pre-treated rats, rats pre-treated with EPO had a slightly elevated haematocrit (P>0.05; Table 1) and a significantly higher serum concentration of rhEPO (P<0.0001; Table 1); however this concentration is less than 0.1 IU/ml.

Table 3.1

	PBS pre-treated rats	EPO pre-treated rats	n	P value
Haematocrit (%)	38.0 ± 1.8	44.5 ± 2.0	6	> 0.05
Serum rhEPO concentration (IU/ml)	0 ± 0	0.0847 ± 0.0231	8	< 0.0001

Table 3.1: Effect of daily EPO pre-treatment on haematocrit and serum rhEPO concentration; in rats treated for three days with either PBS or EPO, measurements were taken 24 h after the final dose. Data are expressed as mean ± SEM for n observations.

3.3.6 Effect of EPO concentration on the percentage of circulating CD34$^+$/flk-1$^+$ cells

When compared with PBS pre-treated rats, pre-treatment with EPO significantly increased the percentage of CD34$^+$/flk-1$^+$ cells present within the mononuclear cell component of the blood (P<0.05; Figure 3.5B). These data are also illustrated in the form of a representative histogram (Figure 3.5A).

Figure 3.5
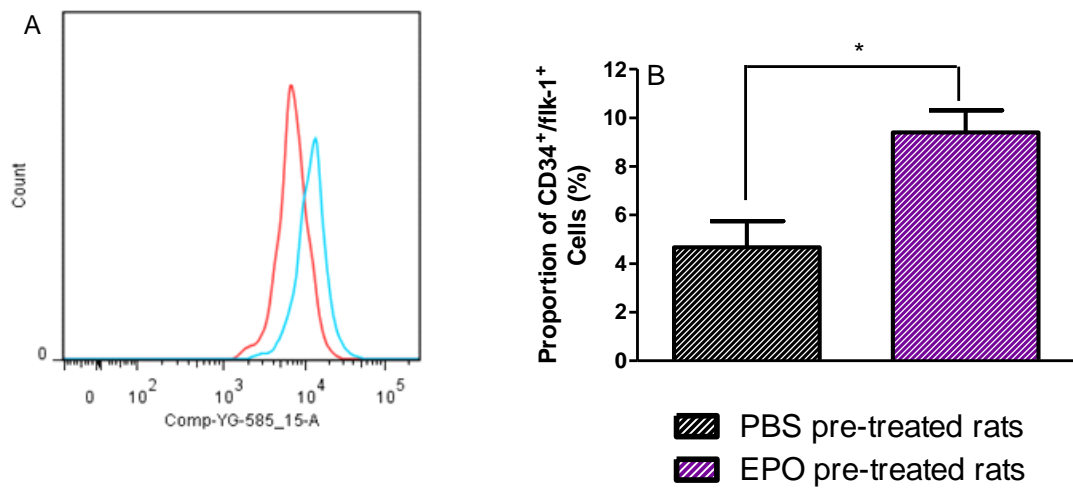
Figure 3.5: Effect of daily EPO pre-treatment on the percentage of circulating CD34$^+$/flk-1$^+$ cells (B); in rats treated for three days with either PBS (n=3) or EPO (n=3) and sacrificed 24 h after the last injection. Data are expressed as mean ± SEM for n observations, *P<0.05 when compared to PBS pre-treated rats. Representative histogram (A), the red line represents PBS pre-treated rats and the blue line represents EPO pre-treated rats.

3.3.7 Effect of EPO pre-treatment on the phosphorylation of Akt in the livers of rats subjected to haemorrhagic shock

EPO is known to activate several cell signalling pathways as a result of its interaction with the EPO-R. In particular, EPO can act on the Akt pro-survival pathway which may confer tissue-protective and anti-inflammatory effects; therefore the effect of EPO pre-treatment on the activation of Akt, and of proteins that interact with Akt such as GSK-3β (Cross *et al.*, 1995) and eNOS (Dimmeler *et al.*, 1999) was investigated.

When compared with sham-operated rats pre-treated with PBS, HS-rats pre-treated with PBS developed a significant reduction in the phosphorylation of Akt on Ser[473] in the liver (P<0.05; Figure 3.6). Pre-treatment with EPO attenuated the decline in phosphorylation of Akt induced by haemorrhage and resuscitation (P<0.05; Figure 3.6). EPO pre-treatment in sham-operated rats had no significant effect on the phosphorylation of Akt on Ser[473] (P>0.05; Figure 3.6).

Figure 3.6

Figure 3.6: Phosphorylation of Akt on Ser[473] in the liver; in rats pre-treated daily over three days and subjected to sham-operation on the fourth day (Sham + PBS 3 day pre-treatment, n=3, Sham + EPO 3 day pre-treatment, n=3) or HS on the fourth day (HS + PBS 3 day pre-treatment, n=3; HS + EPO 3 day pre-treatment, n=3). Data are expressed as mean ± SEM for n observations, *P<0.05 Sham vs. HS + PBS 3 day pre-treatment, #P<0.05, HS + PBS 3 day pre-treatment vs. HS + EPO 3 day pre-treatment.

3.3.8 Effect of EPO pre-treatment on the phosphorylation of GSK-3β in the livers of rats subjected to haemorrhagic shock

When compared with sham-operated rats pre-treated with PBS, HS-rats pre-treated with PBS developed a significant reduction in the phosphorylation of GSK-3β on Ser^9 in the liver (P<0.05; Figure 3.7). Pre-treatment with EPO attenuated the decline in phosphorylation of GSK-3β induced by haemorrhage and resuscitation, although this was not statistically significant (P>0.05; Figure 3.7). EPO pre-treatment in sham-operated rats had no significant effect on the phosphorylation of GSK-3β on Ser^9 (P>0.05; Figure 3.7).
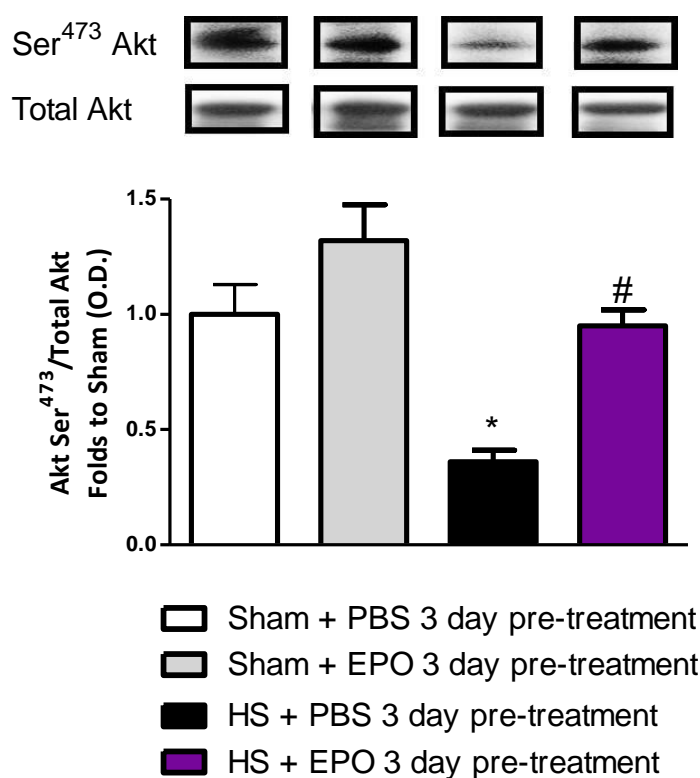
Figure 3.7

Figure 3.7: Phosphorylation of GSK-3β on Ser^9 in the liver; in rats pre-treated daily over three days and subjected to sham-operation on the fourth day (Sham + PBS 3 day pre-treatment, n=3, Sham + EPO 3 day pre-treatment, n=3) or HS on the fourth day (HS + PBS 3 day pre-treatment, n=3; HS + EPO 3 day pre-treatment, n=3). Data are expressed as mean ± SEM for n observations, *P<0.05 Sham vs. HS + PBS 3 day pre-treatment, #P<0.05, HS + PBS 3 day pre-treatment vs. HS + EPO 3 day pre-treatment.

3.3.9 Effect of EPO pre-treatment on the phosphorylation of eNOS in the livers of rats subjected to haemorrhagic shock

When compared with sham-operated rats pre-treated with PBS, HS-rats pre-treated with PBS developed a significant reduction in the phosphorylation of eNOS on Ser^{1177} in the liver (P<0.05; Figure 3.8). Pre-treatment with EPO attenuated the decline in phosphorylation of eNOS induced by haemorrhage and resuscitation (P<0.05; Figure 3.8). EPO pre-treatment in sham-operated rats had no significant effect on the phosphorylation of eNOS on Ser^{1177} (P>0.05; Figure 3.7).
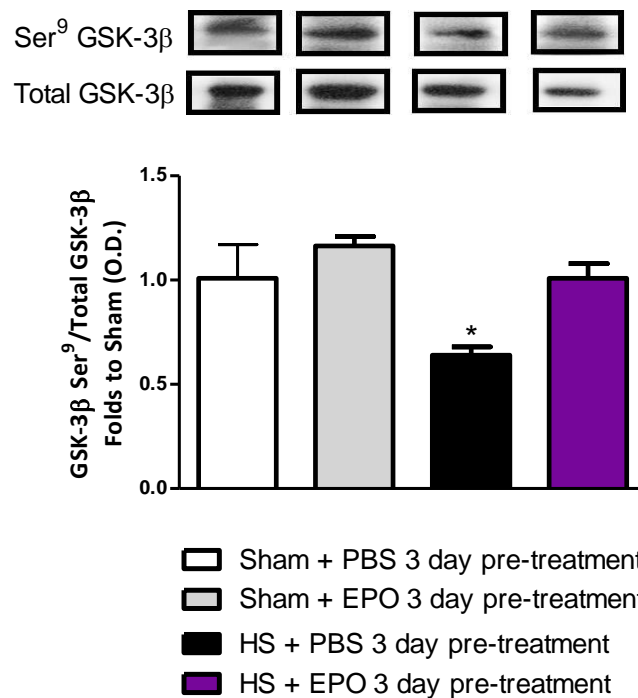
Figure 3.8
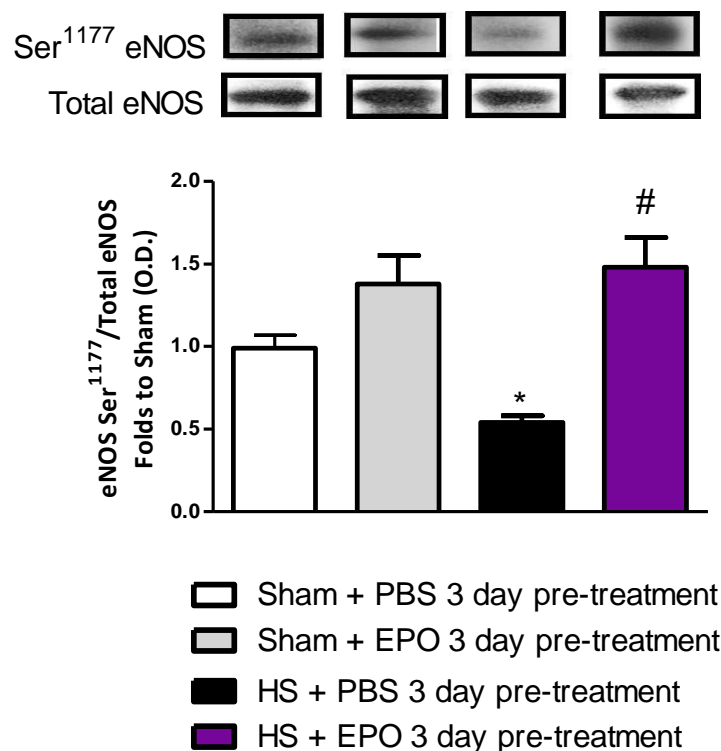
Figure 3.8: Phosphorylation of eNOS on Ser^{1177} in the liver; in rats pre-treated daily over three days and subjected to sham-operation on the fourth day (Sham + PBS 3 day pre-treatment, n=3, Sham + EPO 3 day pre-treatment, n=3) or HS on the fourth day (HS + PBS 3 day pre-treatment, n=3; HS + EPO 3 day pre-treatment, n=3). Data represent mean ± SEM for n observations, *P<0.05 Sham vs. HS + PBS 3 day pre-treatment, #P<0.05, HS + PBS 3 day pre-treatment vs. HS + EPO 3 day pre-treatment.

3.3.10 Effect of EPO pre-treatment on the phosphorylation of the nuclear translocation of p65 in the livers of rats subjected to haemorrhagic shock

When compared with sham-operated rats pre-treated with PBS, HS-rats pre-treated with PBS developed a significant increase in the nuclear translocation of the p65 NF-κB subunit in the liver, indicating the activation of NF-κB ($P<0.05$; Figure 3.9). Pre-treatment with EPO significantly attenuated the increase in NF-κB activation induced by haemorrhage and resuscitation ($P<0.05$; Figure 3.9), and no significant effect in sham-operated rats ($P>0.05$; Figure 3.9).

Figure 3.9
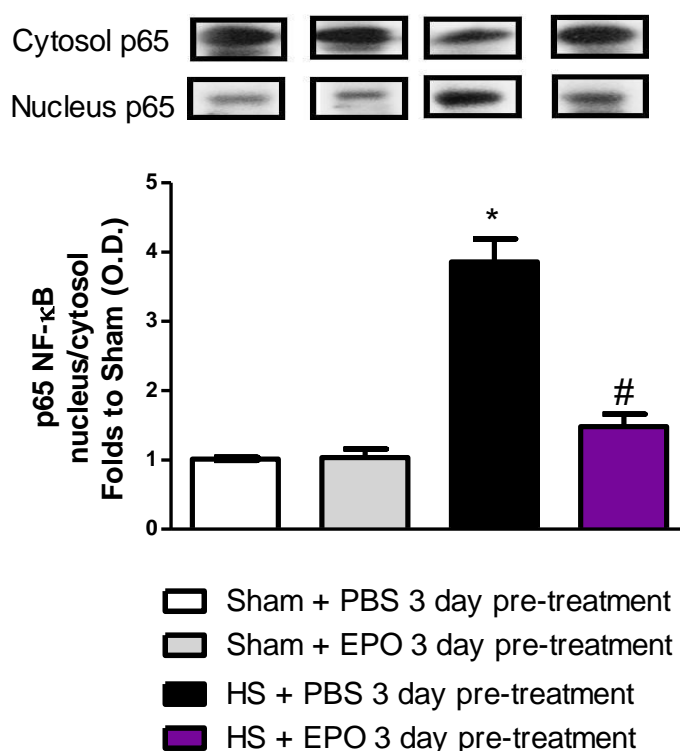
Figure 3.9: Nuclear translocation of the p65 subunit of NF-κB in the liver; in rats pre-treated daily over three days and subjected to sham-operation on the fourth day (Sham + PBS 3 day pre-treatment, n=3, Sham + EPO 3 day pre-treatment, n=3) or HS on the fourth day (HS + PBS 3 day pre-treatment, n=3; HS + EPO 3 day pre-treatment, n=3). Data represent mean ± SEM for n observations, *$P<0.05$ Sham vs. HS + PBS 3 day pre-treatment, #$P<0.05$, HS + PBS 3 day pre-treatment vs. HS + EPO 3 day pre-treatment.

3.4 Discussion

This study was designed to investigate the tissue-protective action of EPO when administered as a daily treatment prior to the induction of haemorrhage. EPO pre-treatment (1000 IU/kg/day i.p for 3 days) significantly attenuated the renal dysfunction and the hepatic and skeletal muscle injury induced by haemorrhage and resuscitation. This is the first time this particular pharmacological 'pre-conditioning' approach has been used as a therapeutic strategy in a pre-clinical model of HS.

Abdelrahman and colleagues have previously reported that administration of EPO prior to resuscitation (after severe haemorrhage) reduces organ injury and dysfunction. Specifically, administration of EPO (300 IU/kg i.v. 5 min prior to resuscitation) reduced the renal dysfunction and hepatic injury caused by haemorrhage and resuscitation in the rat. The beneficial effect of EPO was associated with prevention of the HS-induced increase in caspase activity in the kidney (Abdelrahman *et al.*, 2004b); in particular, caspases – 3, 8 and 9, which are known to play a pivotal role in the process of apoptosis (Du *et al.*, 2000). The degree of protection exerted by EPO in the previous study is comparable to the degree of protection reported here, indicating that EPO is still able to induce a protective effect when administered over a long period prior to haemorrhage. This suggests that during the three day pre-treatment period EPO is able to increase the resistance of tissues to withstand a later insult.

How does EPO exert this tissue-protective (priming) effect? It could be argued that continuous treatment with EPO could result in an increase in basal blood pressure, which could improve the perfusion of organs. It is reported that a third of patients receiving long term EPO treatment develop hypertension (Rossi *et al.*, 2011), however, in this study there was no difference in the baseline MAP values of PBS and EPO pre-treated rats suggesting that EPO pre-treatment did not have a hypertensive effect. Additionally, EPO may improve the perfusion of tissues by promoting erythropoiesis and increasing the oxygen carrying capability of the blood. Long term EPO treatment is reported to increase haematocrit in both experimental animals (Brunner *et al.*, 2009) and in patients (Corwin *et al.*, 2007); however, this is often associated with an

increased incidence of thrombotic events (Corwin *et al.*, 2007). Here, EPO pre-treatment did cause a slight increase in haematocrit although the observed increase in haematocrit was not statistically significant.

As the half-life of EPO is approximately 10 h in the rat when injected intraperitoneally (Gorio *et al.*, 2005), one could argue that EPO has accumulated in the plasma so that significant plasma levels of EPO are still present when the haemorrhage and injury occurs. However, this was not the case as – even with repetitive injections of 1000 IU/kg of EPO per day – the serum concentration of recombinant human EPO (i.e. injected EPO) prior to the onset of haemorrhage was less than 0.1 IU/ml. Although this value is within the plasma concentration range of EPO that is thought to afford tissue-protection (Brines *et al.*, 2008a), it is likely that this serum concentration of EPO would be insufficient to induce a beneficial effect in this model of HS. In previous studies investigating the therapeutic effects of EPO in the context of HS, the lowest dose used is 300 IU/kg (Abdelrahman *et al.*, 2004b; Wu *et al.*, 2010); which would result in a plasma concentration of approximately 5 IU/ml when injected intravenously. This is 50 fold higher than the serum concentration measured following three days of treatment with EPO. Taking these studies into account one would assume that due to the complex nature of ischaemia-reperfusion injury and inflammation that occurs in animal models of HS, serum concentrations lower than 5 IU/ml would be inadequate to exert a tissue-protective effect. As higher concentrations of EPO are needed to exert a tissue-protective effect this serum concentration of EPO may not account for the observed beneficial effects (Brines *et al.*, 2008a).

There is good evidence that EPO acts via the classical EPO-R present on EPCs to promote their mobilisation from the bone marrow in response to hypoxia in order to induce angiogenesis. Additionally, EPCs are known to secrete paracrine proteins which may contribute to the protective effects observed in this study (Heeschen *et al.*, 2003). Repetitive dosing with EPO has been reported to increase the proportion of circulating EPCs (defined using combinations of the following markers: CD34, CD45, CD133, sca-1 and flk-1) in experimental animals (Heeschen *et al.*, 2003) and in patients (Bahlmann *et al.*, 2004). In this study, EPO pre-treatment over a period of three days significantly

increased the proportion of circulating $CD34^+/flk-1^+$ cells. This is approximately a 100% increase which is similar to the magnitude of the effect described by Heeschen and colleagues in mice treated with EPO (1000 IU/kg/day for 3 days prior to cell analysis); although the proportion of positive staining cells appears to be lower in PBS pre-treated mice than in rats (Heeschen *et al.*, 2003). The therapeutic potential of EPCs has been assessed in patients with ST-elevated MI; patients received an autologous cell infusion directly into the left anterior descending coronary artery five days following percutaneous coronary intervention. An average of 4.92 x 10^9 peripheral blood mononuclear cells was injected, of which 0.05% were positive for CD34 and VEGFR2. Cellular infusion resulted in improvement of left ventricular contractile function during the six month follow up period presumably as a result of increased angiogenesis in the infarcted heart (Tatsumi *et al.*, 2007). Studies using cellular infusions are difficult to conduct as large numbers of cells are required in order to ensure sufficient delivery of cells to the diseased tissue. Therefore, the potential of agents such as EPO (Yip *et al.*, 2011) or granulocyte-macrophage colony-stimulating factor (GM-CSF) (Subramaniyam *et al.*, 2009) to promote mobilisation of cells from the bone marrow may be a more attractive option.

EPO is known to enhance the survival of erythroid progenitor cells (by reducing their apoptosis) by interacting with the classical EPO-R to activate several intracellular signalling pathways, in particular JAK/STAT, PI3K/Akt and MAPK pathways [reviewed in (Chateauvieux *et al.*, 2011)]. As the EPO-R is expressed in many non-haematopoietic tissues it is hypothesised that EPO can activate these pathways in other cells in order to induce a similar anti-apoptotic effect.

In this study, HS caused a decline in Akt phosphorylation, hence a decline in activation, which was significantly attenuated by pre-treatment with EPO. Ruscher and colleagues demonstrated a similar effect in the brain; where pre-treatment with EPO up to 48 h prior to an ischaemic insult protected rat cortical neurons from apoptosis by an Akt-dependent mechanism (Ruscher *et al.*, 2002). HS also resulted in a corresponding decline in GSK-3β phosphorylation; which was also significantly attenuated by EPO pre-treatment. This effect has also been confirmed *in vitro* where EPO-induced

phosphorylation of Ser[9] on GSK-3β was attributable to its anti-apoptotic effect in cardiomyocytes (Ohori *et al.*, 2008). In line with the activation of GSK-3β, HS caused a significant rise in the nuclear translocation of the p65 subunit of NF-κB, which was significantly attenuated by pre-treatment with EPO. A similar effect was observed when EPO was administered 24 h prior to induction of MI; here EPO pre-treatment resulted in a significant reduction in infarct size and a decrease in NF-κB activation in the left ventricle (Xu *et al.*, 2005).

Another downstream effect of Akt activation is the phosphorylation and activation of eNOS, which results in an increased production of NO in the endothelium. NO can induce local vasodilation, inhibit adhesion of platelets and neutrophils, and regulate angiogenesis (Luque Contreras *et al.*, 2006). There is good evidence that agents which release NO or enhance the formation of endogenous NO attenuate organ injury and dysfunction induced by HS (Anaya-Prado *et al.*, 2003). Here, the phosphorylation of eNOS at the Akt phosphorylation site; Ser[1177], was significantly reduced by HS, this decline was attenuated by EPO pre-treatment. Su and colleagues demonstrated that EPO is able to activate eNOS in endothelial cells by interacting with the βcR and causing Akt-dependent phosphorylation of eNOS (Su *et al.*, 2011).

Pre-treatment of sham-operated rats with EPO did not cause a significant change in the expression of any of the aforementioned proteins, which may have been expected over the 3 day treatment period. However, there was a slight non-significant increase in the phosphorylation of Akt, GSK-3β and eNOS which may have been sufficient to have a physiological effect in the liver and so contribute to the ability of the EPO pre-treated rats to be more resistant to injury.

The expression of flk-1 on the cell surface of EPCs is of particular interest as activation of this receptor is known to lead to interaction with multiple intracellular signalling pathways, which are similar to those activated by EPO. For example, activation of flk-1 results in phosphorylation and activation of Akt, which promotes cell survival by inhibiting the effects of pro-apoptotic proteins (Gerber *et al.*, 1998a; Gerber *et al.*,

1998b). Additionally, flk-1-mediated phosphorylation of Akt results in the subsequent phosphorylation and activation of eNOS (Dimmeler *et al.*, 1999; Gerber *et al.*, 1998b) which is very similar to the proposed mechanism of anti-apoptotic action of EPO in endothelial cells (Su *et al.*, 2011). Flk-1 is also able to interact with ERK1/2 to promote cell proliferation (Takahashi *et al.*, 2001). It is possible that in combination with EPO's ability to activate pro-survival signalling pathways, the mobilisation of flk-1$^+$ cells is able to contribute to EPO's tissue-protective effects by potentiating the action of these pathways.

The correlation between EPO-induced EPC mobilisation and EPO's beneficial effects is well documented in the literature. Both Bahlmann *et al*. and Heeschen *et al.* have demonstrated EPO's ability to mobilise EPCs in animal models (Bahlmann *et al.*, 2003; Heeschen *et al.*, 2003). This effect has also been observed in stroke patients where EPO treatment resulted in an increased proportion of circulating EPCs which was associated with a significant and independent reduction in the incidence of a major adverse neurological event within 90 days of the initial insult (Yip *et al.*, 2011). Ferrario and colleagues also demonstrated a similar correlation between endogenous EPO production and EPC mobilisation in patients following acute MI (Ferrario *et al.*, 2007). Although the direct inhibition of EPO-induced mobilisation of EPCs has not been investigated it is possible to inhibit EPC mobilisation in animals using pharmacological agents, for example with vascular endothelial growth factor inhibitor (Tian *et al.*, 2009) or dopamine (Chakroborty *et al.*, 2008). Prevention of the EPC mobilisation afforded by EPO by these interventions may allow the differentiation between the EPC-dependent and EPC-independent effects of EPO to be identified. Even if it were possible to block the mobilisation of EPCs afforded by EPO, it is still possible that EPO causes tissue-protection by directly activating pro-survival signalling pathways.

In conclusion, daily pre-treatment with EPO prior to haemorrhage attenuates the early organ injury and dysfunction in rats subjected to severe HS. Pre-treatment with EPO restored phosphorylation and, hence, activation of Akt, which in turn resulted in inhibition of GSK-3β (secondary to phosphorylation on Ser^9) and inhibition of the activation of NF-κB. Activation of Akt also resulted in phosphorylation and activation of

eNOS. Due to the lack of effect on the basal blood pressure, haematocrit and serum EPO concentration, the proposed mechanism behind the protective effects exerted by EPO in this study may involve the mobilisation of $CD34^+/flk-1^+$ cells from the bone marrow, resulting in activation of the Akt pro-survival pathway. The pre-treatment regimen used in this study may provide new therapeutic options for clinical situations where blood loss is foreseeable, for example, prior to major surgery or in military conflicts. Pre-treatment with EPO also offers an alternative to cell-based therapies as it can induce the mobilisation of EPCs, therefore eliminating the problems with stem cell treatments i.e. rejection of injected allogeneic cells and the large number of cells required.

CHAPTER 4

A non-erythropoietic peptide that mimics the 3D structure of
erythropoietin attenuates the organ injury and dysfunction
induced by haemorrhagic shock

4.1   Introduction

As discussed in Chapter 3, EPO has been demonstrated to exert potent tissue-protective effects in the context of ischaemia-reperfusion injury in many animal models of disease.

The positive results from pre-clinical studies encouraged the development of many clinical trials, the findings of which have been varied. For example, in the Gottingen EPO Stroke Study, stroke patients treated with EPO (100,000 IU over 3 days) showed an improvement in neurological activity and infarct size one month following stroke. The authors also reported that haematocrit, haemoglobin and red blood cell counts remained normal despite the high dose of EPO used (Ehrenreich *et al.*, 2002). However, the German Multicentre EPO Stroke Trial, a larger scale follow-on trial from the Gottingen study, demonstrated a high mortality associated with EPO treatment; in particular when it was combined with recombinant tissue plasminogen activator (tPA; used in stroke patients to promote the degradation of the blood clot) (Ehrenreich *et al.*, 2009).

EPO's efficacy has also been investigated clinically in the context of critical care. Trauma and surgical patients often require multiple blood transfusions, however this is associated with a poor prognosis. Therefore, the use of EPO was hypothesised to minimise the number of blood transfusions patients received in order to improve outcome. A prospective, randomised, placebo-controlled trial that enrolled 1,460 critically ill patients reported that treatment with EPO (40,000 IU per week for 3 weeks) resulted in reduced 28 day mortality in a subset of trauma patients without affecting the number of red blood cell transfusions required. However, there was also an increased incidence of thrombotic events despite improving mortality in these patients (Corwin *et al.*, 2007).

The safety concerns associated with EPO treatment in patients have been linked to the EPO-induced stimulation of erythropoiesis via the EPO-R which results in increased red blood cell levels and so contributes to an increased risk of thrombosis. As a result, a

detailed investigation into the interaction of EPO with its receptor was performed and it was found that EPO interacts with EPO-R at two distinct points (labelled on Figure 4.1). The modification of EPO's structure to prevent its association with EPO-R meant that EPO was unable to stimulate erythropoiesis via EPO-R but surprisingly these modified EPOs still possessed tissue-protective actions (Leist *et al.*, 2004). The data from this study suggests that the tissue-protective effects exerted by EPO are a result of EPO's action on a different receptor; a so-called tissue-protective receptor.

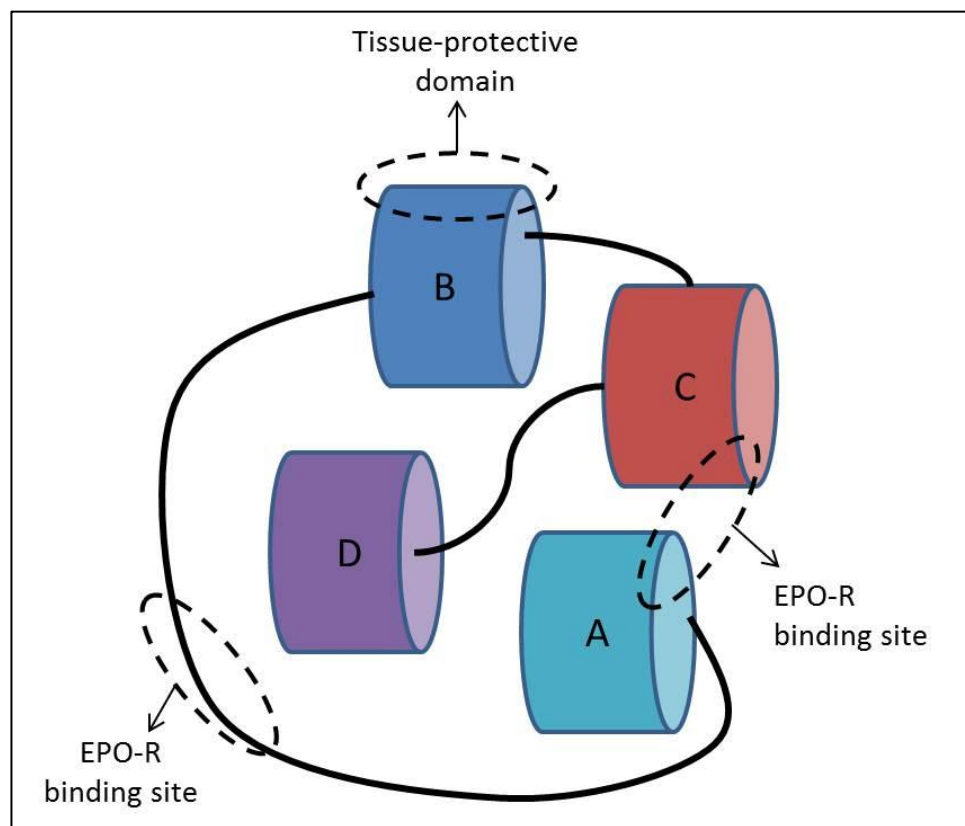Figure 4.1

Figure 4.1: Structure of EPO consisting of four protein domains (A, B, C and D) with EPO-R binding sites and tissue-protective domain highlighted.

Brines and colleagues hypothesised that the tissue-protective receptor is a heteromeric complex consisting of a single monomer of EPO-R and CD131, also known as the βcR. This was based on the knowledge that GM-CSF and IL-3 are able to

synergistically enhance the proliferation of erythroid progenitor cells (Sieff *et al.*, 1989) and that the signal-transduction domain present as part of the receptor complexes for GM-CSF, IL-3 and IL-5, is able to associate with the EPO-R monomer (Jubinsky *et al.*, 1997). Also, as mice that are genetically-deficient for the βcR do not have impaired erythropoiesis (Nishinakamura *et al.*, 1995), it is evident that the function of this complex must be independent of erythropoiesis and so may be responsible for EPO's tissue-protective effects.

Both the βcR and EPO-R subunits have been found to be present in many tissues including the brain and the heart, and immunoprecipitation studies have demonstrated that these monomers are able to co-localise on the membrane of these cells (Brines *et al.*, 2004). This was also demonstrated by Su and colleagues with the co-localisation of the βcR and EPO-R in endothelial cells. The consequential interaction of EPO with the heteromeric receptor resulted in eNOS activation and so an increase in NO production (Su *et al.*, 2011).

In addition to the discovery of the βcR-EPO-R complex, it has also been reported that EPO's beneficial effects are mediated by this receptor. The protection exerted by EPO against spinal cord injury was lost in mice that lacked the gene for βcR when compared to wild-type mice subjected to the same procedure (Brines *et al.*, 2004). This has been further confirmed by the use of antibodies against the βcR and gene silencing techniques in endothelial cells (Su *et al.*, 2011). Despite the constitutive expression of the monomers that form the βcR the expression of this receptor is known to increase following an injurious stimulus (Brines *et al.*, 2008a). Once expressed the βcR has a low affinity for EPO which means that the low levels of circulating EPO are insufficient to activate the receptor and it can only by activated by EPO produced locally in response to injury (Brines *et al.*, 2008a). Taken together, these data highlight that the βcR is indeed an interesting target in disease therapy as it is thought to mediate EPO's tissue-protective effects and it is distinctly different from the EPO-R. This may help to minimise the adverse effects associated with EPO-R stimulation and increased thrombosis.

Numerous analogues have been developed based on the structure of EPO which mimic the tissue-protection exerted by EPO but have a reduced erythropoietic effect, for example, carbamylated-EPO and asialo-EPO (Leist *et al.*, 2004). The focus of this study is an 11 amino acid peptide called pyroglutamate helix B surface peptide (pHBSP). The discovery of this peptide was a result of the investigation into the structure of EPO. Helix B of EPO's tertiary structure is on the aqueous face of the protein and does not interact with the classical EPO-R (Figure 4.1), this was identified as the domain responsible for the protective effects observed with EPO. A series of peptides were synthesised based on the amino acid sequence of this domain culminating in the discovery of the circularised peptide; pHBSP (amino acid sequence: UEQLERALNSS), the amino acid sequence of pHBSP is present on the aqueous face of helix and is thought to consist of the residues which interact with the tissue-protective receptor (Brines *et al.*, 2008b). The modification on the N-terminus of this peptide improves its stability by allowing circularisation of the structure so it can be stored for up to two years at 4°C or one year at 25°C (Robertson *et al.*, 2011).

The peptide pHBSP does not stimulate erythropoiesis, as it had no effect on haemoglobin or haematocrit levels in rats following repetitive dosing over a 28 day period, indicating that pHBSP does not interact with the EPO-R. Additionally, its pharmacokinetic properties vary from those of EPO, as its half-life is approximately 2 min in the rat and rabbit when injected intravenously, whereas EPO's half-life is approximately 4 h (Brines *et al.*, 2008b).

The peptide pHBSP exerts tissue-protective effects against ischaemia-reperfusion injury in multiple disease states including AKI in the mouse (Brines *et al.*, 2008b) and rat (Patel *et al.*, 2012), stroke (Brines *et al.*, 2008b), MI (Ahmet *et al.*, 2011) and traumatic brain injury in combination with HS (Robertson *et al.*, 2011). It has also been suggested to act via the βcR-EPO-R heterocomplex in the context of a mouse model of neuropathic pain. In this study, wild-type mice and mice lacking the gene for the βcR were subjected to sciatic nerve injury; repetitive pHBSP treatment resulted in reduced tactile and cold allodynia in wild-type mice. This protection was lost in βcR knockout

mice treated with pHBSP suggesting that the βcR plays a vital role in the protection observed with pHBSP treatment (Swartjes *et al.*, 2011).

The positive effects observed with pHBSP in animal models of neuropathic pain has led to many clinical trials in this field, for example, pain and retinal oedema in diabetic and neuropathic patients (EudraCT No: 2010-021518-45), complex regional pain syndrome (EudraCT No: 2012-033688-24). There are also ongoing trials investigating the safety and efficacy of pHBSP in rheumatoid arthritis (EudraCT No: 2010-023469-22) and critical limb ischaemia (EudraCT No: 2010-018584-41), however the results are yet to published. Heij and colleagues have reported beneficial effects observed with pHBSP treatment (2 mg in 6 ml saline i.v. infusion, 3 times a week for 4 consecutive weeks) in patients with sarcoidosis. These patients have significant neuropathy and treatment with pHBSP in a small group of patients (n=12) resulted in a reduction in pain, as defined using the small fibre neuropathy screening list (SFNSL) score and quality of life scoring systems (Heij *et al.*, 2012). There are no trials to date that have investigated the use of pHBSP in an acute, emergency setting such as trauma.

The aim of this study was to investigate the tissue-protective effect of pHBSP on the organ injury and dysfunction induced by HS. In particular, establish an ideal time point for administration to ensure maximum protection. As both pHBSP and EPO are thought to act via similar cell signalling pathways, the activation of the Akt pro-survival pathway was investigated in order to understand the mechanism(s) of action behind pHBSP's tissue-protective effects.

4.2 Methods

This study was carried out on 54 male Wistar rats (Charles River Ltd, Margate, UK) weighing 286 ± 4 g. For details on surgical procedure, haemorrhage and resuscitation, quantification of organ injury and function, histological evaluation, determination of MPO activity and statistical analysis refer to sections 2.2.1, 2.2.2, 2.2.5, 2.2.6, 2.2.7 and 2.2.10, respectively. The average volume of blood withdrawn during the haemorrhage period was 10.13 ± 0.25 ml (n=40, across all haemorrhaged groups).

4.2.1 Experimental Groups

Rats were randomly allocated into the following groups:

1.	Sham + Saline	(n=10)
2.	Sham + pHBSP 60 min post resus	(n=4)
3.	HS + Saline	(n=10)
4.	HS + pHBSP 0 min post resus	(n=5)
5.	HS + pHBSP 30 min post resus	(n=10)
6.	HS + pHBSP 60 min post resus	(n=10)
7.	HS + pHBSP 90 min post resus	(n=5)

Rats were treated with either 1 ml/kg saline or 1 μg/kg pHBSP i.v. at 0, 30, 60 or 90 min after the onset of resuscitation. This dose was selected based on a previous study which demonstrated a significant reduction in the renal dysfunction induced by AKI with a dose of 0.8 nmol/kg (Brines *et al.*, 2008b). Assuming the molecular weight of pHBSP is 1258, a dose of 0.8 nmol/kg is equivalent to 1 μg/kg. As the half-life of pHBSP is short, multiple time points of administration were chosen to investigate when the ideal time for administration would be. Rats in groups; Sham + Saline and HS + Saline, are pooled for saline treatment across the 4 time points. Sham-operated rats underwent identical surgical procedures but without haemorrhage or resuscitation.

4.2.2 Western blot analysis

For details refer to section 2.2.8, in addition to the measurement of Akt and GSK-β phosphorylation, p65 nuclear translocation and ICAM-1 expression; the phosphorylation of the MAPKs and STAT3 was also investigated. The following antibodies were used; rabbit anti-total p38 dilution 1:1000; mouse anti-phospho p38 Thr^{180}/Tyr^{182} dilution 1:1000; rabbit anti-total ERK1/2 dilution 1:2000; mouse anti-phospho ERK1/2 Thr^{202}/Tyr^{204} dilution 1:2000; rabbit anti-total STAT3 dilution 1:2000; mouse anti-phospho STAT3 Tyr^{705} dilution 1:2000.

4.2.3 Materials

For details refer to section 2.2.9, additionally pHBSP was supplied by Araim Pharmaceuticals Inc. (Ossining, NY, USA).

4.3   Results

4.3.1 Effect of treatment with pHBSP on the circulatory failure caused by haemorrhagic shock

Baseline MAP values were similar amongst all seven groups. When compared with sham-operated rats, haemorrhage caused a significant decline in MAP which was improved by resuscitation; however MAP was still significantly different from that of sham-operated rats (P<0.05, Figure 4.2). Treatment with pHBSP at any of the four time points was insufficient to increase MAP during resuscitation (P>0.05; Figure 4.2). Treatment of sham-operated rats with pHBSP had no significant effect on the MAP (P>0.05; Figure 4.2).

Figure 4.2

Figure 4.2: Alterations in MAP; in rats subjected to sham-operation or HS and treated with either saline (Sham + Saline, n=10; HS + Saline, n=10), or pHBSP (Sham + pHBSP 60 min post resus, n=4; HS + pHBSP 0 min post resus, n=5; HS + pHBSP 30 min post resus, n=10; HS + pHBSP 60 min post resus, n=10; HS + pHBSP 90 min post resus, n=5). Data are expressed as mean ± SEM for n observations, *P<0.05 Sham + Saline vs. HS + Saline.

4.3.2 Effect of treatment with pHBSP on the renal dysfunction induced by haemorrhagic shock

When compared with sham-operated rats, HS-rats treated with vehicle developed significant increases in serum urea (P<0.05; Figure 4.3A) and creatinine (P<0.05; Figure 4.3B), indicating the development of renal dysfunction. Treatment of HS-rats with pHBSP at both 30 and 60 min post resuscitation significantly attenuated the rise in serum creatinine (P<0.05; Figure 4.3B). However, there was no significant effect on serum levels of creatinine with treatment at 0 or 90 min post resuscitation (P>0.05; Figure 4.3B), or on serum levels of urea following treatment with pHBSP (P>0.05; Figure 4.3A). Treatment of sham-operated rats with pHBSP had no significant effect on either serum urea or creatinine levels (P>0.05; Figure 4.3).

Figure 4.3

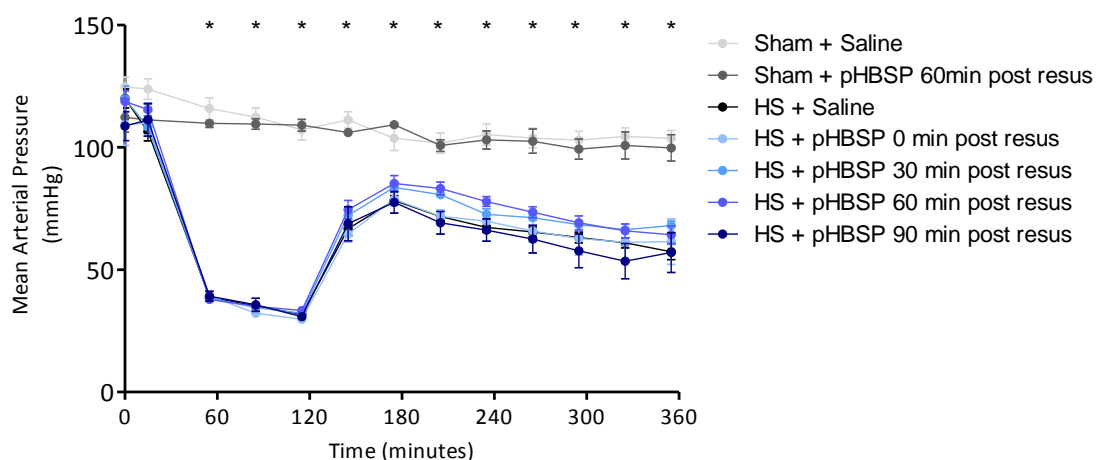**Figure 4.3:** Alterations in serum levels of (A) urea and (B) creatinine; in rats subjected to sham-operation or HS and treated with either saline (Sham + Saline, n=10; HS + Saline, n=10), or pHBSP (Sham + pHBSP 60 min post resus, n=4; HS + pHBSP 0 min post resus, n=5; HS + pHBSP 30 min post resus, n=10; HS + pHBSP 60 min post resus, n=10; HS + pHBSP 90 min post resus, n=5). Data are expressed as mean ± SEM for n observations, *P<0.05 Sham + Saline vs. HS + Saline, #P<0.05 HS + Saline vs. HS treatment groups.

4.3.3 Effect of treatment with pHBSP on the hepatic injury induced by haemorrhagic shock

When compared with sham-operated rats, HS-rats treated with vehicle developed significant increases in serum AST (P<0.05; Figure 4.4A) and ALT (P<0.05; Figure 4.4B), indicating the development of hepatic injury. Treatment of HS-rats with pHBSP at both 30 and 60 min post resuscitation significantly attenuated the rise in serum AST (P<0.05; Figure 4.4A) and ALT (P<0.05; Figure 4.4B). However, there was no significant effect observed on serum levels of AST or ALT with treatment at 0 or 90 min post resuscitation (P>0.05; Figure 4.4). Treatment of sham-operated rats with pHBSP had no significant effect on either serum AST or ALT levels (P>0.05; Figure 4.4).

Figure 4.4

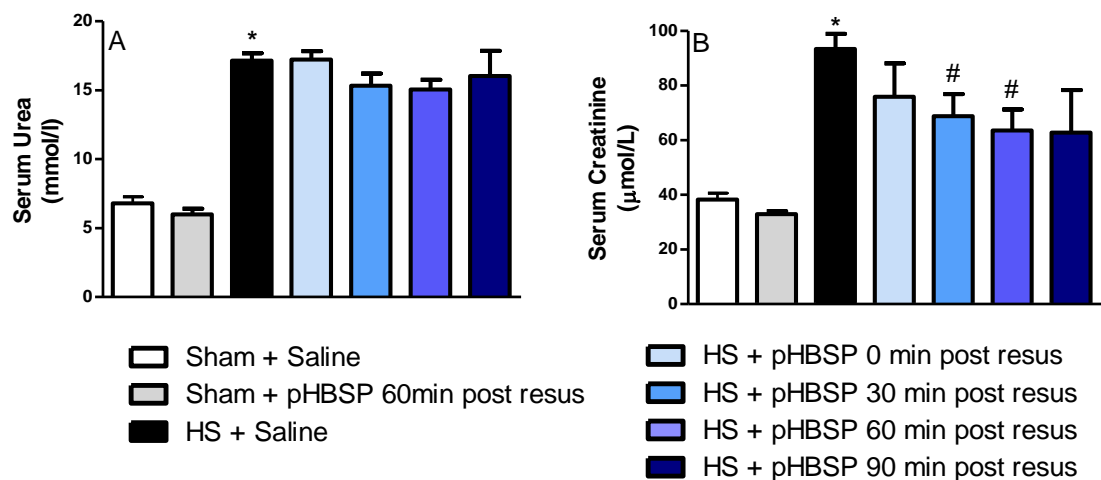**Figure 4.4:** Alterations in serum levels of (A) AST and (B) ALT; in rats subjected to sham-operation or HS and treated with either saline (Sham + Saline, n=10; HS + Saline, n=10), or pHBSP (Sham + pHBSP 60 min post resus, n=4; HS + pHBSP 0 min post resus, n=5; HS + pHBSP 30 min post resus, n=10; HS + pHBSP 60 min post resus, n=10; HS + pHBSP 90 min post resus, n=5). Data are expressed as mean ± SEM for n observations, *P<0.05 Sham + Saline vs. HS + Saline, #P<0.05 HS + Saline vs. HS treatment groups.

4.3.4 Effect of treatment with pHBSP on the skeletal muscle injury induced by haemorrhagic shock

When compared with sham-operated rats, HS-rats treated with vehicle developed significant increases in serum CK (P<0.05; Figure 4.5), which may indicate the development of skeletal muscle injury. Treatment of HS-rats with pHBSP at both 30 and 60 min post resuscitation significantly attenuated the rise in serum CK (P<0.05; Figure 4.5). However, there was no significant effect observed on serum levels of CK with treatment at 0 or 90 min post resuscitation (P>0.05; Figure 4.5). Treatment of sham-operated rats with pHBSP had no significant effect on serum CK levels (P>0.05; Figure 4.5).

Figure 4.5

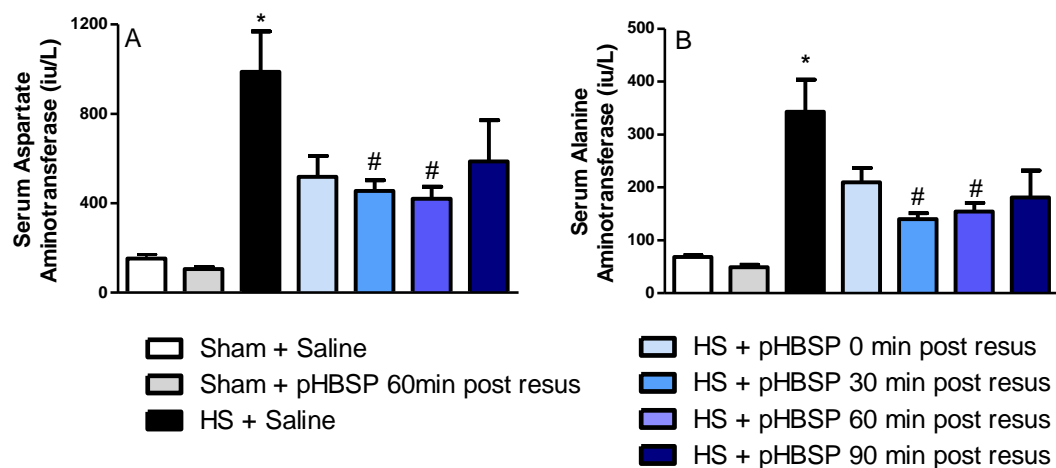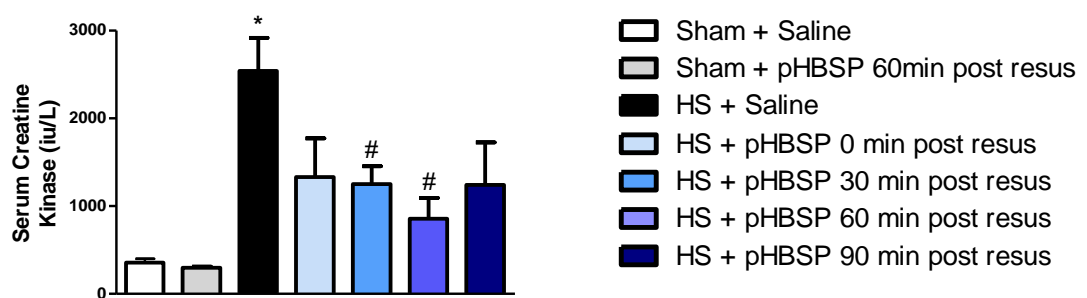**Figure 4.5:** Alterations in serum levels of CK; in rats subjected to sham-operation or HS and treated with either saline (Sham + Saline, n=10; HS + Saline, n=10), or pHBSP (Sham + pHBSP 60 min post resus, n=4; HS + pHBSP 0 min post resus, n=5; HS + pHBSP 30 min post resus, n=10; HS + pHBSP 60 min post resus, n=10; HS + pHBSP 90 min post resus, n=5). Data are expressed as mean ± SEM for n observations, *P<0.05 Sham + Saline vs. HS + Saline, #P<0.05 HS + Saline vs. HS treatment groups.

4.3.5 Effect of treatment with pHBSP on the pulmonary injury and inflammation induced by haemorrhagic shock

When compared with sham-operated rats (Figure 4.6A), the lung histology of HS-rats treated with vehicle confirmed inflammatory cell infiltration ($P<0.05$; Figure 4.6B and E) and alveolar septal thickening ($P<0.05$; Figure 4.6B and F), suggesting development of lung injury and inflammation. Treatment of HS-rats with pHBSP attenuated the degree of inflammatory cell infiltration ($P<0.05$; Figure 4.6C, D and E) and alveolar septal thickening ($P<0.05$; Figure 4.6C and E), with treatment at 30 min post resuscitation most effective.

Figure 4.6
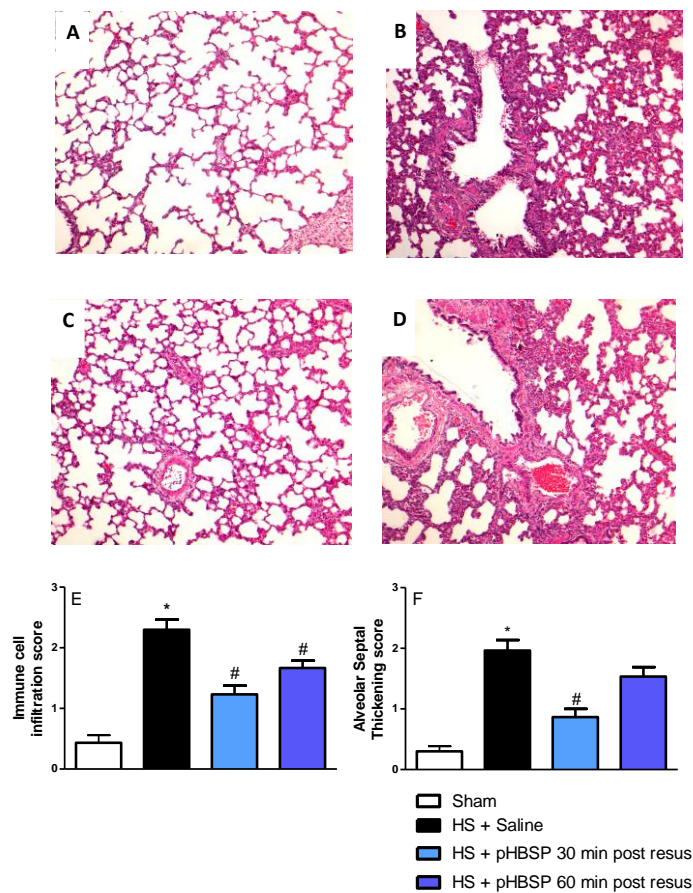
Figure 4.6: Lung histological sections taken from (A) sham-operated rat with inflammatory cell infiltration grade 1 and no observable alveolar septal thickening, (B) HS-rat treated with vehicle with inflammatory cell infiltration grade 3 and widespread alveolar septal thickening, (C) HS-rat treated with pHBSP 30 min post resuscitation with inflammatory cell infiltration grade 1 and minimal alveolar septal thickening and (D) HS-rat treated with pHBSP 60 min post resuscitation with inflammatory cell infiltration grade 2 and focal alveolar septal thickening. Haematoxylin-eosin stain, magnification ×100; figures are representative of at least three experiments performed on different days. Histological scoring for (E) inflammatory cell infiltration and (F) alveolar septal thickening, in rats subjected to sham-operation or HS and treated with either saline (Sham + Saline, n=3; HS + Saline, n=3), or pHBSP (HS + pHBSP 30 min post resus, n=3; HS + pHBSP 60 min post resus, n=3). Data are expressed as mean ± SEM for n observations, *P<0.05 Sham + Saline vs. HS + Saline, #P<0.05 HS + Saline vs. HS treatment groups.

4.3.6 Effect of treatment with pHBSP on the pulmonary congestion induced by haemorrhagic shock

When compared with sham-operated rats (Figure 4.7A), the lung histology of HS-rats treated with vehicle confirmed pulmonary congestion ($P<0.05$; Figure 4.7B and E), suggesting development of lung injury and inflammation. Treatment of HS-rats with pHBSP attenuated the degree of pulmonary congestion ($P<0.05$; Figure 4.7C, D and E), with treatment 60 min post resuscitation most effective.

Figure 4.7
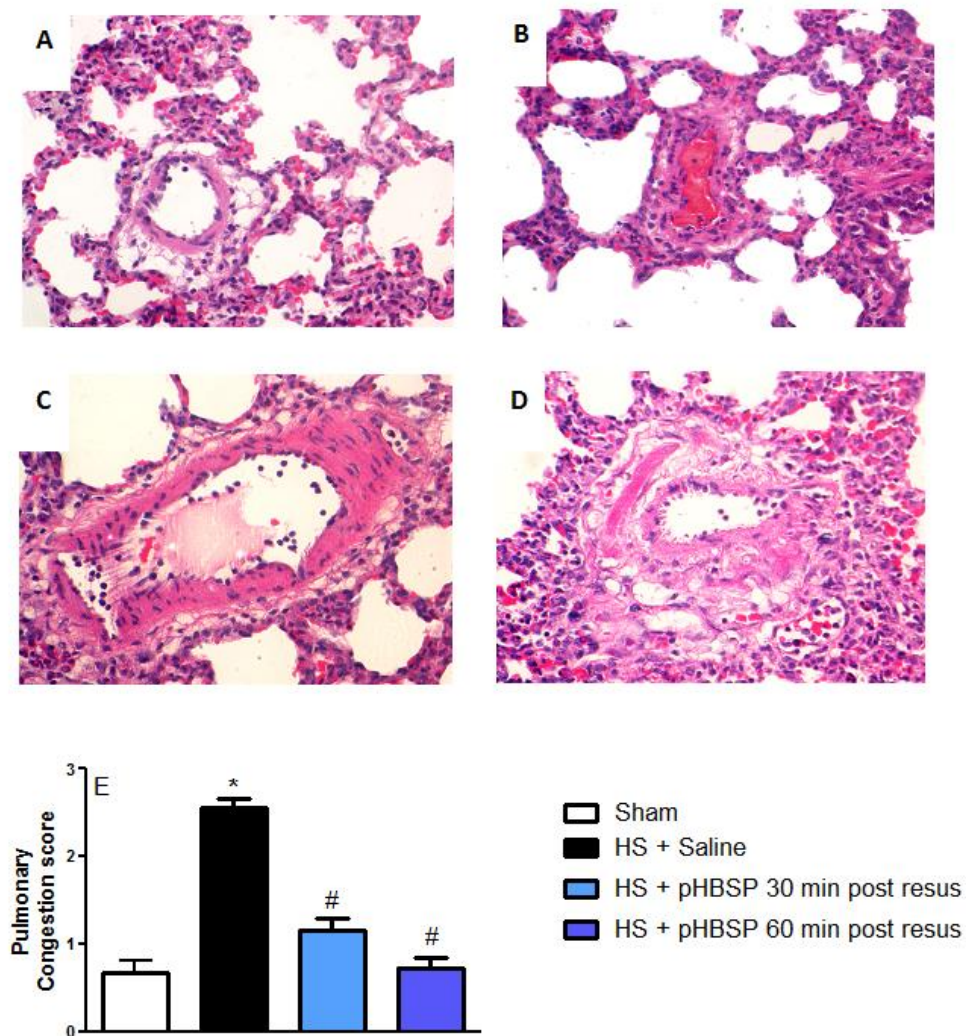
Figure 4.7: Lung histological sections taken from (A) sham-operated rat with pulmonary congestion grade 0, (B) HS-rat treated with vehicle with pulmonary congestion grade 3, (C) HS-rat treated with pHBSP 30 min post resuscitation with pulmonary congestion grade 1 and (D) HS-rat treated with pHBSP 60 min post resuscitation with pulmonary congestion grade 1. Haematoxylin-eosin stain, magnification ×400; figures are representative of at least three experiments performed on different days. Histological scoring for (E) pulmonary congestion, in rats subjected to sham-operation or HS and treated with either saline (Sham + Saline, n=3; HS + Saline, n=3), or pHBSP (HS + pHBSP 30 min post resus, n=3; HS + pHBSP 60 min post resus, n=3). Data are expressed as mean ± SEM for n observations, $*P<0.05$ Sham vs. HS + Saline, $\#P<0.05$ HS + Saline vs. HS treatment groups.

4.3.7 Effect of treatment with pHBSP on the MPO activity in the lungs from rats that underwent haemorrhage and resuscitation

MPO activity was determined to estimate the degree of neutrophil infiltration in the lung. When compared with sham-operated rats, HS-rats treated with vehicle had significantly higher MPO activity in the lung ($P<0.05$; Figure 4.8), suggesting the infiltration of neutrophils into the lung. Treatment of HS-rats with pHBSP at 30 min post resuscitation significantly reduced the rise in MPO activity caused by haemorrhage and resuscitation in the lung ($P<0.05$; Figure 4.8). However, there was no effect observed with treatment 60 min post resuscitation ($P>0.05$; Figure 4.8).

Figure 4.8

Figure 4.8: Activation of MPO in the lung in rats subjected to sham-operation or HS and treated with either saline (Sham + Saline, n=3; HS + Saline, n=3), or pHBSP (HS + pHBSP 30 min post resus, n=3; HS + pHBSP 60 min post resus, n=3). Data are expressed as mean ± SEM for n observations, *$P<0.05$ Sham + Saline vs. HS + Saline, #$P<0.05$ HS + Saline vs. HS treatment groups.

4.3.8 Effect of treatment with pHBSP on the expression of ICAM-1 in the lungs from rats that underwent haemorrhage and resuscitation

The expression of ICAM-1 was measured as an indication of the inflammation occurring in the lung. When compared with sham-operated rats, HS-rats treated with vehicle developed a significant increase in the expression of ICAM-1 in the lung ($P<0.05$; Figure 4.9). Treatment of HS-rats with pHBSP at 30 or 60 min post resuscitation significantly attenuated the increase in ICAM-1 expression caused by haemorrhage and resuscitation in the lung ($P<0.05$; Figure 4.9).

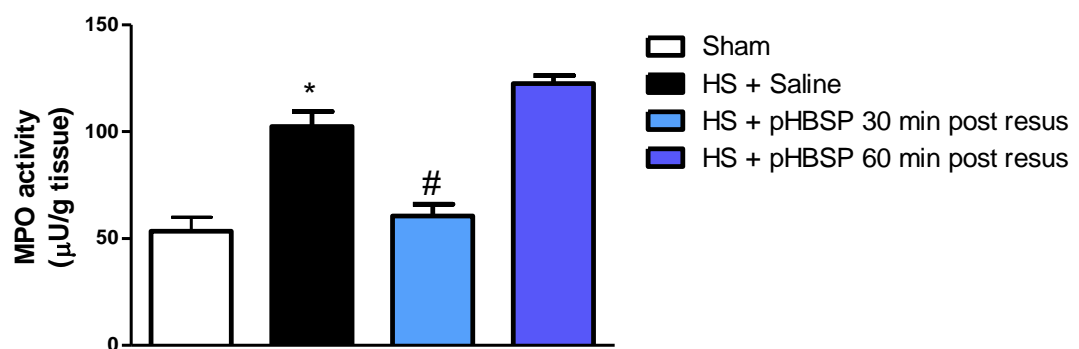Figure 4.9
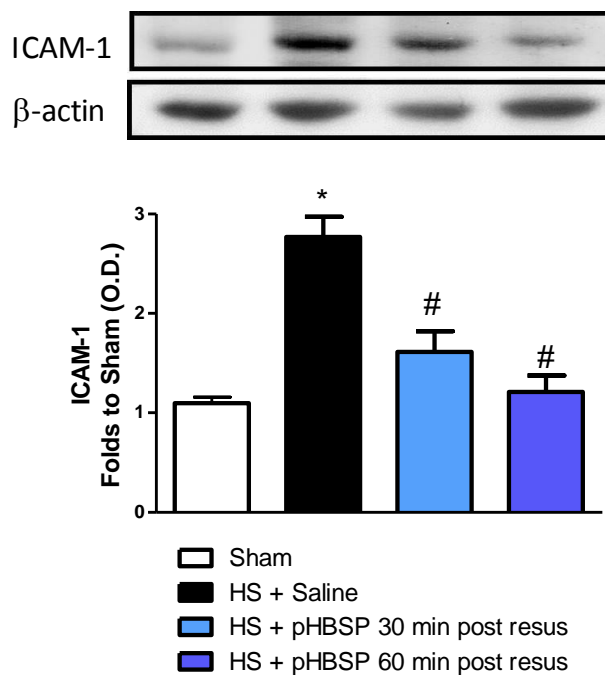
Figure 4.9: Expression of ICAM-1 in the lung; in rats subjected to sham-operation or HS and treated with either saline (Sham + Saline, n=3; HS + Saline, n=3), or pHBSP (HS + pHBSP 30 min post resus, n=3; HS + pHBSP 60 min post resus, n=3). Data are expressed as mean ± SEM for n observations, *$P<0.05$ Sham + Saline vs. HS + Saline, #$P<0.05$ HS + Saline vs. HS treatment groups.

4.3.9 Effect of treatment with pHBSP on the phosphorylation of Akt in the livers and kidneys from rats that underwent haemorrhage and resuscitation

As pHBSP is thought to act via similar cell signalling pathways as EPO, the effect of pHBSP treatment on the activation of the Akt pro-survival pathway and related proteins was investigated. This was investigated in the liver as the studies in Chapters 2 and 3 demonstrated that HS and treatments can modulate this pathway in this organ. Additionally, the effect of pHBSP treatment on these pathways has been investigated in the kidney as pHBSP appears to influence cell signalling in the kidney following ischaemia-reperfusion injury (Patel *et al.*, 2012).

When compared with sham-operated rats, HS-rats treated with vehicle developed a significant decrease in the phosphorylation of Akt on Ser^{473} in both the liver (P<0.05; Figure 4.9A) and kidney (P<0.05; Figure 4.10B). Treatment of HS-rats with pHBSP attenuated the decline in the phosphorylation of Akt caused by haemorrhage and resuscitation in the liver (P<0.05; Figure 4.10A) and kidney (P<0.05; Figure 4.10B).

Figure 4.10

Figure 4.10: Phosphorylation of Akt on Ser^{473} in the (A) liver and (B) kidney; in rats subjected to sham-operation or HS and treated with either saline (Sham + Saline, n=3; HS + Saline, n=3), or pHBSP (HS + pHBSP 30 min post resus, n=3; HS + pHBSP 60 min post resus, n=3). Data are expressed as mean ± SEM for n observations, *P<0.05 Sham + Saline vs. HS + Saline, #P<0.05 HS + Saline vs. HS treatment groups.

4.3.10 Effect of treatment with pHBSP on the phosphorylation of GSK-3β in the livers and kidneys from rats that underwent haemorrhage and resuscitation

When compared with sham-operated rats, HS-rats treated with vehicle developed a significant decrease in the phosphorylation of GSK-3β on Ser[9] in the liver (P<0.05; Figure 4.11A) and kidney (P<0.05; Figure 4.11B). Treatment of HS-rats with pHBSP attenuated the decline in the phosphorylation of GSK-3β caused by haemorrhage and resuscitation in the liver (P<0.05; Figure 4.11A) and kidney (P<0.05; Figure 4.11B).

Figure 4.11
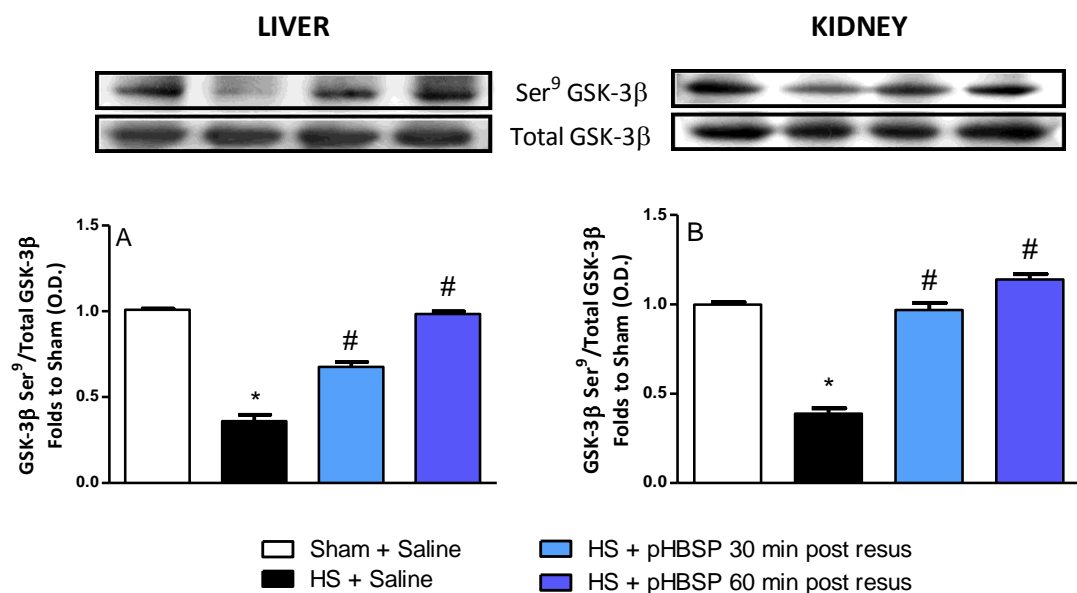
Figure 4.11: Phosphorylation of GSK-3β on Ser[9] in the (A) liver and (B) kidney; in rats subjected to sham-operation or HS and treated with either saline (Sham + Saline, n=3; HS + Saline, n=3), or pHBSP (HS + pHBSP 30 min post resus, n=3; HS + pHBSP 60 min post resus, n=3). Data are expressed as mean ± SEM for n observations, *P<0.05 Sham + Saline vs. HS + Saline, #P<0.05 HS + Saline vs. HS treatment groups.

4.3.11 Effect of treatment with pHBSP on the phosphorylation of eNOS in the livers and kidneys from rats that underwent haemorrhage and resuscitation

When compared with sham-operated rats, HS-rats treated with vehicle had a similar degree of phosphorylation of eNOS on Ser^{1177} in the liver (P>0.05; Figure 4.12A) and kidney (P>0.05; Figure 4.12B). Treatment of HS-rats with pHBSP significantly increased the phosphorylation of eNOS above the level observed in sham-operated rats and HS-rats in both the liver (P<0.05; Figure 4.12A) and kidney (P<0.05; Figure 4.12B).

Figure 4.12

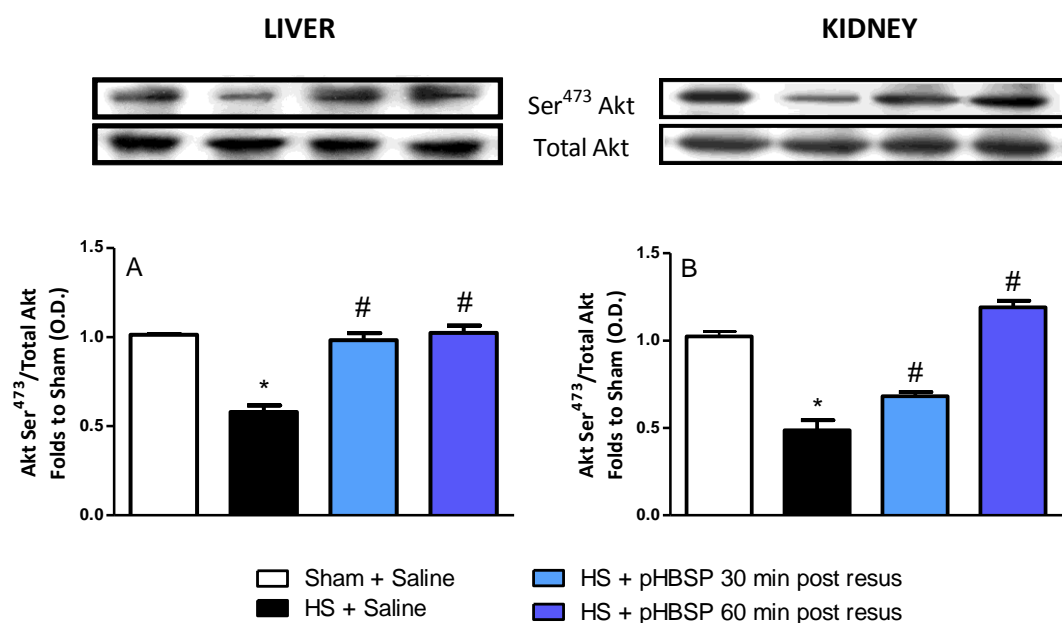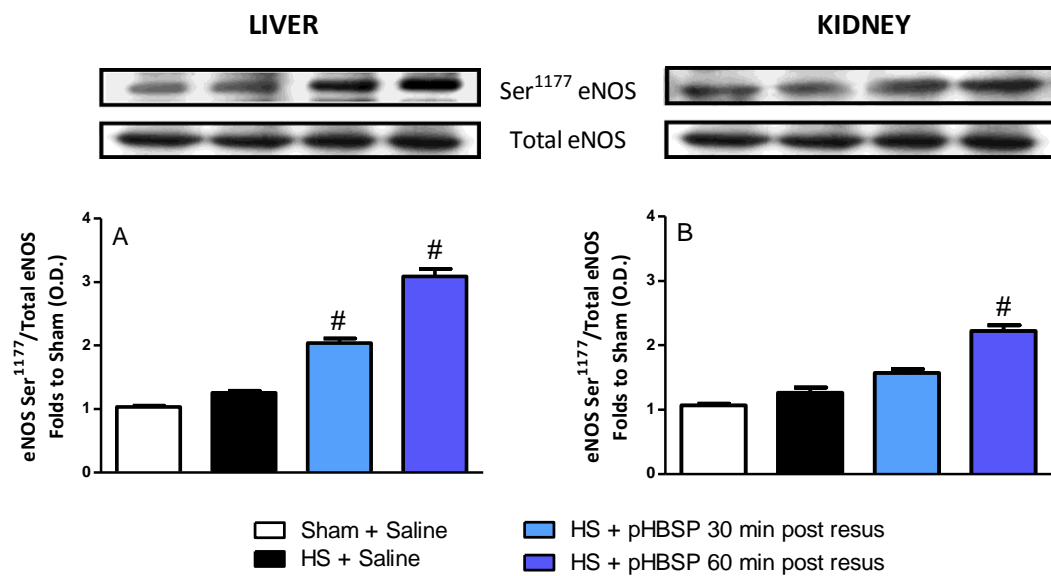**Figure 4.12:** Phosphorylation of eNOS on Ser^{1177} in the (A) liver and (B) kidney; in rats subjected to sham-operation or HS and treated with either saline (Sham + Saline, n=3; HS + Saline, n=3), or pHBSP (HS + pHBSP 30 min post resus, n=3; HS + pHBSP 60 min post resus, n=3). Data are expressed as mean ± SEM for n observations, *P<0.05 Sham + Saline vs. HS + Saline, #P<0.05 HS + Saline vs. HS treatment groups.

4.3.12 Effect of treatment with pHBSP on the nuclear translocation of the p65 subunit of NF-κB in the livers and kidneys from rats that underwent haemorrhage and resuscitation

When compared with sham-operated rats, HS-rats treated with vehicle developed significant increases in the nuclear translocation of the p65 NF-κB subunit in the liver (P<0.05; Figure 4.13A) and kidney (P<0.05; Figure 4.13B), indicating the activation of NF-κB. Treatment of HS-rats with pHBSP resulted in a significant reduction in nuclear translocation of p65 and, hence, the activation of NF-κB in the liver (P<0.05; Figure 4.13A) and kidney (P<0.05; Figure 4.13B).

Figure 4.13
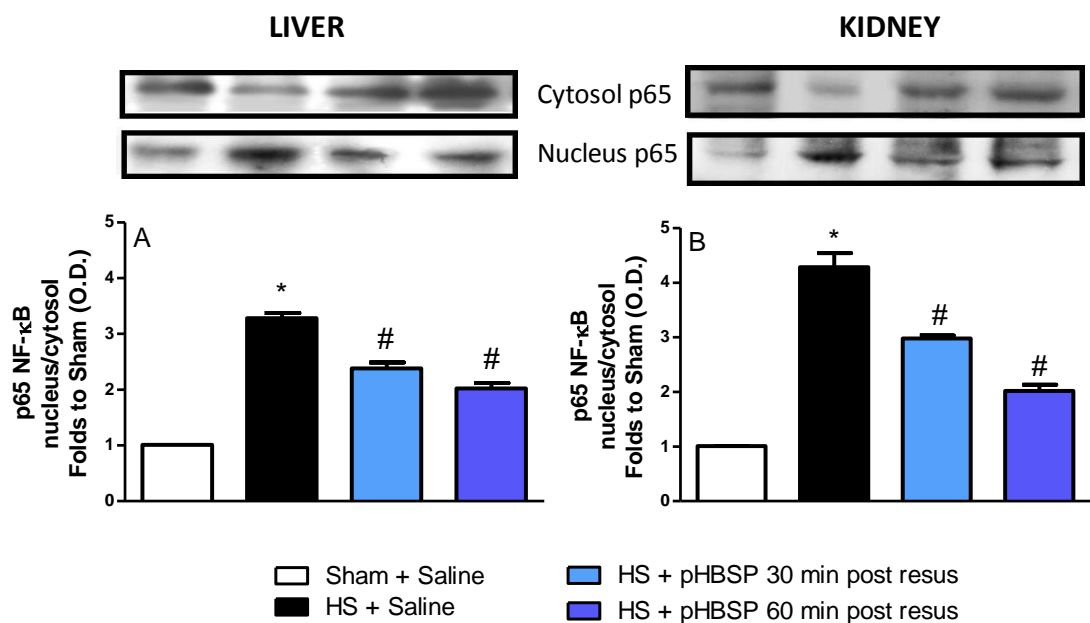
Figure 4.13: Nuclear translocation of the p65 subunit of NF-κB in the (A) liver and (B) kidney; in rats subjected to sham-operation or HS and treated with either saline (Sham + Saline, n=3; HS + Saline, n=3), or pHBSP (HS + pHBSP 30 min post resus, n=3; HS + pHBSP 60 min post resus, n=3). Data are expressed as mean ± SEM for n observations, *P<0.05 Sham + Saline vs. HS + Saline, #P<0.05 HS + Saline vs. HS treatment groups.

4.3.13 Effect of treatment with pHBSP on the phosphorylation of p38 in the livers and kidneys from rats that underwent haemorrhage and resuscitation

When compared with sham-operated rats, HS-rats treated with vehicle developed a significant increase in the phosphorylation of p38 on Thr^{180} and Tyr^{182} in the liver (P<0.05; Figure 4.14A) but not in the kidney (P>0.05; Figure 4.14B). Treatment of HS-rats with pHBSP significantly attenuated the rise in p38 phosphorylation caused by haemorrhage and resuscitation in the liver (P<0.05; Figure 4.14B) and had no effect on p38 phosphorylation in the kidney (P>0.05; Figure 4.14A).

Figure 4.14
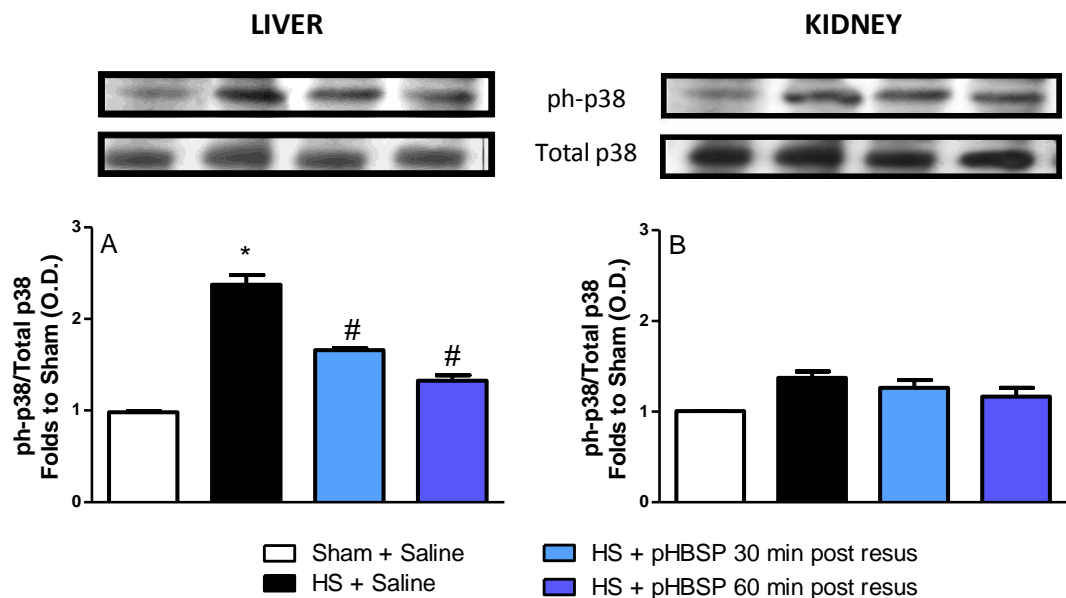
Figure 4.14: Phosphorylation of p38 on Thr^{180} and Tyr^{182} in the (A) liver and (B) kidney; in rats subjected to sham-operation or HS and treated with either saline (Sham + Saline, n=3; HS + Saline, n=3), or pHBSP (HS + pHBSP 30 min post resus, n=3; HS + pHBSP 60 min post resus, n=3). Data are expressed as mean ± SEM for n observations, *P<0.05 Sham + Saline vs. HS + Saline, #P<0.05 HS + Saline vs. HS treatment groups.

4.3.14 Effect of treatment with pHBSP on the phosphorylation of ERK1/2 in the livers and kidneys from rats that underwent haemorrhage and resuscitation

When compared with sham-operated rats, HS-rats treated with vehicle developed a significant increase in the phosphorylation of ERK1/2 on Thr^{202} and Tyr^{204} in the liver (P<0.05; Figure 4.15A) and kidney (P<0.05; Figure 4.15B). Treatment of HS-rats with pHBSP significantly attenuated the rise in ERK1/2 phosphorylation caused by haemorrhage and resuscitation in the liver (P<0.05; Figure 4.15A) and kidney (P<0.05; Figure 4.15B).

Figure 4.15
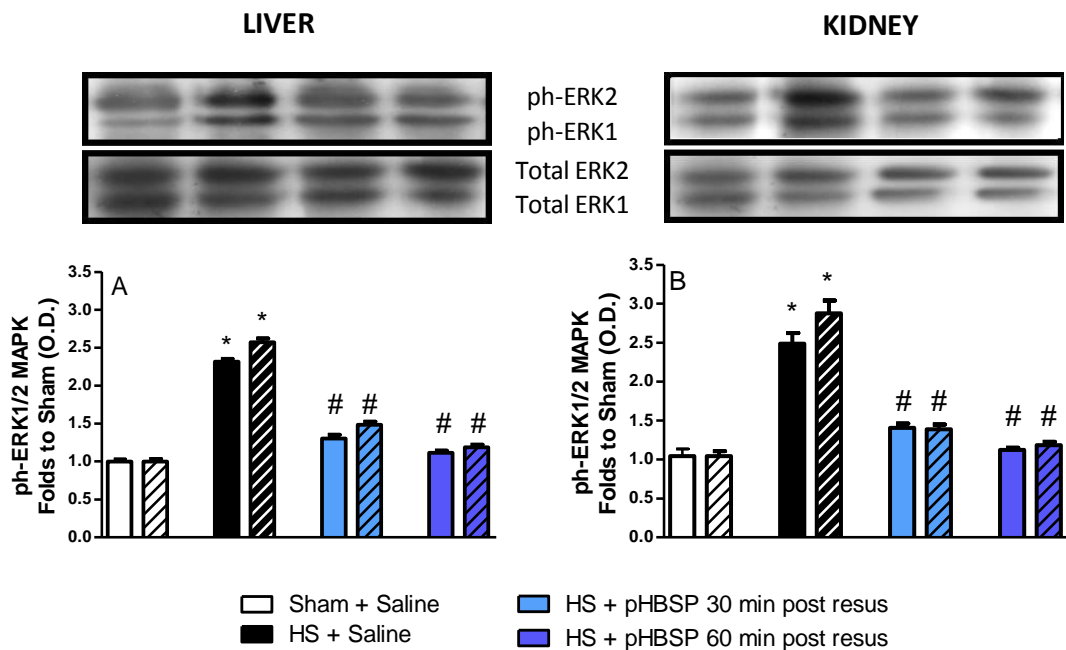
Figure 4.15: Phosphorylation of ERK1/2 on Thr^{202} and Tyr^{204} in the (A) liver and (B) kidney; in rats subjected to sham-operation or HS and treated with either saline (Sham + Saline, n=3; HS + Saline, n=3), or pHBSP (HS + pHBSP 30 min post resus, n=3; HS + pHBSP 60 min post resus, n=3). Coloured bars represent ERK1 and patterned bars represent ERK2. Data are expressed as mean ± SEM for n observations, *P<0.05 Sham + Saline vs. HS + Saline, #P<0.05 HS + Saline vs. HS treatment groups.

4.3.15 Effect of treatment with pHBSP on the phosphorylation of STAT3 in the livers and kidneys from rats that underwent haemorrhage and resuscitation

When compared with sham-operated rats, HS-rats treated with vehicle had a similar degree of phosphorylation of STAT3 on Tyr^{305} in both the liver (P>0.05; Figure 4.16A) and kidney (P>0.05; Figure 4.16B). Treatment of HS-rats with pHBSP significantly increased the phosphorylation of STAT3 above the level of sham-operated rats in the liver (P<0.05; Figure 4.16A) but had no effect in the kidney (P>0.05; Figure 4.16B).

<u>Figure 4.16</u>
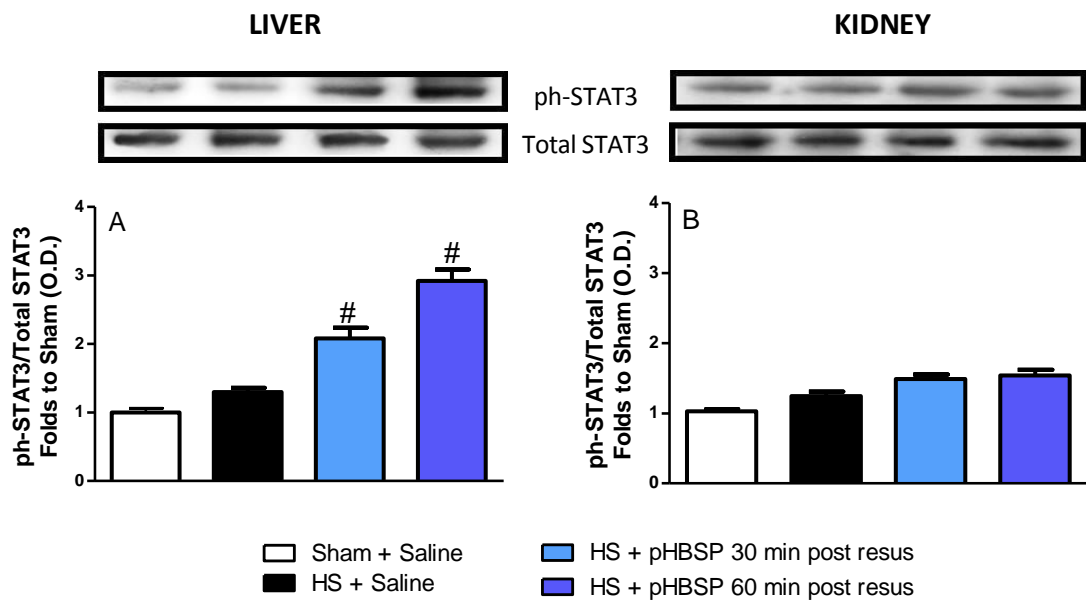
Figure 4.16: Phosphorylation of STAT3 on Tyr^{305} in the (A) liver and (B) kidney; in rats subjected to sham-operation or HS and treated with either saline (Sham + Saline, n=3; HS + Saline, n=3), or pHBSP (HS + pHBSP 30 min post resus, n=3; HS + pHBSP 60 min post resus, n=3). Data are expressed as mean ± SEM for n observations, *P<0.05 Sham + Saline vs. HS + Saline, #P<0.05 HS + Saline vs. HS treatment groups.

4.4 Discussion

This study was designed to investigate the effect of pHBSP treatment on the organ injury and dysfunction induced by severe haemorrhage and resuscitation. Treatment with pHBSP (1 µg/kg) significantly attenuated the renal dysfunction, hepatic injury, skeletal muscle injury, and pulmonary injury and inflammation induced by HS. Administration of pHBSP either 30 or 60 min after the onset of resuscitation exerted the greatest degree of protection.

The protective effects of EPO against the tissue injury induced by various ischaemic insults have been extensively investigated and there is a general consensus that EPO can exert its anti-apoptotic effects in many organs including the brain (Siren *et al.*, 2001), kidney (Sharples *et al.*, 2004) and heart (Brunner *et al.*, 2009). These beneficial effects are downstream of EPO's ability to modulate the expression of anti-apoptotic proteins such as Bcl-2 and reduce the expression of pro-apoptotic proteins such as Bax and Bad, as well as reducing the activation of caspase enzymes (Abdelrahman *et al.*, 2004b). Whether these anti-apoptotic actions are also attributable to pHBSP's protective effects is unclear.

There is some evidence that the beneficial effects of EPO are secondary to the activation of Akt (Burger *et al.*, 2009; Fliser *et al.*, 2006). In this study, HS resulted in a significant reduction in the phosphorylation of Akt in both the liver and kidney. This was attenuated by treatment with pHBSP, even when pHBSP was given as late as 60 min into resuscitation. This corroborates with evidence that both EPO and pHBSP also enhance the phosphorylation of Akt in cardiomyocytes treated with TNF-α (Ueba *et al.*, 2010). GSK-3β phosphorylation in the liver and kidney was also significantly reduced by HS. Treatment with pHBSP restored the degree of Ser^9 phosphorylation on GSK-3β to the levels seen in sham-operated rats even when pHBSP was given as late as 60 min into resuscitation. Interestingly, inhibition of GSK-3β also mediates the cardioprotective effects of EPO (Nishihara *et al.*, 2006). In line with the activation of GSK-3β, HS caused a significant rise in the nuclear translocation of the p65 subunit of

NF-κB, which was significantly attenuated by treatment with pHBSP. All of the above findings support the view that both EPO and pHBSP act via a similar signalling pathway in order to restore the activation of Akt, resulting in inhibition of GSK-3β and inhibition of the activation of NF-κB.

In addition to inhibiting the activation of GSK-3β, activation of Akt results in the phosphorylation and activation of eNOS. In this study, HS did not affect eNOS phosphorylation on Ser^{1177}. However, administration of pHBSP at 60 min post-resuscitation caused a pronounced increase in eNOS phosphorylation and, hence, activity. This increase in eNOS activation may be indicative of the interaction of βcR with Akt and eNOS (Su *et al.*, 2011), therefore suggesting that activation of eNOS (possibly secondary to activation of Akt) may contribute to the beneficial effects of pHBSP.

Activation of p38 MAPK promotes cellular stress responses such as proliferation, differentiation and production of pro-inflammatory cytokines. This occurs in response to ischaemia and haemorrhage in a number of organs (Donnahoo *et al.*, 1999; Guo *et al.*, 2003). HS results in the activation of p38 MAPK, while inhibition of its activity in animal models of HS attenuates renal, cardiac and lung injury (Sato *et al.*, 2008; Sato *et al.*, 2011; Sato *et al.*, 2010). Here, HS results in activation of p38 MAPK in the liver, which was attenuated by treatment with pHBSP. It is well documented that activation of p38 peaks 1 h after haemorrhage in the kidney and liver, and then gradually declines (Sato *et al.*, 2011; Sato *et al.*, 2012). So it is possible that a more marked activation of p38 MAPK occurred early after haemorrhage in the kidney which may have been attenuated by pHBSP. However, the activation of p38 MAPK in the liver also peaks 1 h after haemorrhage and this was attenuated by pHBSP; this difference in the effect between organs may be due to differential degrees of activation.

Severe HS results in the activation of ERK1/2 and agents that prevent its activation in HS exert beneficial effects (Fukudome *et al.*, 2010). Here, HS caused significant activation of both ERK1/2 in the liver and kidney which was attenuated by pHBSP treatment. Both EPO and pHBSP have been demonstrated to inhibit the activation of

ERK1/2 in the context of the ischaemic injury that occurs in MI (Ueba *et al.*, 2010). In contrast EPO has also been described to induce ERK phosphorylation in cardiomyocytes, and this is proposed to contribute to EPO's beneficial effects in myocardial ischaemia-reperfusion injury (Teng *et al.*, 2011).

STAT proteins are small downstream cell signalling proteins that can influence gene expression in response to tyrosine kinase receptor activation. STATs are primarily activated by phosphorylation by JAK kinases associated with the cell membrane receptor (Aaronson *et al.*, 2002). STATs are also strongly associated with EPO signalling as the classical EPO-R is a kinase receptor associated with various STATs including STAT3 and STAT5 (Lacombe *et al.*, 1998). Here, HS had no effect on STAT3 phosphorylation; however pHBSP treatment resulted in pronounced phosphorylation of STAT3 in the liver. Both EPO and pHBSP have been found to promote phosphorylation of STAT3 in the heart (Ahmet *et al.*, 2011; Ueba *et al.*, 2010) suggesting that STAT signalling may contribute to the tissue-protective effects observed with EPO and pHBSP as well as the erythropoietic action of EPO.

This is not the first study that has investigated the effect of pHBSP in the context of HS, Robertson and colleagues used a rat model of combined traumatic brain injury and HS. pHBSP was found to significantly reduce the contusion volume associated with brain injury as well as improving the cerebral blood flow and neurological function (Robertson *et al.*, 2011). However, they did not investigate the effect of pHBSP on the ischaemic injury induced by HS in other organs like the liver and kidney, so this has been investigated for the first time here.

The gain in efficacy observed with late treatment may be attributable to the pharmacokinetics of pHBSP and the expression of its proposed target. The short half-life indicates that pHBSP is rapidly degraded and is only present for a short period of time. There is also evidence that the proposed tissue-protective receptor is inducible by injury and inflammation, and that it is either up-regulated or exposed following an insult (Brines *et al.*, 2008a), as a result treating with pHBSP at 60 min post resuscitation means it is able to act on its target at the ideal time. Similar effects have been

observed in other disease models, for example, administration of pHBSP in a model of AKI 6 h after the onset of reperfusion (Patel *et al.*, 2012) and in a model of MI 24 h after left anterior descending coronary artery occlusion (Ahmet *et al.*, 2011) both resulted in beneficial effects. However in this study, treatment at 30 min post-resuscitation appeared to offer the better protection with respect to lung injury scoring as well as MPO activity. In fact, treatment at 60 min post-resuscitation actually increased MPO activity when compared to HS-rats treated with vehicle. This lack in protection may be due to the inability of the late treatment to prevent the early infiltration of neutrophils.

In conclusion, treatment with pHBSP significantly attenuated the organ injury and dysfunction induced by severe haemorrhage and resuscitation in rats. pHBSP treatment resulted in activation of the Akt pro-survival pathway in the liver and kidney, which in turn resulted in inhibition of GSK-3β (secondary to phosphorylation on Ser^9) and inhibition of the activation of NF-κB. Activation of Akt also resulted in phosphorylation and activation of eNOS. Treatment with pHBSP caused an inhibition of both p38 and ERK1/2 MAPKs in the liver and kidney and was able to induce significant phosphorylation of STAT3 in the liver. The effects exerted by pHBSP on these cell signalling pathways are similar to those reported in the literature with EPO treatment. Therefore, it is likely that both pHBSP and EPO act via similar cell signalling pathways to exert their tissue-protective effects. In addition, as EPO treatment in patients is associated with an increased risk of thrombotic events, non-erythropoietic analogues of EPO, such as pHBSP, could be viable therapeutic agents. Especially as pHBSP can be administered later into the resuscitation period, it extends the "golden hour" in which treatment can be administered to prevent tissue injury following haemorrhage.

CHAPTER 5

General discussion

5.1 Summary of results

In this thesis, the tissue injury that occurs following HS has been effectively modelled using a clinically relevant and scientifically reproducible rat model of severe haemorrhage and resuscitation. The effects of three different experimental therapeutic approaches on the organ injury and dysfunction induced by HS have been investigated using this model. In particular, the effects of these interventions on the HS-induced dysfunction of the Akt pro-survival pathway have been investigated, as modulation of this pathway is hypothesised to reduce inflammation and cell death.

In Chapter 2, the acute administration of BMMNCs significantly attenuated the organ injury and dysfunction induced by HS and restored the activation of the Akt pro-survival pathway resulting in an inhibition of the pro-inflammatory transcription factor; NF-κB. It is possible that the mechanism by which BMMNCs exerted their beneficial effect in this study is, in part, by modulation of the Akt pro-survival pathway. As the model is acute it is likely that these positive effects are a result of mediators secreted by the stem cells which act in a paracrine manner to protect cells against the ischaemia-reperfusion injury that occurs in HS. However, injection of large numbers of cells is not an ideal therapeutic option, therefore the possibility of stem cell mobilisation was investigated in the next chapter.

In Chapter 3, pre-treatment with EPO over a period of three days prior to the induction of haemorrhage resulted in a significant reduction in the organ injury and dysfunction induced by HS. Repetitive dosing with EPO also resulted in mobilisation of EPCs (defined as $CD34^+$/flk-1^+ cells) and an increased activation of the Akt pro-survival pathway, in particular the phosphorylation of eNOS at the Akt phosphorylation site was significantly increased. The increased activity of the Akt pro-survival may have been a result of EPC mobilisation as EPCs are proposed to exert paracrine effects as well as direct effects by interacting with host cells by the markers on their cell surface. Additionally, EPO is reported to promote the activation of Akt and eNOS possibly by acting via the βcR, therefore this may also be responsible for the observed effect. However, clinical use of EPO, particularly in trauma patients, has been associated with

an increased risk of thrombotic events, therefore the effects of a non-erythropoietic analogue of EPO were investigated in the next chapter.

In Chapter 4, treatment with pHBSP significantly attenuated the organ injury and dysfunction induced by HS. The peptide pHBSP also appeared to restore the function of the Akt pro-survival pathway resulting in a pronounced activation of eNOS. Additionally, pHBSP attenuated the rise in phosphorylation of the p38 and ERK1/2 MAPKs and caused pronounced phosphorylation of STAT3 in the liver. As the best effects on organ injury and cell signalling were observed with treatment as late as 60 min into resuscitation it is possible that pHBSP's effects are mediated via the βcR as its expression is increased following injury.

5.2 Future work

In order to improve the therapeutic potential of stem cell therapy, it would have been interesting to investigate the paracrine mediators released by the BMMNCs used in this study. This could be done by collecting conditioned medium from BMMNCs in culture and analysing the protein content for cytokines and growth factors. This supernatant could then be used as an intervention in the model of HS to investigate its effects on the tissue injury induced by haemorrhage and resuscitation.

The effects of EPO and pHBSP are proposed to be mediated by their interaction with the tissue-protective receptor, investigating this in more detail would lead to better understanding the mechanism of action. In order to do so, mice lacking the gene for βcR could be subjected to HS and treated with either EPO or pHBSP and the resultant effects observed. This was attempted, however HS is very difficult to model in mice as the size of the blood vessels makes surgery very challenging and blood volume is much lower than rats (58.5 ml/kg in mice vs. 70 ml/kg in rats). Also, anaesthesia needs to be maintained for the entire duration of the experiment (approximately 6 h) however this caused depression of respiration which led to significant mortality when combined with the ischaemic insult of haemorrhage.

Additionally, although the three interventions investigated here appear to mediate their beneficial effects by the activation of Akt, it has not been proven that the mechanisms are Akt-dependent. In order to investigate this, inhibitors of PI3K/Akt such as wortmannin and LY290042 could be administered prior to treatment to investigate whether the beneficial effect would be lost. However, administration of these inhibitors is usually 30 min prior to treatment and this is difficult to do in this particular model as the administration of the inhibitor would usually occur during the haemorrhage period which is not advantageous as it would affect the blood volume and pressure response.

5.3   Final conclusions

The three interventions investigated in this thesis all produced significant tissue-protective effects in the context of HS and demonstrated that modulating the activation of the Akt pro-survival pathway may be a good treatment target in HS. As discussed in the introduction (see section 1.6) it has been identified that the genomic response to tissue injury varies greatly between rodents and humans, therefore the positive data presented here may not be directly translatable to the clinical situation. The potential of these interventions as therapeutics is varied; the administration of stem cells in the emergency setting of trauma is not entirely practical and the increased risk of thrombosis associated with EPO treatment is not advantageous in the critical care setting. pHBSP has the most potential as a therapeutic intervention as it is designed to act on a specific molecular target where expression is increased following injury. Also the option to administer pHBSP later into the resuscitation period is attractive. Although there have not been any clinical trials to date which have tested the safety or efficacy of pHBSP in the context of trauma, in the past year there have been a number of trials registered to investigate the effects of pHBSP in the context of other diseases. This may be the start of the application of pHBSP to the clinic, albeit in another disease area.

Author's declaration

I confirm that all the work presented in this thesis is the work of the author, unless otherwise stated. Western blot analysis and myeloperoxidase assays were completed in collaboration with Dr Massimo Collino, University of Turin; histological analysis was performed in collaboration with Professor Fred Wong, University of Singapore and the isolation and characterisation of bone marrow-derived mononuclear cells was completed by Dr Kunihiko Takahashi during his time at the William Harvey Research Institute. No part of this work has been submitted for a degree at this or any other University. Copyright for this thesis rests with the author. Information derived or cited from this work cannot be published without consent and should be acknowledged.

Kiran Kaur Nandra

Citation

Nandra KK (2013) Novel therapeutic approaches for experimental trauma-haemorrhage. PhD thesis. Queen Mary University of London.

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APPENDIX 1

Arterial blood gas analysis of rats subjected to

haemorrhagic shock

Table viii.1

	Prior to haemorrhage	End of haemorrhage	End of experiment
pH	7.36 ± 0.01	7.04 ± 0.03 *	7.32 ± 0.03
P_{CO_2} (kPa)	7.02 ± 0.31	3.94 ± 0.17 *	4.87 ± 0.23 *
P_{O_2} (kPa)	10.46 ± 0.46	17.80 ± 0.52 *	12.93 ± 0.50 *
Haematocrit (%)	39.80 ± 0.77	19.56 ± 1.46 *	24.00 ± 1.41 *
cBase (mmol/L)	2.63 ± 0.07	-21.01 ± 0.73 *	-6.37 ± 1.72 *
Blood lactate (mmol/L)	1.85 ± 0.19	8.77 ± 1.01 *	1.63 ± 0.50

Table viii.1: ABG analysis was performed prior to haemorrhage, at the end of the haemorrhage period and at the end of the experiment, in rats subjected to surgical procedure and haemorrhage for 90 min followed by resuscitation with 20 ml/kg Ringer's lactate for 10 min and 50% of the shed blood for 50 min (n=10). Data are expressed as mean ± SEM for n observations, *$P<0.05$ when compared to value prior to haemorrhage.