The Effects of Blast on Vascular Endothelium

Emma Mary Davies

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Abstract

Extremity injury is a significant burden to those injured in explosive incidents and local ischaemia can result in poor functionality in salvaged limbs. This study examined whether blast injury to the limbs resulted in in activation of, or damage to the endothelium and its subsequent effects on the surrounding tissues.

The hind limbs of terminally anaesthetized rabbits were subjected to one of four blast exposures (high, medium, low, or no blast). Blood samples were analyzed for circulating endothelial cells pre-injury and at 1, 6, and 11 h postinjury as well as analysis for endothelial activation pre-injury and at 1, 6, and 12 h post injury. Post-mortem tissue (12 h post-injury) was analysed for both protein and mRNA expression and also for histopathology. The high blast group had significantly elevated levels of circulating endothelial cells 6 h post injury. This group also had significantly elevated tissue mRNA expression of IL-6, E-selectin, TNF-α, HIF-1, thrombomodulin, and PDGF. There was a significant correlation between blast dose and the degree of tissue pathology (haemorrhage, neutrophil infiltrate, and oedema) with the worst scores in the high blast group. This study has demonstrated that blast injury can activate the endothelium, and in some cases, cause damage that in turn leads to pathological changes in the surrounding tissue. For the casualty injured by an explosion the damaging effects of haemorrhage and shock could be exacerbated by blast injury and vice versa so that even low levels of blast become damaging, all of which could affect tissue functionality and long-term outcomes.

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Abbreviations

Ach	acetyl choline
AP-1	activator protein 1
ARDS	acute respiratory distress syndrome
BSA	bovine serum albumin
bTBI	blast traumatic brain injury
CEC	circulating endothelial cell
CINC-1	cytokine induced neutrophil chemoattractant 1
CO2	carbon dioxide
DCBI	dismounted complex blast injury
EBM-2	endothelial cell growth basal medium
EGL	Endothelial glycocalyx layer
ELISA	enzyme-linked immunosorbent assay
eNOS	endothelial nitric oxide synthase
EPC	endothelial progenitor cell
ET-1	endothelin 1
FITC	fluorescein isothiocyanate
FMD	flow mediated dilatation
GAG	glycosaminoglycan
HIF-1a	hypoxia inducible factor 1 alpha
ICAM	intercellular adhesion molecule
IED	improvised explosive device
IL	interleukin
IR	ischaemia reperfusion

MAP kinase	mitogen activated protein kinase
MIP-2	major intrinsic protein 2
mRNA	messenger ribonucleic acid
NBF	neutral buffered formalin
NF-KB	nuclear factor kappa light chain enhancer of activated B cells
NO	nitric oxide
Nrf2	nuclear factor erythroid 2 related factor 2
PBLI	primary blast lung injury
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PGI2	prostacyclin
PMN	polymorphonuclear neutrophil
QRT-PCR	real time quantitative reverse transcription polymerase chain reaction
ROS	reactive oxygen species
SDF-1	stromal derived factor 1
THMB	thrombomodulin
TNFa	tissue necrosis factor alpha
TNT	trinitrotoluene
tPA	tissue plasminogen activator
UEA-1	Ulex-Europaeus-Lectin-1
uPA	urokinase plasminogen activator
VEGF	vascular endothelial growth factor
VCAM	vascular cell adhesion molecule
vWF	von Willebrand factor
W-PB	Weibel-Palade body

Chapter 1: Introduction

1.1 Background

In recent years injury due to explosives has become a global epidemic. During the conflicts in Iraq and Afghanistan the weapons used evolved from predominantly ballistic missiles, to the widespread use of improvised explosive devices (IEDs). The IED is now the commonest weapon deployed against coalition troops (Ramasamy et al., 2009b). Usually comprised of a combination of weapons-grade explosives, incendiaries, and household goods such as fertilizer, electrical wiring, batteries, or mobile phone components, buried IEDs can target both vehicles and foot soldiers on patrol (Schoenfeld et al., 2013b). They can also be brought into crowded locations when worn by a suicide bomber. IEDs were responsible for over half of combat deaths and injuries sustained in Iraq and Afghanistan (Singleton et al., 2013b, Russell et al., 2014). For soldiers on the ground this increased to 70% of all injuries, whereas gunshot wounds only accounted for 18% (Schoenfeld et al., 2013a). Unfortunately, such injuries are not confined to military targets; civilians are often injured during conflicts, and afterwards due to the explosive remnants of war (ICRC, 2014). They are also targeted in terrorist attacks seen around the world each year. In 2014 there were 16,800 terrorist attacks worldwide, leading to more than 43,500 deaths and injuring a further 40,900. Of these, 8,800 incidents involved bombs or explosives (Miller, 2015).

An understanding of injury mechanisms, patterns of wounding, and subsequent pathophysiology helps us to attain optimal management of blast-injured casualties.

Military care providers are usually more familiar with blast injuries, but in the current climate of terrorism, it is essential that lessons learned are remembered and translated into civilian practice. Over the past two decades improvements in treatment, right from the point of wounding to definitive care mean that severely injured blast casualties, who would previously have died, are now surviving (Brown et al., 2012, Brown and Clasper, 2013, Russell et al., 2011).

The incidence of vascular injury to a named blood vessel in those wounded in recent conflicts was found to be between 9 and 12%, and 73% of these were due to a blast mechanism (Stannard et al., 2011, White et al., 2011). Also, over 70% of blast injuries sustained during this period were to the extremity (Ramasamy et al., 2008), involving muscle, bone, and if not major blood vessels, then certainly the microvasculature. Much is already known about how the shock wave from an explosion affects different body systems. However, a lot about the response to blast injury at a cellular level is still to be discovered. In particular, it is not known whether shock wave has an effect on the endothelium. Endothelium lines every blood vessel in the body, and is often described as an organ system in itself, performing a wide range of functions. Therefore, it would be most interesting to know how the endothelium is affected by blast, as it may guide strategies for reperfusion and limb salvage in the blast-injured casualty of the future.

1.2 The Physics of Blast

An explosion is the phenomenon that results from a sudden release of energy. The energy release may come from many sources, but improvised explosive devices and military ordinance typically contain high performance explosives e.g. gunpowder, TNT or semtex. These are chemical compounds that decompose rapidly to release energy in an intense exothermic reaction (Cullis, 2001). Explosions generate blast waves. When a charge detonates, a detonation wave is formed, that travels through the explosive material. When it reaches the limits of the explosive, pressure from the detonation wave compresses the surrounding air molecules, causing an instantaneous rise in pressure – the peak overpressure. This positive peak overpressure then falls rapidly into a longer negative pressure phase before returning to the baseline ambient pressure (Figure 1).

The positive pressure rise compresses the surrounding medium (air or water) and results in the propagation of a shock wave, which extends outwards from the explosion in a radial fashion (Horrocks, 2001, Elsayed and Atkins, 2008). Air is highly compressible and a thin layer is compressed by the detonation wave to a very high density, pressure and temperature (up to 10,000°C). Initially after detonation there is a hot, high-pressure volume of explosive product gases surrounded by a thin, very hot layer of air. The particle velocity of the product gases and the air can be as high as 2.5km/s. However, as the explosive products expand, they cool down and their pressure, density, temperature and velocity fall. The shocked air layer detaches from the product cloud, and at larger distances from the charge it is shock wave and rapidly moving air behind it that constitute the blast wave (figure 2).

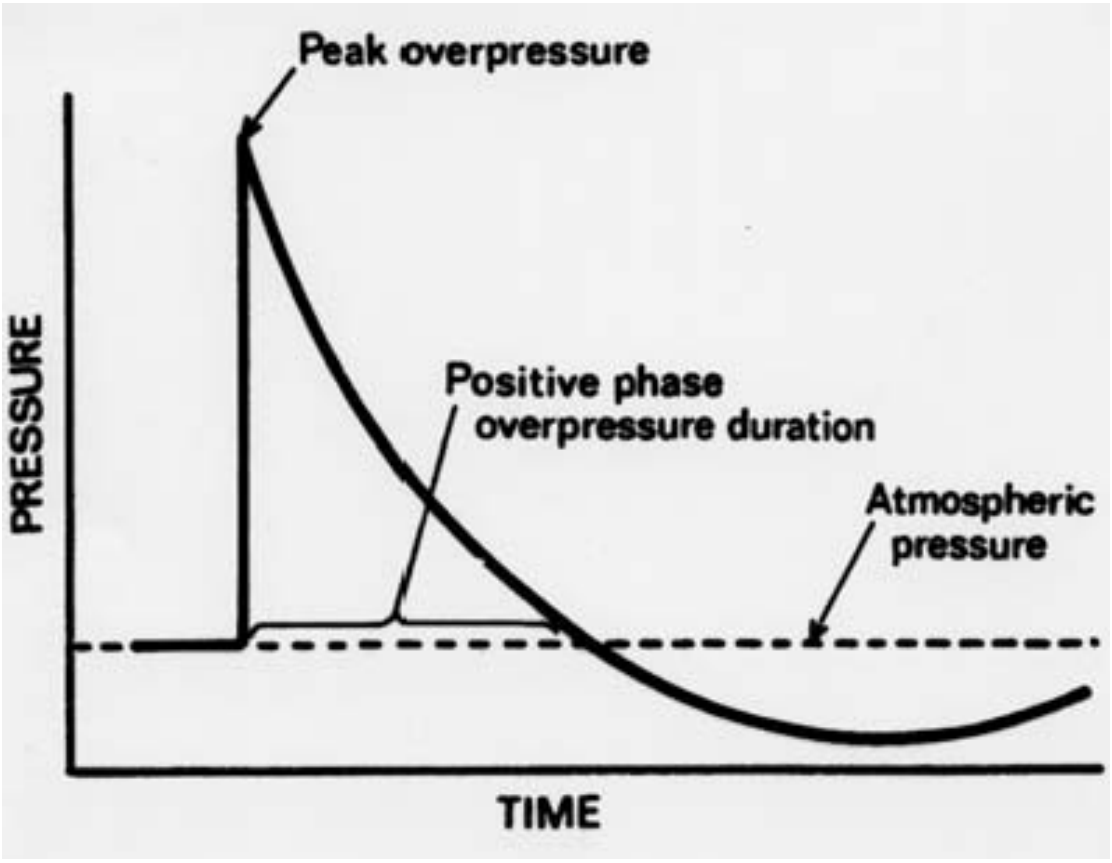


Figure 1. Idealised blast overpressure waveform. Reproduced with permission from BMJ Publishing Group Ltd.

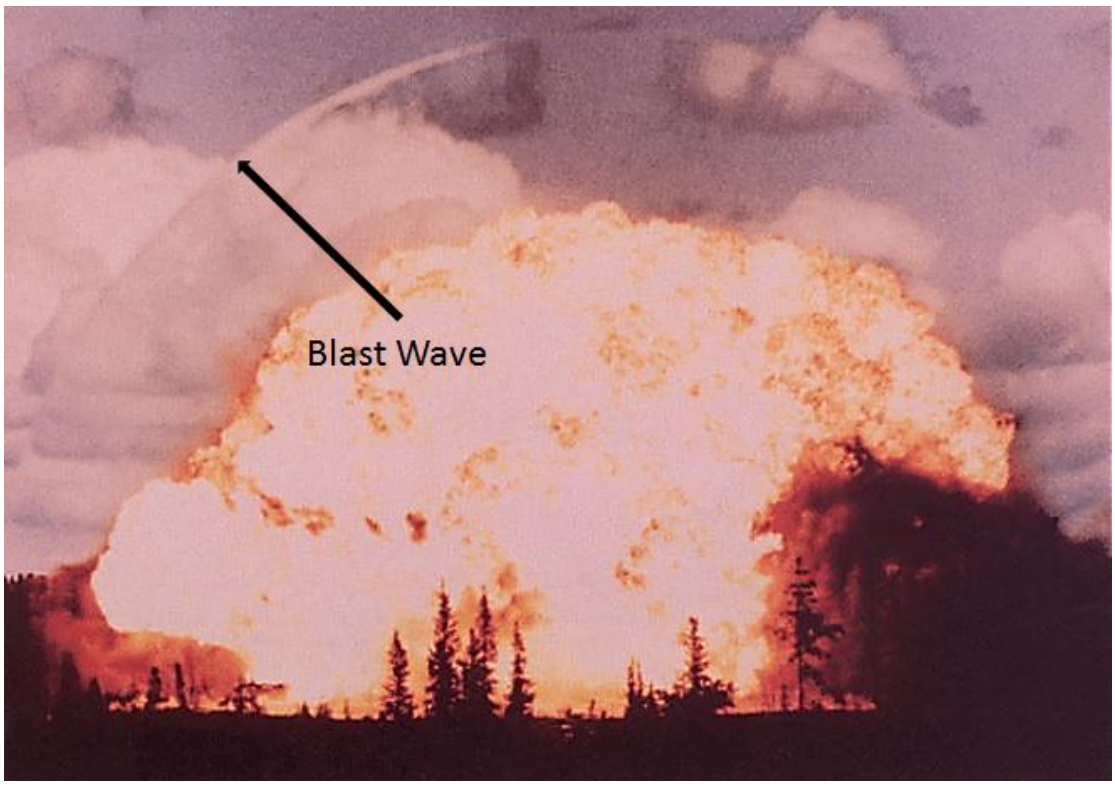


Figure 2. An example of a blast wave separated from the cloud of explosive gases.

Therefore, a blast wave consists of two parts: a high-pressure shock wave followed by rapidly moving air of decaying pressure known as the blast wind.

If they are unimpeded, blast waves extend outward from an explosion in a radial fashion. However, if a blast wave comes into contact with a structure (building or human) it is both propagated through the structure and reflected from it. The damage to that structure is dependent on the peak pressure, the time over which that force is applied, and the properties of the structure itself. The physics of blast waves is non-linear and complex. If an explosion takes place in a confined space, such as a vehicle or underground station, the damage or injury severity can be much greater due to reflections of the blast wave increasing the total force of the positive pressure phase

1.3 Classification of Blast Injury

Blast-related injury can be divided into four groups depending on the mechanism by which they occur. These are known as primary, secondary, tertiary and quaternary. More recently a fifth type of injury pattern was described following a bombing incident in Tel Aviv (Kluger et al., 2007) and a quinary group was added.

1.3.1 Primary Blast Injury

When the blast wave interacts with the body it causes anatomical and physiological changes to the tissues that represent Primary blast injury. Gas-containing structures such as ears, lungs and gastrointestinal tract are predominantly affected, but fractures can also occur. There are also effects at a cellular level. Primary blast injury is the focus of this thesis and the injuries to different body regions are discussed in more detail below.

1.3.2 Secondary Blast Injury

Secondary blast injury is caused entirely by projectiles accelerated by the explosion. These can be objects from within the bomb itself such as nuts, bolts and nails, or can be environmental debris including glass, gravel, dirt, and even bone and items such as boot eyelets. These projectiles can cause fractures, contusions and lacerations. They can cause multiple penetrating wounds, and there is often extensive wound contamination. Secondary injuries are the commonest type of injury seen in survivors from explosions and can occur at distances further from the blast than primary injuries.

1.3.3 Tertiary Blast Injury

Tertiary blast injury occurs when somebody becomes displaced and accelerated by the blast wind. Blunt poly trauma can occur when the body decelerates rapidly on impact with a stationary object. This can include closed head injury, fractures, and amputation of already fractured limbs due to flail. Crush injury, from displaced objects and structural collapse caused by blast wind, has also been included in some classifications of tertiary injuries.

1.3.4 Quaternary Blast Injury

Quaternary blast injury encompasses all other miscellaneous explosion-related injuries. This includes burns, inhalational injuries from hot or toxic gas, injury from environmental contamination, psychological effects, and exacerbation of pre-existing co-morbidities. Crush injury is sometimes also included in this category.

1.3.5 Quinary Blast Injury

Quinary blast injury refers to a hyperinflammatory state first observed after a bombing in Tel Aviv. Casualties displayed tachycardia, fever, low central venous pressure, and an excessive requirement for fluids to maintain adequate tissue perfusion. This was out of proportion to any other injuries sustained. It was thought to be due to absorption of the explosive (in this case DETA; pentaerythritoltetranitrate) which possesses vasodilatory properties (Elsayed and Atkins, 2008). The US Department of Defense (DoD) Directive 6025.21E also includes among quinary injuries, those from bacteria or radiation added to the explosive device and released on detonation.

1.4 Pathophysiology of Primary Blast Injury

Primary blast injuries are caused by the interaction of the blast wave with the body and are a type of non-penetrating trauma. They most commonly involve air-filled organs and air–fluid interfaces. Organs are damaged by dynamic pressure changes at tissue-density interfaces. The blast wave enters the body in two forms, known as stress waves, and shear waves. One or the other of these waves predominates, depending on the characteristics and location of the blast. Injuries are caused by these transmitted waves and can occur at locations spatially separated from the site of body wall displacement. Rupture of the tympanic membranes, pulmonary damage, air embolization, and rupture of hollow viscera are the most significant primary forms of blast injury (Horrocks, 2001, DePalma et al., 2005).

Stress waves are longitudinal pressure waves, like sound waves, that move at supersonic speeds, with higher amplitudes than sound waves. They generate high local forces with small, but rapid distortions. Pathophysiological effects are at the micro-vascular level; gross lacerations are not typical. Stress waves create a "spalling" effect at air-tissue interfaces, thus hollow or gas containing organs such as ear, lung and bowel tend to be most severely affected. Reflection and reinforcement of stress waves occurs at interfaces of different tissue density, which means the site of worst injury can be distant from the site of body impact. When a stress wave encounters an interface between two media of different physical properties, for example when it attempts to propagate from bowel wall into the gas filled lumen, a component of the compressive stress wave is reflected back at the interface as a tension wave. Most materials are weaker in tension than compression and disruption at the interface (tissue damage) may result. A stress wave may also compress a gas-

containing structure such as an alveolus or bowel segment (implosion) and then subsequent expansion damages the wall of the structure (Horrocks, 2001, Cooper and Taylor, 1989).

Shear waves are transverse waves of long duration and low velocity that result from deformation of the body wall and compression of visceral structures. The asynchronous movement of tissues with different inertia can cause disruption of solid organs and tearing of structures from their attachments. Injuries occurring in close proximity to the region of body wall deformation are generally due to local shear. Shear waves are probably responsible for primary blast injury of solid abdominal viscera, mesenteries and the large bowel (Horrocks, 2001, Cooper and Taylor, 1989).

1.4.1 Limb Injuries Secondary to Blast

Among casualties in recent conflicts a blast mechanism of injury accounts for around 75 per cent of all limb injuries (Owens et al., 2007), and 70 per cent of blast injuries sustained are to the extremeties (Ramasamy et al., 2009b). However, comparably little research has been carried out, with regard to delineating the specific pathophysiological effects of blast on the limb, when compared to other body areas such as the chest or head.

One difficulty is that many of the injuries encountered are a combination of primary, secondary and tertiary blast injury, and the contribution of each component varies depending on distance from the centre of the blast, and whether the individual is on foot, or within a vehicle or building. There are also multiple types of tissue involved

(bone, muscle, blood vessel, nerve), which may be affected differently, but all contribute to functional outcome of an injured limb.

Traumatic amputation after blast exposure was originally thought to be a wholly tertiary effect caused by blast wind-induced limb flailing and avulsion. Initial comparisons were made with fast jet pilots ejecting into a slipstream at speeds in excess of 1000km/hr. However, the pilots suffered predominantly ligamentous injuries and fracture-dislocations at joints (joints being the expected area of weakness). These injuries were sometimes associated with more proximal fractures, but not with traumatic amputation. Hull et al looked at survivors of explosions between 1979 and 1990 that sustained traumatic amputations and found that only 3 out of 41 (7.3%) occurred through a joint (Hull, 1992). They also looked at a series of fatalities in which only 1 out of 73 (1.4%) blast induced traumatic amputations occurred through a joint (Hull et al., 1994). Hull and Cooper then used a computer model of a bone exposed to blast forces, and a goat hind limb model to confirm their hypothesis that the primary mechanism of the bone injury was the direct coupling of the blast wave into the tissues, leading to a fracture from the resulting axial stresses in the bone. Following fracture it is the subsequent blast wind that flails the fractured extremity leading to amputation (Hull and Cooper, 1996). More recently Singleton et al reported on a series of traumatic amputations from Afghanistan. They found that 75.9% of the amputations occurred at the site of a fracture, not through a joint, supporting the role of primary blast in traumatic amputation. The remainder of the amputations (24.1%) occurred through a joint indicating that flail is also likely to play an important role in the mechanism of amputation. (Singleton et al., 2014).

Not all blast-injured limbs sustain traumatic amputation. Significant proportions sustain fractures and/or soft tissue injuries, including neuro-vascular damage. Primary blast is typically considered to cause an oblique diaphyseal fracture pattern, whereas multi-fragmentary or shattering type fractures are usually attributed to injury from secondary projectiles. Multiple dirty fragmentation wounds often complicate the management of other injuries by introducing a source of infection and leaving deficits in soft tissue cover. Arterial injury, or prolonged tourniquet application to the injured limb to prevent exsanguinating haemorrhage, may lead to ischaemia of the muscle tissue which in turn may compromise the functional outcome. All this needs to be taken into account when assessing the salvageability of a limb. Limbs that are initially salvaged may still require amputation later on. Gwinn et al. demonstrate this in a study of 26 blast-injured lower limbs with open fractures and arterial injury. Following initial salvage on the battlefield with wound debridement, vascular repair and bony stabilisation, 14 limbs required early amputation for reasons of vascular compromise, overwhelming infection, or inability to provide soft tissue cover. A further 6 limbs underwent late amputation for bony non-union, chronic infection or neuropathic pain, giving a high overall amputation rate of 76.9% (Gwinn et al., 2011).

Additionally, in a casualty with multiple injuries, saving life has to take precedence over saving limb. As previously described casualties may be hypotensive and suffering from haemorrhagic shock, and tourniquets may need to be applied to injured limbs to prevent exsanguinating haemorrhage. This saves life, but potentially contributes to limb ischaemia, which not only affects the functional outcomes following limb salvage, but also makes it difficult to determine the extent of the injury caused by the blast per se. It has been noted that blast-injured limbs do worse post-

revascularisation than those injured by other mechanisms (Midwinter, M.J. Personal communication), and it is possible that blast wave causes additional effects on the tolerance of the tissues to ischaemia. Looking at what is already known about the way blast affects other organ systems, especially at a cellular level, may give an indication of its likely effects on the limb, it's vessels and endothelium.

1.4.2 Blast Lung Injury

The lungs, due to their unique structure and function are one of the most susceptible organs to primary blast injury. The shock wave generated by an explosion can cause widespread damage in the lungs, characterized by rupture of alveolar capillaries and intrapulmonary haemorrhage. The presence of free blood and haemoglobin in the alveoli leads to the formation of free radicals, oedema and an augmented early inflammatory response, which leads to a compromise in pulmonary gas exchange, and hypoxia that can worsen over several hours(Kirkman and Watts, 2011). This in turn leads to leucocyte accumulation and subsequently epithelial cell damage (at 12–24h), endothelial cell damage (24–56h) and the late oedema typical of acute respiratory distress syndrome (ARDS) (Gorbunov et al., 2005, Smith, 2011b).

Marked congestion of pulmonary arteries, arterioles, veins, venules, and alveolar capillaries accompanied by vascular engorgement is another frequent histological finding (Elsayed and Atkins, 2008). Other notable features of blast lung include the unequal distribution of severity within the thorax. This is due to the reflection and augmentation of the blast waves. Certain regions of the lung are consistently more severely affected, such as the lung in proximity to the mediastinum and costo-phrenic

angles. “Rib markings” are typical on the lateral lung surfaces, but actually represent the areas beneath the intercostal spaces being more severely injured (Horrocks, 2001).

1.4.3 Abdominal Blast Injury

Primary blast injury affects predominantly gas-containing parts of the gastro-intestinal tract, it is uncommon and is more likely to be seen in cases of immersion blast, or blast in an enclosed space, than in an open air explosion. The overall incidence among survivors of explosions is reported in the literature at around 3.0 per cent (ranging from 1.3-33 per cent)(Owers et al., 2011). At high blast overpressures the bowel wall can rupture, causing haemorrhage and faecal contamination of the peritoneal cavity. At lower blast overpressures bleeding occurs within the intestinal wall, and can range from small petechiae to large confluent haematomas. Haemorrhage is initially confined to submucosal regions but becomes transmural with increasing severity. Some of these contusions progress to necrosis of the bowel wall and lead to late perforation (Horrocks, 2001). Small bowel contusions >15mm diameter, and colonic contusions >20mm diameter are at higher risk of late perforation and therefore warrant resection, whilst smaller lesions can be treated conservatively (Cripps and Cooper, 1997). Histologically the contusions are similar to those seen in blunt abdominal trauma. The terminal ileum and caecum are the most commonly affected organs (Owers et al., 2011). High blast loads can also cause damage to solid organs such as liver and spleen ranging in severity from subcapsular haematomas to lacerations or fractures.

1.4.4 Blast Injury to the Brain

Primary blast injuries to the brain include concussion as well as barotrauma caused by acute gas embolism. Fatalities are associated with subdural and subarachnoid bleeding and contusions throughout the brain in the absence of skull fracture (Bass et al., 2012). Loss of consciousness and coup and contre-coup injuries were previously considered secondary or tertiary injuries, but with the increased use of body armour in the military protecting the chest from lethal pulmonary blast injuries, damage to the central nervous system after an explosion has been increasingly attributed to the direct effects of the blast wave (DePalma et al., 2005, Bass et al., 2012). In patients who sustain significant blast traumatic brain injury (bTBI), hyperaemia and severe cerebral oedema occur frequently. Penetrating or closed head injury caused by the explosion may be associated with vasospasm and pseudoaneurysm formation. Mild bTBI can occur without loss of consciousness and can present with cognitive deficits and similar symptoms to posttraumatic stress disorder (Matsumoto et al., 2010).

The mechanism of blast-induced neurotrauma has been demonstrated to involve oxidative injury. Oxidative stress activates matrix metaloproteinases that cause the loosening of the vascular unit. The vascular unit is formed by endothelial cells, their associated blood brain barrier tight junctions, basal lamina, pericytes and parenchymal cells including astrocytes, neurons and interneurons. Reduction of the tight junction proteins damages the blood brain barrier, which leads to cerebral vascular leakage, neuroinflammation and neurodegeneration (Abdul-Muneer 2013).

1.4.5 Autonomic Effects of Primary Blast Injury

Primary blast injury to the thorax causes a characteristic response consisting of bradycardia, hypotension and apnoea or shallow breathing. This has been demonstrated in a number of experimental models (Irwin et al., 1998, Jaffin et al., 1987, Kirkman and Watts, 2011). It is a reflex response mediated via the autonomic nervous system, with efferent, and possibly afferent, pathways conducted via the vagus nerve (Ohnishi et al., 2001). Other contributing factors to the hypotension in response to thoracic blast are thought to be rapid release of nitric oxide from the pulmonary circulation, which leads to systemic vasodilatation (Zunic et al., 2000, Zunic, 2005), and myocardial impairment leading to reduction in cardiac output.

Following blast injury, the physiological response to haemorrhage and to fluid resuscitation is altered. The usual response to haemorrhage consists of an initial tachycardia (via vagal inhibition, activation of sympathetic efferent activity) and maintenance of blood pressure (via the arterial baroreceptor reflex). Once 20-30% of circulating blood volume has been lost the tachycardia changes to a vagally mediated bradycardia (depressor reflex), peripheral resistance is reduced and arterial blood pressure falls (Elsayed and Atkins, 2008). Thoracic blast augments the bradycardic, hypotensive response to haemorrhage. This is thought to be via augmentation of the depressor reflex as it can be attenuated by administration of morphine, which reveals a tachycardic response in its place (Sawdon et al., 2002).

1.4.6 Effects of Blast at a Cellular Level

Most of the research regarding effects of blast wave at a cellular level has been carried out using animal models of blast lung injury, so it is uncertain whether these effects are unique to the lung, or whether they can be extrapolated to other tissues of the body, in particular muscle, blood vessels and endothelium. Elsayed and Gorbunov demonstrated that a number of biochemical alterations take place, which lead to oxidative stress, which causes cellular damage, dysfunction or death (Figure 3) (Elsayed et al., 1997, Elsayed and Gorbunov, 2003).

They showed that in haemorrhagic blast lung injury, iron is released from damaged red blood cells in the form of catalytically active ferric iron complexes. There is depletion of glutathione, vitamins C and E, and other water and lipid soluble antioxidants, causing a decline in the total pulmonary antioxidant reserve and leading to increased oxidative injury. This in turn instigates redox signalling in the lung parenchyma cells, resulting in pro-inflammatory changes in the lung microvasculature. There is an increase in microvascular permeability, with a decrease in endothelial integrity provided by the VE-cadherin adhesion junctions. Phagocytic leucocytes (PMNs and monocytes) are induced to migrate across the endothelium into the surrounding tissues by increased expression of cellular adhesion molecules (VCAM-1 and ICAM-1). This is regulated at the transcriptional level by redox-sensitive transcriptional factors (NF-KB, Nrf2, Ref-1, AP-1), and at the post-transcriptional level via redox-sensitive MAP kinases (Gorbunov et al., 2007).

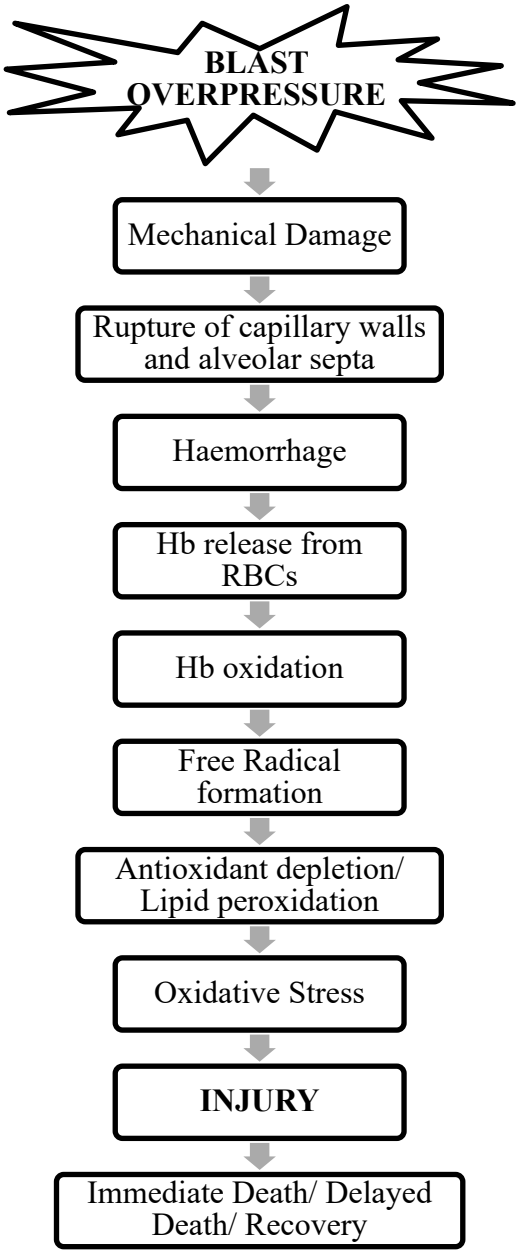


Figure 3. Flow chart summarizing the sequence of events involved in Blast Overpressure induced injury (Elsayed et al., 1997, Elsayed and Gorbunov, 2003)

Systemically there is an increase in pro-inflammatory cytokines and chemokines including IL-6, MIP-2 (rat equivalent of the human IL-8), CINC-1 and TNF-α. This is accompanied by hypoferraemia in the peripheral blood, which is mediated by IL-6 and is commonly seen in the acute phase response following major trauma. Iron is sequestered in the bone marrow where it plays an essential role in the acute phase activation of haemopoiesis and leucocyte maturation (Gorbunov 2007, Elsayed 2008). There is also release of nitric oxide causing systemic hypotension. This may be a compensatory response to the microcirculatory insult. Nitric oxide has been shown to act as either an oxidant or an antioxidant depending on the circumstances, and may therefore either promote or mitigate against oxidative stress (Elsayed and Gorbunov, 2003, Elsayed and Atkins, 2008).

Neutrophils (PMNs) are recruited into the peripheral blood initially following the blast injury, with rapid rises in both total numbers, and the expression of pro-inflammatory adhesion molecules on their surfaces (e.g. CD11b). They are then sequestered into the lung microvasculature where they migrate across the endothelium to the alveolar sites of the lung lesions. Here they degranulate and release myeloperoxidase, which is implicated in the inflammatory lung injury, and they accelerate lysis of the extravasated erythrocytes by erythrophagacytosis.

1.5 Epidemiology of Blast Injury

The sections above describe the physics of blast and how it translates to a mechanism of injury, and the known pathophysiology of those injuries. By also looking a little at the epidemiology of blast injury it becomes apparent that this remains a significant problem both for the military and the civilian population. Improvements in body armour and the process of trauma care have seen improved survival from blast injury, but there is yet to be any specific treatments that mitigate directly against the effects of blast.

Some aspects of blast injury have been known about for many years, with case reports dating back as far as 1768. During the First World War there were reports of "shell shock"; men in the field of an explosion were found dead, with no external wounds visible, and often blood stained fluid coming from their nose or mouth (Hill, 1979, Zuckerman, 1940). Casualties displaying bradycardia and hypotension after blast highlighted potential autonomic mechanisms during the Second World War, and the first experimental models of blast lung were described (Zuckerman, 1940). Since then observational data have been collected from various conflicts and civilian bombing incidents, which have been combined with experimental findings to give us the understanding of blast injury we have today. Repeated use of similar mechanisms of wounding allowed identification of injury patterns and levels of blast at which they were likely to occur.

1.5.1 Northern Ireland

Between 1969 and 1977, 5600 bomb blast incidents in Northern Ireland resulted in the death of 495 people. One aspect of explosive-related injuries that became apparent was the difference in injury pattern depending on the placement of the explosive device. Proximity to the explosion always resulted in more serious injuries. However, primary blast injuries such as blast lung and bowel were exacerbated by explosions in enclosed spaces, due to the reflection of the blast wave. Also, explosions that resulted in the fragmentation of projectiles caused far greater numbers of fractures and soft tissue injuries (Hill, 1979)(Mellor, 1992).

Mellor studied the 828 servicemen injured by explosions and found that of the 26% who died, two thirds were exposed to what he classified as "very severe" blast with overpressures > 550kPa (Mellor, 1992). The 5 most commonly observed factors in the fatally injured undergoing post mortem were brain damage, typically sub arachnoid haemorrhage (66%), skull fracture (51%), diffuse lung contusions/ blast lung (47%), eardrum rupture (45%) and liver laceration (34%). Lower limb fractures and traumatic amputations of multiple limbs were also very common; approximately 30% had a fracture and 30% had an amputation of at least one limb (Hill, 1979).

In survivors, injuries were predominantly caused by secondary missiles, rather than the blast wave itself. Body armour was worn by 90% of the servicemen and probably reduced the number of fatal secondary missile injuries. Less than 20% of the soldiers wore helmets which could potentially have reduced the number of head injuries (Mellor, 1992).

1.5.2 Iraq and Afghanistan

More than a decade of fighting in Iraq and Afghanistan saw a large number of coalition forces and Afghan Nationals injured and killed by explosive devices. Among UK troops there were 517 deaths due to hostile action between January 2002 and November 2013, of which explosive mechanisms of injury were responsible for 55.65% (Russell et al., 2014). This is a similar proportion to that identified by an American study looking at combat deaths in American, Canadian and British soldiers between 2006 and 2010 (Schoenfeld et al., 2013b).

During this conflict the weapons deployed have changed, from predominantly ballistic missiles (which are initially powered and guided into the air then fall onto their target under gravity), to the widespread use of buried IEDs, which target both vehicles and foot soldiers on patrol. This led to noticeable changes in the patterns of wounding, with a greater number of traumatic amputations (Ramasamy et al., 2009b). Foot soldiers in particular suffered injuries due to blast in 70% of cases (Schoenfeld et al., 2013a). They repeatedly presented with a characteristic group of injuries consisting of bilateral lower limb amputations (usually proximal trans-femoral), upper limb injury or amputation (usually the non-dominant side due to position of weapon being held), pelvic, genito-urinary and abdominal trauma that has been named "severe Dismounted Complex Blast Injury" (DCBI) (Andersen et al., 2012). Andersen noted that among US casualties, in 2010 there were twice as many triple and quadruple amputees than in the years 2001-2009 combined. The power of the explosive device underfoot seemed to roughly translate into the extent of the limb injuries received; smaller ones causing disruption of a single limb below the knee, and more powerful devices leading to much more proximal amputations. By 2010 it appeared that the

insurgents were making more powerful devices. Brown et al also found a statistically significant increase in traumatic amputations caused by IEDs when comparing the injuries of surviving British casualties prior to 2008 with those injured from 2008 to 2010. Those in the latter period also had higher injury severity scores and greater numbers of abdominal, pelvic and perineal injuries, but there was no difference in the rate of head, chest and spine injuries (Brown and Clasper, 2013). This fits with the placement of the IEDs in the ground transferring energy upwards through the lower limbs in the first instance. Increasingly proximal limb amputation was associated with reduced survival, as was increasing injury burden from head, chest and abdominal injuries (Morrison et al., 2012), and likely a larger blast load.

The prevalence of primary blast lung injury (PBLI) varied greatly between casualties that survived and those that did not. As expected, it was much higher among those that died (Smith, 2011b, Aboudara et al., 2014, Singleton et al., 2013a).

1.5.3 Terrorist Bombing Incidents

The Global terrorism database has recorded 88,254 terrorist attacks using bombs/explosives between 1970 and 2017, with numbers rising sharply to reach a peak of over 16,000 in 2014, then beginning to fall between 2015-2019. National Consortium for the Study of Terrorism and Responses to Terrorism (START). (2018). Global Terrorism Database [Data file]. Retrieved from http://www.start.umd.edu/gtd. (Muggah 2019). Some of the larger incidents in the last twenty years include the bombings in Madrid (2004), London (2005), Boston (2013) and Manchester (2017), from which important lessons were learned regarding the management of mass casualties, as well as the treatment of blast injuries.

During all these incidents, and in line with the military experience of explosion-related injury, severely wounded casualties presented with specific patterns of injury unusual in other mechanisms of trauma (Turegano-Fuentes et al., 2008). This included ear drum perforation, maxillo-facial and long bone fractures. Blast lung, blast bowel, injury to solid abdominal viscera and traumatic lower limb amputation were also seen. The mortality was higher in those explosions occurring in enclosed spaces e.g. the train carriages where the doors were closed when the bomb detonated, compared to when the doors were open, or the Boston bombings which took place outside; further demonstrating the reflection and amplification of the shock wave in an enclosed space. Other factors also contributed to the survival of the critically injured. These included emergency services efficiently triaging and distributing the injured among the major trauma centres and other nearby hospitals to prevent any one facility being overwhelmed. In Boston lessons learned from military experience in Iraq and Afghanistan were also put to good use with the early application of tourniquets to prevent exsanguination among casualties with amputations and mangled extremities (Caterson et al., 2013, Kellermann and Peleg, 2013). Whilst these improvements in the stream lining of trauma system care and preventing exsanguinating haemorrhage have improved survival, it again highlights there is no specific medical treatment that mitigates against the effects of blast injury or specifically the cellular effect of the shock wave. As these effects become better understood it may be possible to identify treatments that reduce the damaging effect of blast to cells and thus reduce the incidence of blast lung, or improve the success rate of limb revascularisation.

1.6 Ischaemia-Reperfusion Injury

Restoration of blood flow to ischaemic tissue is essential to prevent irreversible damage. However, the act of reperfusion may result in a local and systemic inflammatory response that may augment tissue injury in excess of that produced by the ischaemia alone. Cellular damage after reperfusion of previously viable ischaemic tissues is defined as ischaemia–reperfusion injury (IR injury). IR injury is characterized by oxidant production, complement activation, leucocyte–endothelial cell adhesion, platelet–leucocyte aggregation, increased microvascular permeability and decreased endothelium-dependent relaxation. In its severest form, IR injury can lead to multiorgan dysfunction or death (Eltzschig and Collard, 2004).

1.6.1 Neuromuscular Recovery following Ischaemia-Reperfusion

The need for reperfusion of ischaemic limbs following combat injury remains significant, with the incidence of named vessel vascular injury among combat casualties reported at between 9 and 12 per cent in Iraq and Afghanistan (Stannard et al., 2011, White et al., 2011). This is higher than in previous conflicts. Reduced evacuation times, combined with use of tourniquets and blood product resuscitation means that there is now increased opportunity for attempted limb salvage. However, a recent study has shown that salvaging an injured limb does not always give the best functional outcome in the long term when compared with amputation (Doukas et al., 2013). For this reason, a better understanding of the ischaemic threshold of the limb, and the factors that may alter this threshold will help guide clinical decision making when it comes to limb salvage.

Traditionally, surgical doctrine has taught that restoration of blood flow in trauma should be performed in less than 6 hours. More recently it has become apparent that functional recovery is compromised with increasing ischaemic time. Hancock et al used a porcine model of extremity ischaemia-reperfusion injury to look at the functional recovery of limbs following different periods of ischaemia, both with and without concomitant haemorrhagic shock (Hancock et al., 2011). They found that animals with less than 1 hour of ischaemia (control) had clinically normal limb function by the end of a 2-week observation period, with minimal muscle and nerve changes on histology. Contralateral, non-experimental limbs also revealed normal histology and function. Limbs exposed to 3 hours of ischemia showed impaired functional recovery, with moderate-to-severe degeneration of nerve and muscle noted on histology. Limbs exposed to 6 hours of ischaemia or permanent iliac vessel ligation had minimal electromyelographic response and the animals showed severe systemic inflammation, which correlated with severe muscle and nerve degeneration. Concurrent class III hemorrhagic shock worsened neuromuscular recovery across all groups but was greatest in groups undergoing greater than 3 hours of extremity ischaemia ($P < .01$), demonstrating that the presence of haemorrhagic shock compounds the effect of extremity ischemia and reduces the ischemic threshold of the limb to less than 3 hours. Whilst no study has yet demonstrated this, anecdotal evidence has suggested that blast injury is also likely to reduce the ischaemic threshold of the limb, and compound the local and systemic inflammatory response to ischaemia-reperfusion.

1.7 Vascular Endothelium

The endothelium lines all blood vessels of the vascular tree. It is a highly active organ system that participates in many physiological processes. Endothelial cells manufacture, store and release a wide range of factors that control vasomotor tone, cellular adhesion, trafficking of cells and nutrients, inflammatory response, thrombo-resistance, and the growth of new blood vessels. The key position endothelium holds between the blood and the tissues allow it to react to many physical and chemical stimuli. Such stimuli can cause a change in phenotype from a quiescent state to one of activation that involves the host defence response (Aird, 2004, Deanfield et al., 2007). This section examines the unique features of endothelial structure that allow it to perform such a wide range of functions, and then looks at some of the functional changes that it undergoes following activation, and potentially damage, by injurious stimuli in different disease states. This will help to guide the study of effects on the endothelium where blast is the injurious stimulus involved.

1.7.1 Structure of Endothelium

Forming a single, fairly uniform layer, endothelial cells are attached to an underlying basement membrane, which overlies a layer of smooth muscle in most vessels, except capillaries where there is a discontinuous lining with pericytes. The endothelial cells measure approximately 25-50μm long and 10-15μm wide. Their long axis is oriented in the direction of the blood flow. Each cell is thicker in the middle near its nucleus (approximately 3μm) and becomes thinner at the edges (ranging from 0.1μm in the aorta to less than 0.01μm in capillaries and veins)(Thorgeirsson, 1978, Florey, 1966). Examination using scanning electron microscopy has shown that appearance of the

luminal surface of blood vessels differs. For example, the aorta is generally described as fairly smooth with longitudinal folds, whereas the endothelial lining of the pulmonary artery is covered by a meshwork of irregular projections or microvilli (250-350nm in diameter and 300-3000nm long) which vastly increase the surface area of the cells (Smith et al., 1971).

There are two main types of endothelium. Continuous endothelium is found in arteries, veins, and muscular capillaries, whereas visceral capillaries have fenestrated endothelium (Thorgeirsson, 1978). Each segment of the circulation has a distinct, characteristic arrangement of inter-endothelial junctions. At one end of the spectrum, extensive tight junctions and a deficiency in caveolae characterize cerebral capillaries, which are impermeable to even small molecules. At the other end, fenestrated endothelium, such as that found in the hepatic sinusoid and renal glomerulus does not present a morphologic barrier and is freely permeable to large molecules (Schwartz and Benditt, 1972).

In vascular endothelium there are no junctional complexes like those encountered in other types of epithelium (Simionescu et al., 1975). Endothelial cells have only tight junctions and gap junctions. Tight junctions seal neighbouring cells together and are impermeable to macromolecules. They confine transport proteins to their domains on either the luminal or the basal surface of the endothelial cell and prevent diffusion of actively transported substances back between cells. Gap junctions allow inorganic ions and small water-soluble molecules to pass directly from the cytoplasm of one cell to the cytoplasm of the cell next to it, so the cells are coupled both electrically and metabolically. They are composed of transmembrane proteins called connexons, each

formed from 6 identical subunits called connexins. Two connexons join across the intercellular gap to form an aqueous channel connecting the 2 cells (Alberts et al., 1994).

Transport of macromolecules across the capillary wall occurs in caveolae (formerly known as plasmalemmal vesicles), which represent the structural equivalent of large pores. These invaginations of the plasma membrane, which form vesicles of ~70 nm diameter, are found in most mammalian cell types. They are found in large numbers in the continuous endothelium of certain microvascular beds (e.g. heart, lung, and muscles). George Palade, who originally discovered caveolae, used tracers of different sizes to demonstrate transcytosis (Farquhar, 2012). These studies showed that all tracers above 2nm diameter were restricted to caveolae and that their transport was inhibited by agents known to interfere with the mechanism of membrane fusion with target membranes (Stan et al., 1997). Patent transendothelial channels formed by several vesicles that open simultaneously on both endothelial cell fronts were also demonstrated. These transendothelial channels are the structural equivalents of small pores because they are continuous, water-filled passages. In continuous endothelium, small molecules cross the vascular wall via caveolae that can either function as isolated units shuttling across the endothelium, or fuse with one another to form patent transendothelial channels. No evidence was obtained for transport of any of these tracers through the tight junctions (Farquhar, 2012).

The Weibel-Palade Body (W-PB)

Endothelial cells contain a prominent oval nucleus and all the common cytoplasmic organelles such as golgi apparatus and endoplasmic reticulum. There is also a unique

structure, known as the Weibel-Palade body (W-PB). It is a rod-shaped cytoplasmic organelle, which measures approximately 0.1μm wide and 3μm long, and is made up of a number of small tubules arranged in the long axis of the rod and surrounded by an outer membrane (Thorgeirsson, 1978, Weibel and Palade, 1964). Initially the function of the W-PB was uncertain, but their uniqueness to endothelial cells served as an unambiguous structural marker in cell culture (Weibel, 2012). It has since been discovered that they store von Willebrand factor (vWf) and play a role in the blood coagulation system. The tubular structures seen inside the W-PB on electron microscopy are in fact large, high molecular weight, multimers of vWf. These molecules are most effective in mediating platelet adhesion (Wagner and Frenette, 2008, Lip and Blann, 1997). W-PBs also contain P-selectin (a receptor for leukocytes), the vasoconstrictor endothelin, and tissue plasminogen activator. They are secretory granules produced in response to secretagogues produced by injury or inflammation. The secretion from these organelles provides a very rapid way for the activated endothelium to become adhesive for platelets and leukocytes and plays an important role in endothelial biology by regulating haemostasis, vascular tone, inflammation, and angiogenesis (Wagner & Frenette 2008).

The Endothelial Glycocalyx Layer (EGL)

On the luminal surface of the endothelium lies the endothelial glycocalyx layer (EGL), a complex layer of membrane-bound proteoglycans, glycoproteins and glycolipids, with additional soluble components attached including superoxide dismutase, hyaluronan and albumin. The proteoglycans consist of a core protein that carries one or more covalently attached glycosaminoglycan (GAG) chains (Sieve 2018). Its inner margin is indistinguishable from the outer leaflet of the plasmalemma

and is generally considered an intrinsic part of it (Pries 2000). The EGL was first identified by Luft in 1966 who applied the ruthenium red technique to visualize it (Luft, 1966). Prior to this electron microscopy had failed to demonstrate its existence because it was routinely removed during tissue processing.

The EGL has many functions. It forms an interface between the vessel wall and the blood stream and maintains the colloid osmotic gradient. It regulates vascular exchange of water and solutes and acts as a sieve for plasma proteins. The EGL forms a barrier to leucocyte-endothelium adhesion, and provides binding sites for antithrombin III and tissue factor pathway inhibitors, giving an overall antiadhesive and anticoagulant effect. It also acts as a shear stress sensor and regulator of mechanotransduction (Johansson et al., 2011. Schott, 2016). The EGL can be damaged easily and is vulnerable to insults from a variety of sources, such as inflammation, trauma, haemorrhagic shock, hypovolemia and ischaemia-reperfusion. Damage to the EGL commonly precedes further damage to the vascular endothelium (Schott, 2016).

1.7.2 Endothelial Function in Health

As mentioned previously, the endothelial lining of blood vessels presents a large surface area for exchange of materials between blood and tissues, and is critically involved in many other processes such as regulation of blood flow, inflammatory responses and blood coagulation. Quiescent endothelial cells normally maintain an anticoagulant, fibrinolytic, anti-inflammatory state within the vasculature, but can become activated and switch to a pro-coagulant, anti-fibrinolytic, and pro-inflammatory phenotype in response to a variety of stimuli (Aljada, 2003).

Thromboresistant Surface

In the normal healthy state endothelial cells maintain a thromboresistant surface and intravascular coagulation does not occur. They produce a large number of both anticoagulant and procoagulant substances, which achieve a balance between the promotion and the inhibition of coagulation and fibrinolysis.

Anticoagulant products include nitric oxide (NO), prostacyclin (PGI_2), protein C, protein S, thrombomodulin, heparan sulphate, tissue factor pathway inhibitor, urokinase type plasminogen activator (uPA) and tissue type plasminogen activator (tPA). The release of NO and PGI_2 increases the activity of guanylate- and adenylate-cyclase, respectively, raising c-GMP and c-AMP levels. This is followed by inhibition of platelet aggregation and thrombosis. Expressed at the endothelial cell surface, thrombomodulin binds thrombin, causing a configurational change that inhibits the conversion of fibrinogen into fibrin and permits the activation of protein C by thrombin, followed by inactivation of factor Va and VIIIa. Fibrinolysis is enabled by secretion of tPA, which leads to active plasmin formation and subsequent fibrin degradation (Hartge et al., 2007).

In contrast, the endothelium also secretes plasminogen-activator inhibitor-1 (PAI-1), which inhibits tPA and functions as an anti-fibrinolytic agent. Other procoagulant products of endothelial cells include von Willebrand factor (vWF), thromboxane A_2, interleukin-1 (IL-1), tissue factor, platelet activating factor and phospholipids (Aljada, 2003).

Regulation of Vasomotor Tone

Another key function of the endothelium is to regulate blood flow by maintenance of vascular tone. Production and secretion of several vasoactive molecules that relax or constrict the vessel allow a balance between metabolic demand and tissue oxygen supply to be achieved. Nitric oxide (NO) and prostacyclin I2 (PGI_2) are the two main vasodilators, which also act to inhibit platelet aggregation; others include endothelium-derived hyperpolarising factor and C-type natriuretic peptide. The endothelium also secretes vasoconstrictors, including endothelin-1 (ET-1), angiotensin II (Ang II), thromboxane A_2 and reactive oxygen species (ROS)(Hartge et al., 2007, Deanfield et al., 2007).

Endothelium derived relaxing factor, now known to be NO, was not discovered until 1980. Pioneering work by Furchgott and Zawadski showed that endothelium is required for acetyl choline (Ach) to cause relaxation of vascular smooth muscle. When the endothelium is removed, Ach application results in vasoconstriction of vascular smooth muscle, however, if it is preserved then the resultant action is vasodilation (Furchgott and Zawadzki, 1980, Ignarro et al., 1987). NO was later shown to be synthesized from L-arginine by endothelial cells in culture by the action of endothelial NO synthase (eNOS) in the presence of cofactors such as hydrobiopterin (Palmer et al., 1988). NO diffuses from the endothelial cells to vascular smooth muscle cells and activates NO-sensitive guanylyl cyclase, leading to the conversion of GTP to cGMP, which induces relaxation by modifying intracellular calcium concentrations (Fadini and Avogaro, 2010).

NO modulates vascular and myocardial contraction. It has a very short biological half-life, which enables it to coordinate changes in vascular diameter throughout the vascular bed in response to changes in flow, and to optimize tissue perfusion relative to cardiac work under differing hemodynamic conditions (Henderson, 1998).

Inflammation

Inflammation is a host defence response intended to eliminate any cause of cellular injury, plus any necrotic cells and tissues resulting from the insult, and to initiate the process of repair. Under normal physiological conditions endothelial cells maintain an anti-inflammatory phenotype. Nitric oxide plays a major role in this by limiting adhesion of platelets and white blood cells to the endothelium and inhibiting cytokine-mediated release of prothrombotic substances such as P-selectin and vWF from the Weibel-Palade body (Libby et al., 2006). However, in response to pro-inflammatory cytokines and chemokines endothelial cells increase expression of adhesion molecules (E-selectin, P-selectin, ICAM-1, VCAM-1) on their surface. This causes leukocytes to bind to the endothelial surface, roll along it and then transmigrating into the surrounding tissues where they can act. The endothelium also secretes more cytokines, which further amplify the inflammatory response. There is NO mediated vasodilation, increasing blood flow to the affected area, and an increase in vascular permeability leading to tissue oedema. Endothelial cells have receptors for histamine, serotonin, bradykinin, thrombin and leukotriene C4. When these inflammatory mediators bind to their receptors there is an increase in cytosolic free calcium within the endothelial cells, which leads to contraction of the cytoskeleton and the opening of the intercellular junctions in specific segments of the vasculature, particularly postcapillary venules (Simionescu and Antohe, 2006).

The main transcription factor involved in the expression of pro-inflammatory proteins is nuclear factor kappa B (NF-κB). In the normal state it remains bound to an inhibitor protein, which restricts it to the cytoplasm, and NO plays a role in limiting its activation. However, exposure to cytokines such as IL-1 or TNF-α, ROS or endotoxin results in the release of active NF-κB and its translocation into the nucleus, where it induces the transcription of pro-inflammatory cytokines, adhesion molecules and chemokines (Aljada, 2003, Libby et al., 2006).

1.7.3 Changes to Endothelial Function in Disease

The pathophysiology of endothelial dysfunction is complex and involves multiple mechanisms. Endothelial cells respond to various stimuli, not simply as a target for injury, but by undergoing specific alterations in phenotype that change their function, metabolism, and structure. Some of these alterations are increases and decreases in normal constitutive functions, and others are due to induction of new functions and new molecules. This may directly influence both the evolution and the outcome of the response to injury (Cotran, 1987). Much discussion has taken place regarding whether the changes in endothelial function witnessed in various pathologies represents endothelial dysfunction or merely endothelial activation (Deanfield et al., 2007). The terms may be interchangeable, or may lie on a spectrum of severity that begins with endothelial activation, but can lead to endothelial dysfunction, damage and ultimately cell death.

A change in endothelial biology to exhibit a pro-coagulant, anti-fibrinolytic, and pro-inflammatory phenotype has been documented in many disease processes including atherosclerosis, hypertension, diabetes mellitus and cancer. Ongoing endothelial

activation leads to progression of disease. Conditions we recognize as risk factors for cardiovascular disease such as hyperglycaemia, obesity or metabolic syndrome, dyslipidaemia, sedentary lifestyle and smoking all act on the endothelium as pro-inflammatory triggers leading to a chronic state of activation. There are also more acute triggers including infection and trauma (Hartge et al., 2007, Aljada, 2003, Morganti et al., 2002).

Central to this common mechanism of endothelial activation or dysfunction is nitric oxide. The different injurious stimuli lead to a reduced bioavailability of NO, which firstly affects the ability of the endothelium to induce vasodilation. It also leads to a switch in signaling from NO-mediated silencing of cellular processes toward activation by redox signaling. Mitochondrial activity increases and transcription factor NF-κB enters the nucleus and induces the synthesis of adhesion molecules and inflammatory cytokines. The deficit in NO can be due to either defective eNOS expression or activity, or excessive NO degradation (Fadini and Avogaro, 2010). If there is a deficiency of the substrate L-arginine or the co factor tetrahydrobiopterin then eNOS generates reactive oxygen species (ROS) instead of NO. This is known as eNOS uncoupling and means that eNOS plays a major role in both the quiescent and the activated endothelial cell (Deanfield et al., 2007) (Figures 4 and 5).

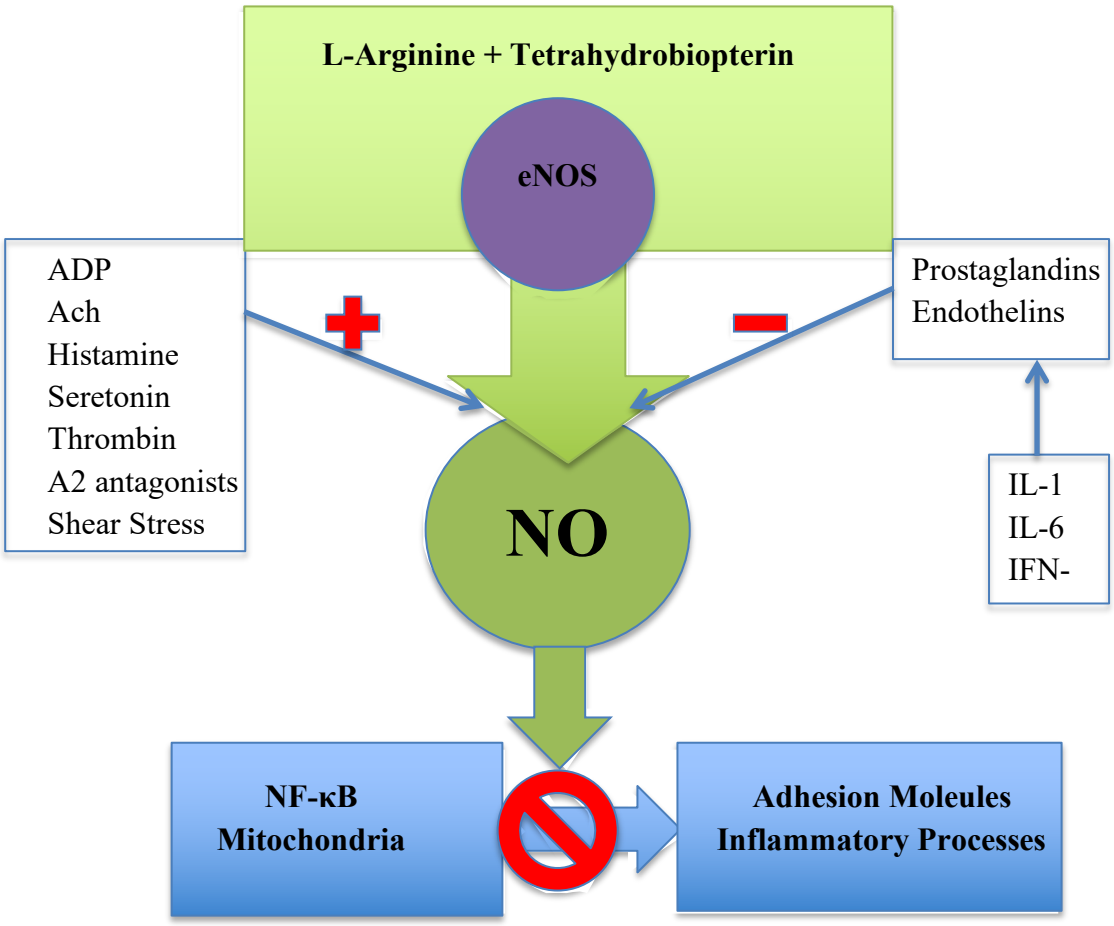


Figure 4. Normal production of NO by eNOS from the substrate L-Arginine in the presence of tetrahydrobiopterin. NO production is stimulated by a number of chemokines and shear stress, and inhibited by many prostaglandins and endothelins. NO targets cysteine groups in key regulatory proteins such as NF-κB and mitochondria which leads to silencing of cellular processes

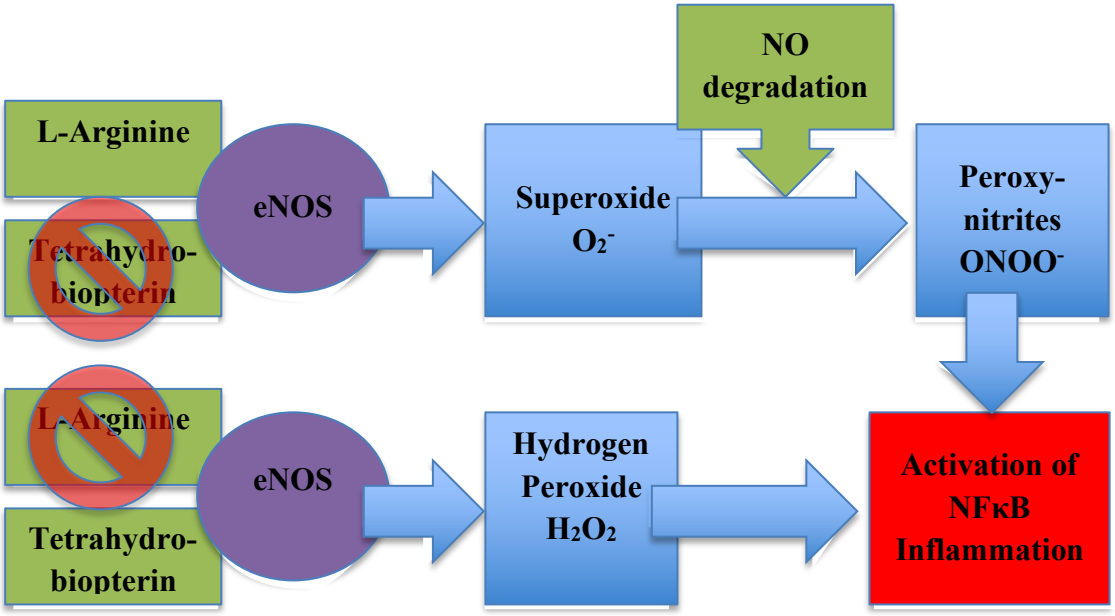


Figure 5. eNOS uncoupling leads to generation of reactive oxygen species instead of NO when either the substrate L-arginine or the key cofactor tetrahydrobiopterin are deficient. ROS then activate pro-inflammatory processes within the endothelial cells.

Circulating Endothelial Cells (CECs)

The presence of CECs in the blood of patients has become established as a useful marker of vascular damage. Current findings suggest that CECs are driven from the intima after vascular insult, and are thus the consequence (rather than the initiator) of a particular pathology. CEC detachment from the endothelium involves multiple factors including mechanical injury, alteration of endothelial cellular adhesion molecules, defective binding to anchoring matrix proteins and cellular apoptosis. The net effect is a reduced interaction between the EC and basement membrane proteins, with subsequent cellular detachment (Goon et al., 2006).

CECs are usually undetectable in the blood of healthy individuals, but counts become elevated in diseases hallmarked by the presence of vascular insult, such as cardiovascular disease (Makin et al., 2004, Lee et al., 2005, Mutin et al., 1999), ANCA (antineutrophil cytoplasmic antibody) associated small vessel vasculitis (Woywodt, 2002), Mediterranean Spotted Fever (George et al., 1993), septic shock (Mutunga et al., 2001) and neoplastic processes (Goon et al., 2006). Makin et al (2004) showed that numbers of CECs in peripheral blood became highly elevated in acute events such as myocardial infarction (MI), but were also elevated in chronic conditions such as ischaemic rest pain, above levels seen in both stable peripheral vascular disease and controls. In addition to this Lee et al (2005) showed a relationship between severity of insult and rise in number of CECs; levels were highest in ST elevation MI, then Non-ST elevation MI, then angina over controls. They also showed that failure of CEC levels to reduce to normal levels by 48 hours after acute MI was a predictor of poor outcome and major cardiac events at 30 days and one year. Higher CEC numbers were also demonstrated in patients that died of

septic shock than those that survived (Mutunga et al., 2001). In vasculitis and Mediterranean spotted fever CECs proved a useful marker of both disease severity, and of disease remission or response to treatment (Woywodt, 2002, George et al., 1993).

Endothelial Progenitor Cells (EPCs)

EPCs originate from the bone marrow, rather than from vessel walls, and are, like CECs, seen in low numbers in healthy individuals. Their numbers tend to increase following vascular injury (Goon et al., 2006), and they have been shown to home in to sites of endothelial damage and promote restoration of both anatomical and functional integrity (Fadini and Avogaro, 2010). Asahara et al first introduced the term EPC in 1997 (Asahara et al., 1997). They isolated CD34+ cells from human umbilical vein blood and cultured them under defined conditions. After several days, the cells displayed phenotypical and functional characteristics of endothelial cells. In an immuno-incompetent animal model of hind limb ischaemia, systemic injection of these cells significantly improved post-ischaemic function, and structure of the reperfused tissue. Microscopic analysis showed incorporation of the injected cells into the vessels' intima. This was the first time that vasculogenesis had been documented to occur in an adult vertebrate organism (Patschan et al., 2011).

1.7.4 Measuring Markers of Endothelial activation and damage as clinical indicators

EC activation is characterised by the synthesis and secretion of cytokines such as IL-1, IL-6, TNFα, angiotensin II and VEGF, the expression of cellular adhesion molecules (ICAM-1, VCAM-1, E selectin and P selectin), the release of chemokines, such as the monocyte chemoattractant protein-1 (MCP-1)(Sima et al., 2009), and pro-coagulants such as vWF (Kayal et al., 1998) and tissue factor (Makin et al., 2004). Components of the glycocalyx such as syndecan-1 and heparan sulphate are shed into the circulation (Schott 2016). There is also reduced NO-dependent vasodilation. Damaged endothelial cells can become detached from the vessel wall and enter the circulation whereupon they are known as circulating endothelial cells (CECs). In response to endothelial damage, endothelial progenitor cells (EPCs) are mobilized from the bone marrow to facilitate repair. All of the above can be measured as markers, allowing the degree of endothelial injury to be quantified. The methods which might be used in a study of response to trauma are discussed below.

Plasma and Tissue Markers

Levels of the markers of endothelial activation mentioned above can be measured in plasma or homogenised muscle tissue by enzyme-linked immunosorbent assay (ELISA). It is also possible to examine these markers by measuring the mRNA expression levels for the genes by QRT-PCR, following RNA extraction from muscle tissue samples.

NO-dependent vasodilation

Testing endothelial-dependent vasodilation involves physiological and/or pharmacological stimulation of endothelial release of NO. The response can then be compared to vascular responses when endothelium independent dilators such as nitroglycerine or specific NO antagonists such as L-NMMA (N-Monomethyl arginine) are used. More invasive measurements of coronary arteries have largely been superseded by flow-mediated dilatation (FMD) of the brachial artery. In this method brachial artery diameter is measured using ultrasound, before and after an increase in shear stress that is induced by reactive hyperaemia. A sphygmomanometer cuff is placed on the forearm distal to the brachial artery and is inflated to 200 mm Hg for 4 to 5 minutes then released. FMD then occurs predominantly as a result of the local endothelial release of NO (Deanfield et al., 2007).

Circulating Endothelial Cells (CECs)

Two main methods have evolved by which CECs can be enumerated; CD 146-based immunomagnetic separation and flow cytometry.

Immunomagnetic separation uses paramagnetic beads coated with antibodies directed against the CD146 molecule. The beads bind to CD146 bearing cells, allowing them to be separated from other cell types in a blood sample using a magnet. CD146 is a trans-membrane glycoprotein, which is constitutively expressed not only on endothelial cells, but also on pericytes, trophoblasts, mesenchymal stem cells, malignant tissues, and a subpopulation of activated T cells. Therefore, in order to reliably identify CECs immunomagnetic separation needs be accompanied by a

second step such as fluorescence microscopy after staining with endothelial cell specific Ulex-Europaeus-Lectin-1 (UEA-1) (Woywodt et al., 2006). Woywodt et al have devised a consensus protocol for CEC enumeration using this method. They define a CEC as a nucleated or anuclear cell that exceeds 10 μm in size and has more than 5 immunomagnetic beads attached. The rosetted cell stains positive with at least two endothelial markers (for example CD146 and UEA-1) and is also negative for leukocyte markers (e.g. CD14 and CD45). A CEC is negative for CD133 (unlike EPCs) and cannot give rise to colonies with a high proliferative potential.

The alternative method of CEC identification is flow cytometry. In this method all mononuclear cells from each blood sample are analysed for size, nuclear complexity, and binding of specific antibodies conjugated to different fluorochoromes (Fadini and Avogaro, 2010). Cell debris is excluded using a morphological gate. CECs are identified as positive for the nuclear stain Syto16 (excludes platelets), negative for CD45 (excludes hematopoietic cells), negative for CD133 (a progenitor cell marker), and positive for CD31 and CD146 (endothelial cell markers)(Mancuso et al., 2009).

The advantage of flow cytometry is that it is an automated process and is therefore less time consuming than immunomagnetic separation, which requires microscopic enumeration of the CECs. The disadvantage is that CECs only represent between 0.01% and 0.0001% of mononuclear cells, and as such, they are a relatively rare event. For accurate detection of rare events in flow cytometry, background “noise” must be substantially less than the frequency of the sought events. Background noise is the term used for other signals perceived by the cytometer to be fluorescent, in the absence of antibodies or fluorescent probes specific to the cells of interest. Thus, for

CECs, the cytometric assay needs to be greater than 99.999% free of background noise. It requires significant expertise, a period of preliminary standardization and a large number of cells to be counted in order to achieve this (Khan et al., 2005).

Endothelial Progenitor Cells (EPCs)

EPCs within peripheral blood can be enumerated by flow cytometry using CD34, CD133, and KDR (VEGFR2) as surface markers (Lekakis 2011). They are distinguished from CECs by their ability to form colonies in vitro. There are two major populations of EPCs which were originally referred to as "early outgrowth" and 'late outgrowth" EPCs due to the time it took for them to appear in culture. These terms have now largely been replaced by Haematopoietic and Non-haematopoietic EPCs as more about their functions have become known (Chong 2016). Haematopoietic EPCs are cultured on fibronectin-coated plates and appear after 5-7 days. Culturing the cells is a two-step procedure, in which the cells are first plated on fibronectin for two days. Nonadherent cells are then re-plated where they give rise to colonies. The colonies consist of rounded cells in the centre, surrounded by spindle-shaped cells in the periphery (Figure 6). These cells can be isolated from the circulation of humans and different animals. They express immature or "stem" cell marker molecules (CD133, c-Kit, and CD34), and markers of the endothelial lineage (KDR or Flk-1, CD31, eNOS). After systemic cell treatment of recipient animals, direct cell incorporation into the endothelial layer is rarely seen. These cells play a primarily supportive role in angiogenesis. They travel to sites of tissue ischaemia and secrete vasoprotective substances, thus acting indirectly. Angiogenic factors secreted by haematopoietic EPCs include CXCL12, CXCL1, and VEGF, with migration inhibitory factor, a potent cytokine known to induce endothelial and smooth muscle

differentiation. Their anti-ischaemic or pro-angiogenic benefit has been well documented in conditions such as ischemic heart disease, peripheral artery disease and cerebrovascular disease. (Patschan et al., 2011, Russell and Petters, 2010, Chong 2016).

Non-haematopoietic EPCs share more characteristics with mature endothelial cells. They can also be cultured from blood mononuclear cells. Culturing is performed on collagen type 2 coated plates in EBM-2 media and the cells appear in culture after a period of 2-3 weeks (Figure 7). Non-haematopoietic EPCs express endothelial cell marker molecules, but not hematopoietic ones. After systemic injection, non-haematopoietic EPCs are incorporated into the endothelial layer. The only difference between mature endothelial cells and non-haematopoietic EPCs is the much more pronounced in vitro proliferation of the latter. These EPCs are derived from non-haematopoietic tissue such as the walls of vessels residing in the bone marrow, from which they were shed into the circulation. Nevertheless, non-haematopoietic EPCs have been documented to be involved in neovascularization under both physiological and pathological conditions. In contrast to the indirect effects of haematopoietic EPCs, non-haematopoietic EPCs predominantly mediate direct endothelial regeneration, by incorporating into the vessel walls (Patschan et al., 2011).

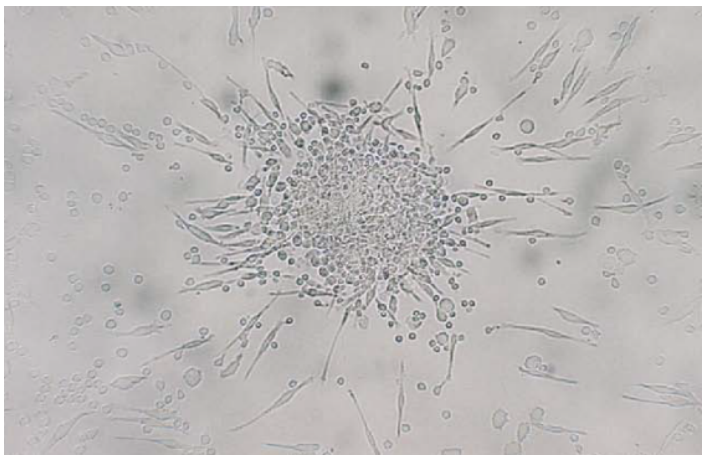


Figure 6. Early outgrowth (haematopoietic) EPC colony, also known as CFU-Hill Colony

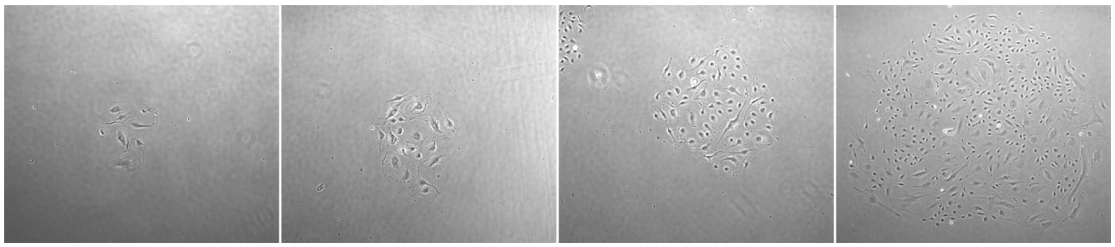


Figure 7. Late Outgrowth (non-haematopoietic) EPC colony.

1.8 Hypothesis

The Hypothesis of this study is that blast causes activation of and damage to vascular endothelium. We aim to test this hypothesis using an animal model of primary blast injury to the lower limbs. Markers of endothelial injury will be measured both systemically from blood samples and locally in the tissues at post mortem. It is postulated that if endothelial injury is identified, then treatments that improve endothelial function and repair in other disease states may be appropriate in the management of blast-injured casualties.

Chapter 2: Model Development and Methods

2.1 Introduction

In order to investigate the effects of primary blast injury on vascular endothelium a model was required that would provide localized injury to muscular tissue, its microvasculature and larger supplying vessels. It was desirable to avoid the systemic effects of a more global primary blast injury. The model also needed to provide sufficient blood volume and tissue samples for examination by several modalities.

2.2 Delivery of Blast wave

Choosing the right experimental model of blast injury

Large animal models of blast have been described (Garner et al., 2009, Garner et al., 2010), where a pig is exposed to a bare charge on an outdoor range. In this model the animal is protected from secondary and tertiary blast effects by Kevlar blankets. However, this is most definitely a global blast injury with widespread systemic effects, and the method would not be amenable to causing only a localized muscular injury.

Inside the laboratory several methods of delivering a blast wave have been described. These include chartaceous electricity detonators containing the explosive diazodinitrophenol (Ning et al., 2012, Ning et al., 2014), a shock tube (Gorbunov et

al., 1997) and a compressed air blast wave generator (Jaffin et al., 1987). The explosion caused by the chartaceous electricity detonators has been reported to cause a localized but severe blast injury, which included bone fractures, skin burns and some systemic sequaelae. The shock tube produces a pure blast shock wave by releasing compressed air when a firing pin pierces an acetate sheet. This, however, provides a global injury to smaller animals such as rabbit, rat and mouse.

For these reasons a compressed air blast wave generator was chosen to deliver the blast wave in this study. It is a bench top device that was developed at DSTL Porton Down (see figure 1 and 2). It is particularly useful at providing blast waves focused on a specific area of the body and allows detailed continuous physiological monitoring whilst the blast is being applied. It has previously been shown to cause typical patterns of primary blast injury when directed over the chest or abdomen of animals (Jaffin et al., 1987, Ohnishi et al., 2001, Sawdon et al., 2002, Guy et al., 1998). The injuries can be reliably reproduced, safely in a laboratory environment, at relatively low cost.

Administration of Blast Wave

The compressed air device uses a cylinder of compressed air to pressurize a chamber to a predetermined level (120kPa). A solenoid-controlled valve is then operated to release this pressure into the blast nozzle, which is sealed with an aluminium disc at the base. The pressure causes the aluminium disc to rupture, directing a shock wave towards the subject beneath (see figures 8 and 9).

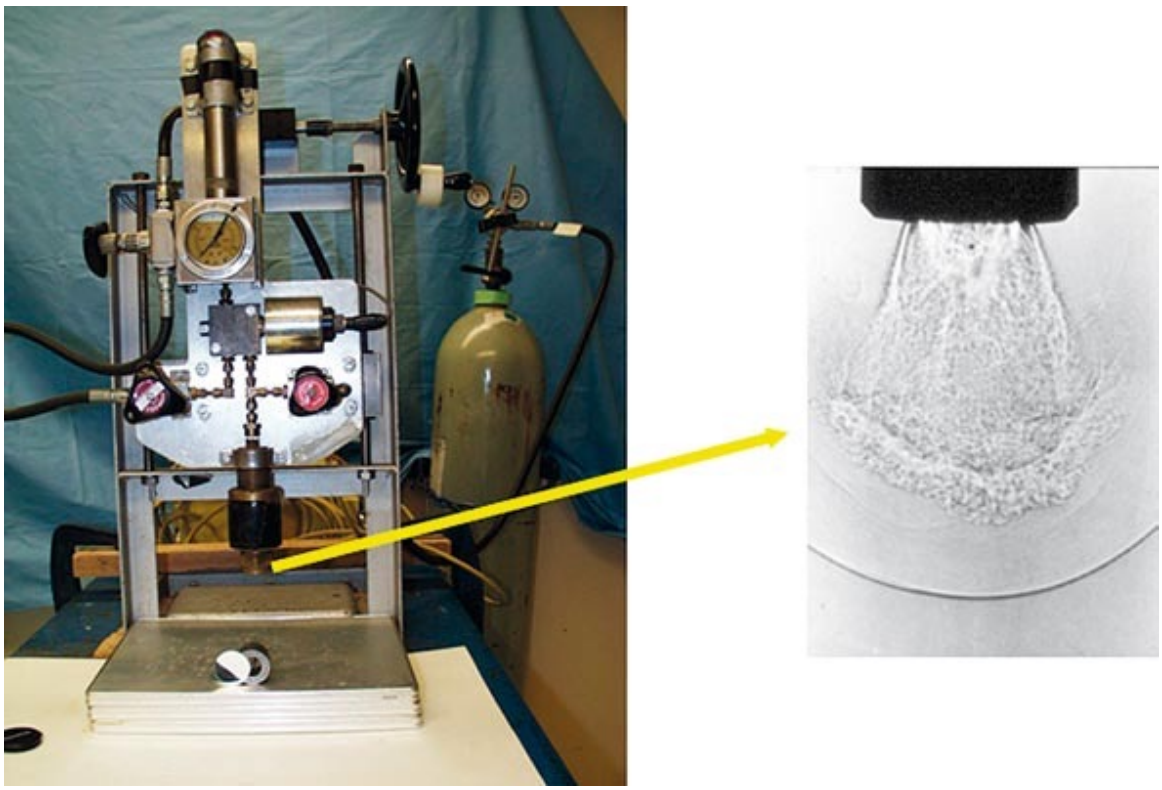


Figure 8. The Bench top blast wave generator used at DSTL Porton Down, and the shockwave it generates captured by high-speed photography.

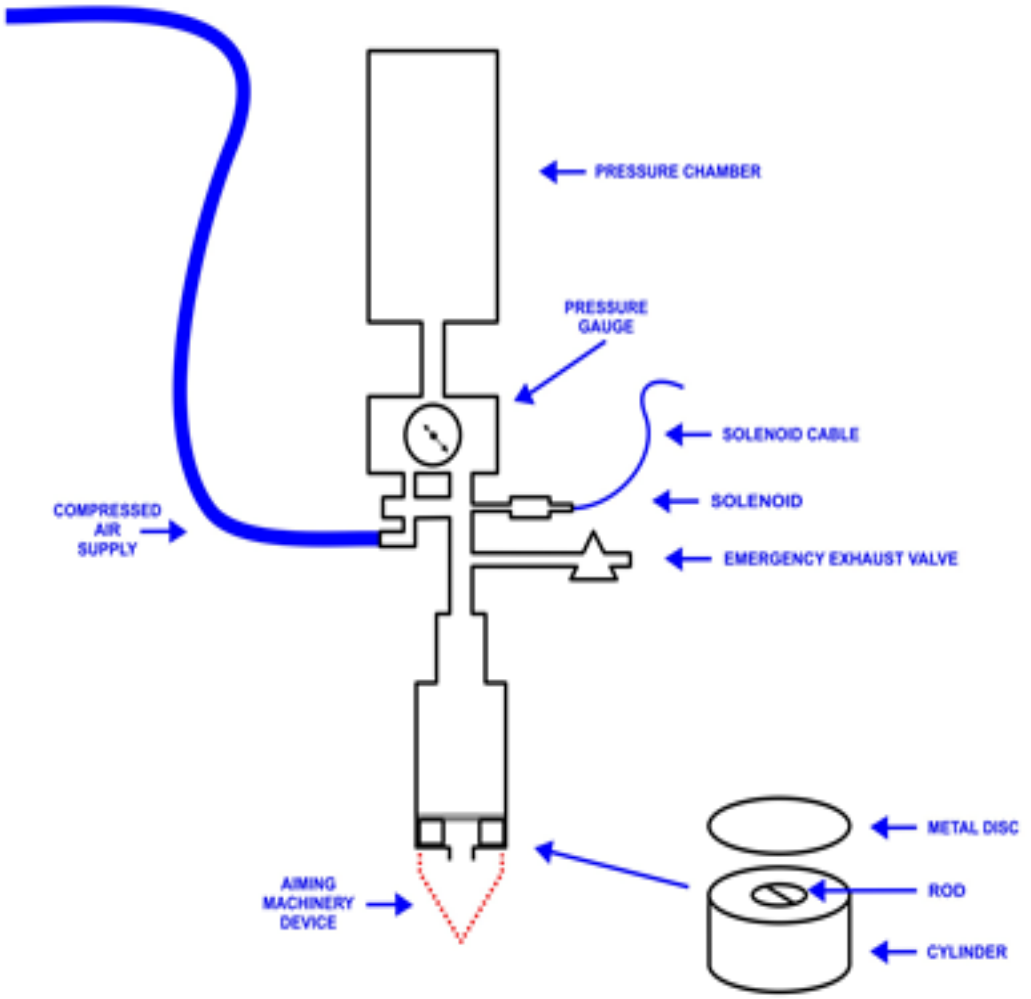


Figure 9. Schematic drawing of the blast wave generator.

In order to alter the force of the blast to which the subject is exposed, the distance between the subject and the blast nozzle can be changed (Jaffin et al., 1987). A smaller distance from the blast nozzle confers a higher blast exposure. In this study the subject was 1.5cm from the blast nozzle for the high blast dose, 2.5cm for the medium blast dose and 5cm for the low blast dose. On each experimental day blast pressures from the compressed air device were checked to ensure consistency at the randomised blast distance (minimum n=3 exposures per distance per day). This calibration was carried out using a "plastic rat" – a high frequency piezoelectronic pressure transducer (MQ20) within a rat-shaped casing - amplified by a 10mV amplifier and analyzed via a data capture system (Sigma 30 digital oscilloscope). Figure 10 and table 1 show the mean values and 95% confidence intervals of all the peak overpressures recorded for the 3 different blast doses/ distances from the blast nozzle.

Animals were randomised (via Excel randomisation table) to receive 1 of 4 (sham, low, medium or high) blast loads to the hind limbs. With the animal in the prone position, each hind limb of each animal received 5 shock waves of the same magnitude directed at specified points over the gastrocnemius muscle. The shock waves were directed approximately 1cm apart in a pre-marked 'T' shape in order to expose the whole caudal aspect of the limb between the stifle (knee) and hock (ankle)(figure 11).

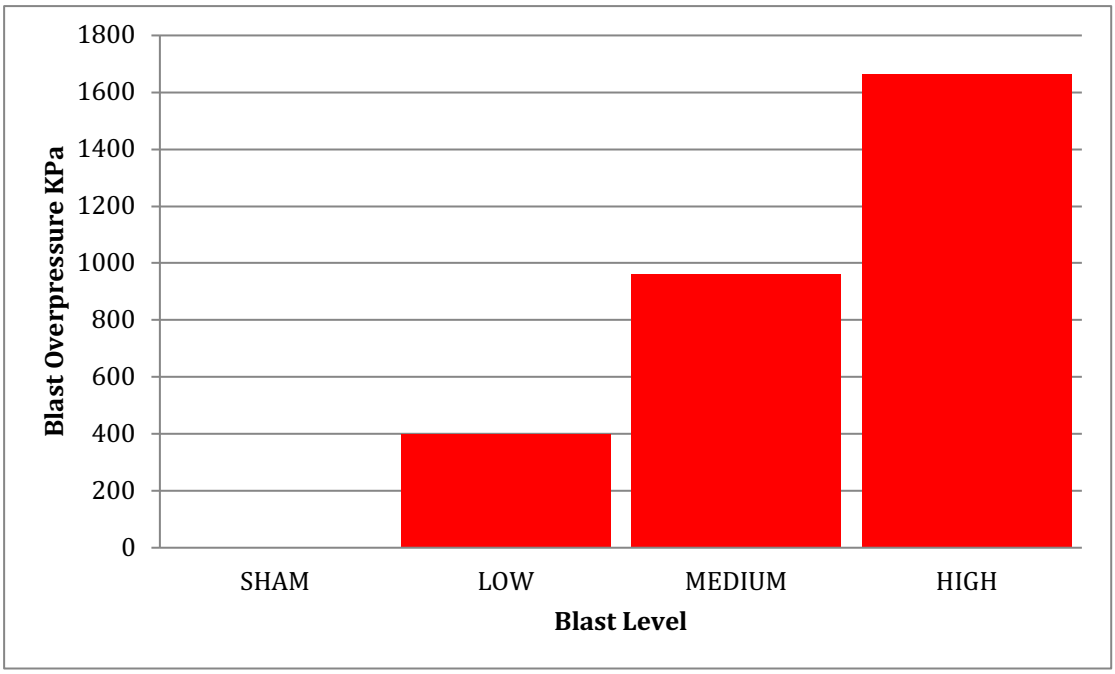


Figure 10. Graph depicting the mean blast overpressures for each level of blast exposure, as captured by pressure sensor on each experimental day. A corresponding table of values can also be found in the appendix.

Group	Mean Peak Overpressure (95% CI) kPa	Mean Impulse at 1.5mS duration (95% CI) sPa
High	1665 (1604-1725)	420.5 (412.8-428.2)
Medium	962.6 (897.2-1028)	256.4 (241.7-271.2)
Low	398.1 (356.4-439.8)	119.7 (110.3-129.2)
Sham	0	0

Table 1. *Measurement of the blast overpressure and impulse output from the compressed air blast wave generator*

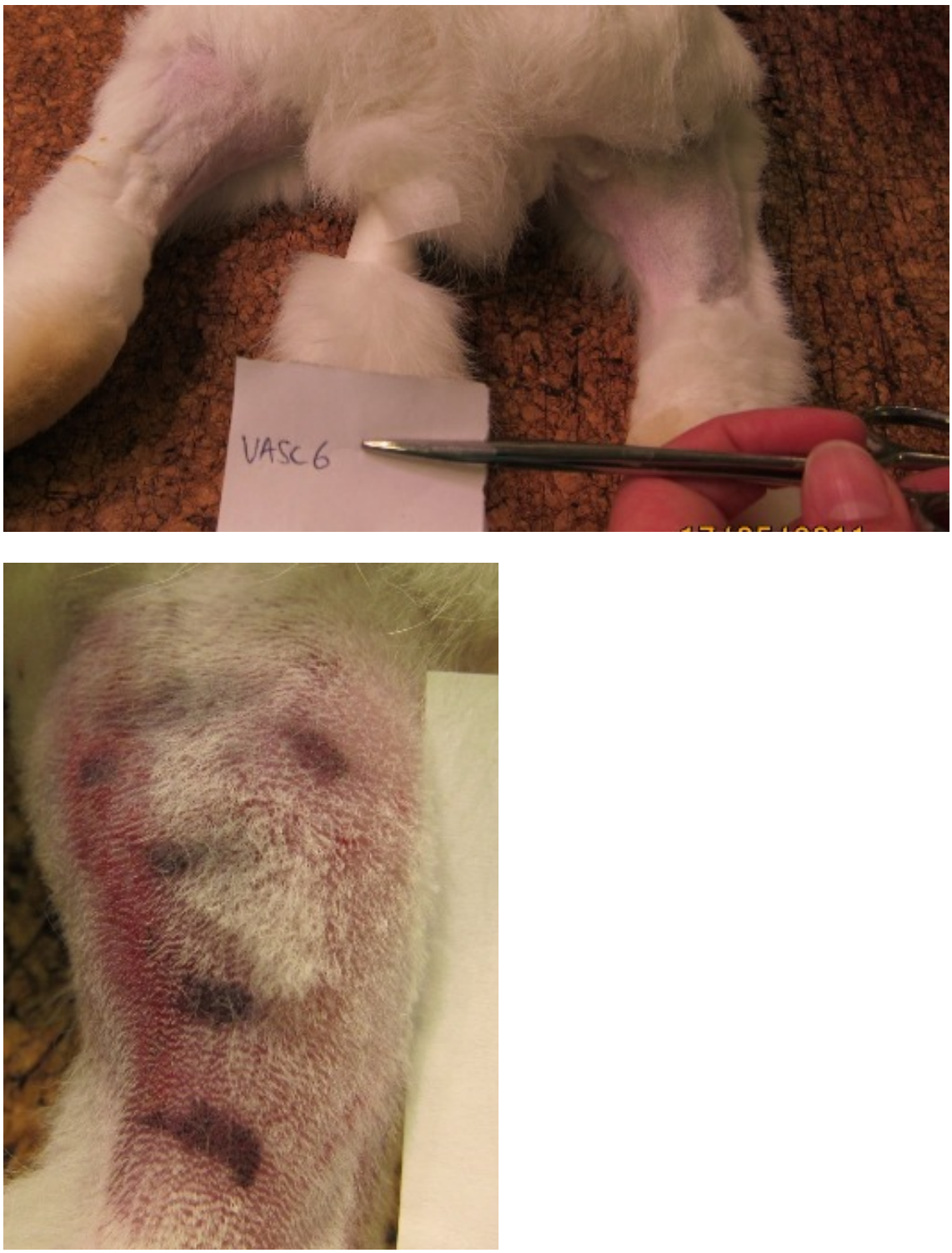


Figure 11. The rabbits were placed in the prone position, and the fur of the hindlimbs overlying the gastrocnemius was shaved. Five shock waves of the same magnitude were directed at the marked points 1cm apart.

2.3 Animal Selection and Husbandry

Rabbits were selected for this study. Smaller animals were discounted as they were unable to provide sufficient samples of blood and tissue for examination. Using a rabbit meant that it was still possible to administer blast via a compressed air device, with the advantages of giving a localized injury to muscle tissue, plus having adequate blood and tissue volumes for sampling. The cost implications and potential systemic interactions of using a larger mammal model of blast injury were thus avoided. The only disadvantage in choosing a rabbit model is the potential limitation of available test kits for biomarkers, as many antibodies are raised in rabbits and therefore cannot be used to process rabbit samples.

Female New Zealand white rabbits with an average weight of 2.52Kg (range 2.00-3.52Kg) were used for this study. Females were chosen as they can be housed together with less risk of fighting and therefore injury prior to the study. Each animal spent a minimum of one week acclimatisation at DSTL Porton Down before use. They were housed in pairs in a large comfortable floor pen, with sawdust and hay bedding mix, at a temperature of 16 to 20°C. There was a natural day/night light cycle with 12 hours light/dark with 30 minutes dawn and dusk. Animals had free access to water and rabbit food (Harlan Teklad 2030 irradiated global rabbit diet), with a tropical forage mix scattered through bedding for enrichment.

The research was subject to formal ethical review process and conducted under a United Kingdom Home Office Licence and in accordance with the Animals (Scientific procedures) Act, 1986. This author and all other investigators were in

possession of an appropriate Home Office Licence. All experiments were carried out under terminal anaesthesia with the assistance of a qualified veterinary surgeon.

2.4 Anaesthesia

Prior to induction of anaesthesia the rabbits received a premedication of midazolam (1-2mg/Kg) by intramuscular injection and were placed under a heat lamp for 15 minutes to allow vasodilation of the ear veins whilst the sedative took effect. The ear was then shaved and a 24G cannula placed into the lateral ear vein and secured. The rabbits were preoxygenated via a face mask and then anaesthetized with an intravenous injection of alfaxalone at 1-2mg/Kg. A size 3 uncuffed endotracheal tube was then passed into the trachea and secured. Correct positioning was confirmed by a positive end tidal CO_2 trace. A surgical plane of anaesthesia was maintained throughout the study period using inhaled Isofluorane (a volatile anaesthetic agent), which was delivered alongside oxygen and nitrous oxide (50:50) via an anaesthetic circuit. At the end of the 12 hour study the animals were culled using IV injection of phenobarbitone without ever regaining consciousness.

2.5 Instrumentation

Using aseptic technique, the left carotid artery was surgically cannulated to allow blood pressure monitoring and blood sampling. Neck dissection was performed to expose the carotid sheath and a 6F catheter was inserted directly into the carotid artery via an arteriotomy. The catheter was flushed and secured, and once its functionality was established the neck wound was closed. Invasive blood pressure, heart rate and end-tidal CO_2 were monitored using a Propac®CS system

(WelchAllyn, Buckinghamshire, UK). An infusion of 0.9% sodium chloride was infused at a rate of 6ml/kg/hr to replace insensible losses. Body temperature was monitored and maintained using homoeothermic blanket system incorporating real-time feedback loop via a flexible rectal thermometer and heat mat (Harvard Apparatus Ltd, Kent, UK).

2.6 Blood Sampling

Following a period of 30 minutes post-surgery acclimatisation, baseline pre-injury blood samples were taken via the carotid catheter. Blood samples were taken at pre-determined time points for the analysis of markers of endothelial damage. Blood samples for CECs were taken at baseline, 1hour post-injury, 6 hours post-injury and 11 hours post-injury. Blood samples for EPCs were taken at baseline and 11 hours post-injury. Blood samples for all other analysis were taken at baseline, 1, 3, 6 and 12 hours post-injury (see table 2).

Time Point	HAEM 0.5ml EDTA	CECs 1.0ml EDTA	EPCs 1.0ml EDTA	Markers 2.0ml Hep
surgery				
30 min post surgery				
Baseline				
1hr post blast				
2hr post blast				
3hr post blast				
4hr post blast				
5hr post blast				
6hr post blast				
7hr post blast				
8 hr post blast				
9hr post blast				
10 hr post blast				
11 hr post blast				
12 hr post blast				

Table 2. Schedule of blood sampling time points.

2.7 Post Mortem Examination

At the end of the study post mortem tissue samples were collected and stored as appropriate for ELISA, RNA extraction and histological examination by light microscopy (see table 2). The heart, lungs, kidney, liver and spleen of each animal were collected and placed into 10% neutral buffered formalin (NBF) for histological examination to exclude any systemic blast effects. Muscle tissue from the forelimb that had not been subjected to blast was also taken and placed in NBF.

The gastrocnemius muscle from each hind limb and the overlying saphenous neuro-vascular bundle were excised and divided as follows for further examination (see table 3).

Tissue	Storage Method	Tissue Analysis
Left Gastrocnemius (Half)	3 x 0.25cm pieces in RNAlater	RT-PCR
Left Gastrocnemius (Half)	3 x 1cm pieces for freezing -80C	ELISA
Right Gastrocnemius (Half)	Whole in NBF	Histology
Right Gastrocnemius (Half)	3mm strips in Glutaraldehyde	EM
Left Saphenous Vessels	Whole in NBF	Histology
Right Saphenous Vessels	Whole in Glutaraldehyde	EM

Table 3. Storage of tissues taken at post mortem for further examination

2.8 Isolation and Enumeration of Circulating Endothelial Cells

1 ml blood was taken to determine levels of circulating endothelial cells (CECs), using a modified CD146-based immunomagnetic separation method as described by Woywodt (Woywodt et al., 2006)(figure 12). Blood (1 ml) was diluted with the same volume of buffer A (0.1% (v/v) BSA, 0.1% (v/v) sodium azide, 0.6% sodium citrate (v/v) in PBS) and incubated with a mouse anti-rabbit CD146 antibody (Millipore, UK) for 20 min at 4°C with gentle end-over-end rotation. The cells were then centrifuged (300 x g, 8 min) and resuspended in 2 ml buffer B (0.1% (v/v) BSA in PBS) and incubated for 20 min at 4°C with 25 µl pan mouse-IgG Dynabeads (Invitrogen, UK) with rotation as before. The volume of buffer B was then doubled and tubes placed in a magnet for 2 min. The supernatant was discarded and cells washed 4 times in buffer B. The cells were flushed through a 100 µl pipette tip 10 times during the final wash. The cells were then resuspended in 500 µl ice-cold methanol and incubated at 4°C for 10 min with gentle mixing as before. The cells were then washed 3 times as before, resuspended in 1 ml buffer B and stored at 4°C for staining and visualisation the following day. For staining and enumeration cells were placed in a magnet for 1 min, the supernatant discarded and they were resuspended in 100 µl 2mg/ml FITC-coupled UEA-1 solution (Sigma-Aldrich) and incubated for 1 hour at 4°C with gentle mixing as before (in the dark). The sample was then washed twice in buffer B and the cell-bead suspension finally dissolved in 200 µl buffer B. CECs were counted with fluorescence microscopy at 553 nm using a haemocytometer. They were defined as cells 10-50 µM in size with 5 or more magnetic beads attached and stained with UEA-1-FITC (see figure 13).

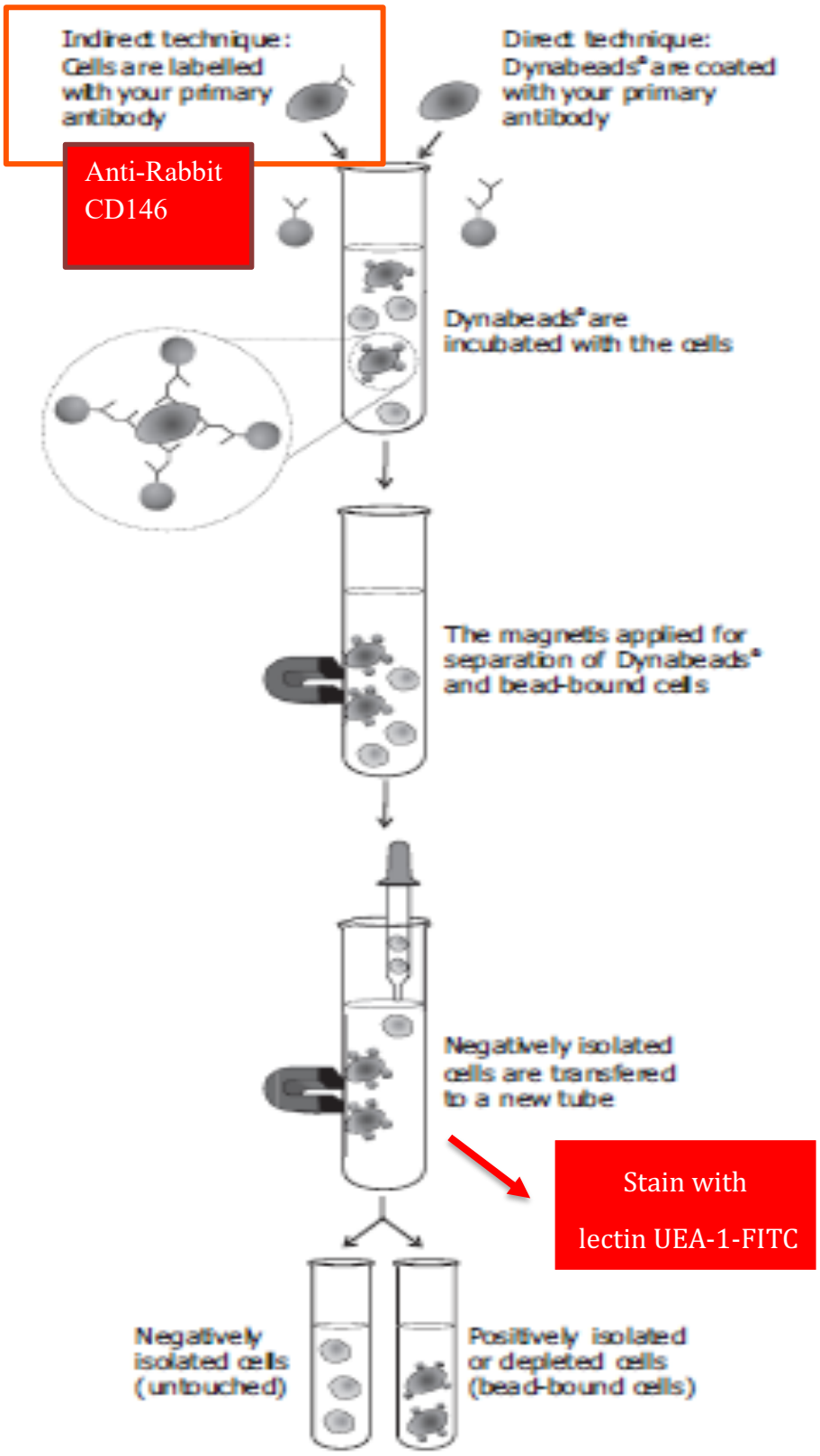


Figure 12. Immunomagnetic separation technique for CECs

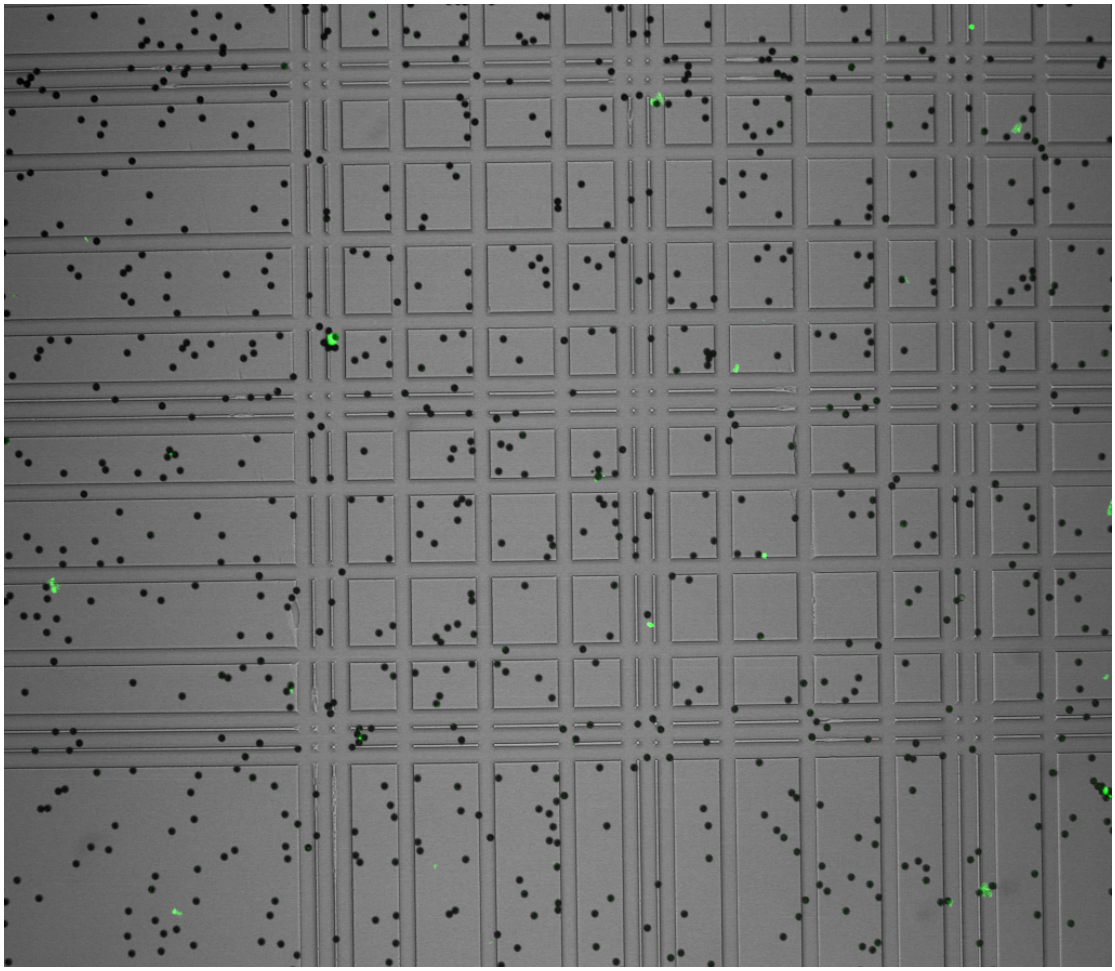


Figure 13. Gridded haemocytometer with visible dynabeads and fluorescing cells.

2.9 Isolation and Enumeration of Endothelial Progenitor Cells

1ml of blood was taken for assessment of endothelial progenitor cells. This was diluted 1:1 with PBS. 10µl cells were removed into an eppendorf for total cell count. Mononuclear cells were obtained by Ficoll separation (Healthcare, 2008). The sample was overlaid onto Ficoll-Premium in FACS tubes and centrifuged at 400 x g for 40 minutes at 18-20°C. The upper layer of plasma and platelets was then removed, and the layer of mononuclear cells transferred to a Falcon tube using a sterile pipette. The mononuclear cells were then suspended in 3 volumes of PBS and centrifuged at 100 x g for 15 min at 18-20°C. The supernatant was removed and the mononuclear cells were resuspended in 5ml CFU-Hill growth medium (Stemcell technologies Ltd.) and centrifuged at 100 x g for 10 minutes at 18-20°C. Again, the supernatant was removed and the cell pellet was resuspended in 2ml CFU-Hill growth medium. A further 10µl of the sample was removed into an eppendorf for counting. The mononuclear cells were then plated onto a 6-well plate coated with fibronectin at 5 x 10^6 cells per well, and the total volume of media made up to 3ml per well. The plates were incubated at 37°C, 5%CO_2, >95% humidity for 48 hours.

After 48 hours, non-adherent cells were removed from the 6-well plate, counted and re-plated, 10^6 cells per well, in a 24-well plate coated with fibronectin, in 1ml CFU-Hill growth medium. They were then incubated at 37°C, 5%CO_2, >95% humidity for a further 3 days for growth of CFU-Hill colonies (early outgrowth EPCs).

Adherent cells were kept in the 6-well plate for growth of endothelial cell forming colonies (ECFCs/ late outgrowth EPCs). 3mls of ECFC complete medium was added

and the plates were incubated at 37°C, 5%CO_2, >95% humidity, with media change every few days for up to 30 days.

After 5 days the media was removed from the CFU-Hill cells in the 24-well plate. The cells were washed with PBS prior to fixation in 300µl pre-chilled methanol. After 5 minutes the methanol was removed and 300µl Giemsa staining solution diluted 1/20 in dH_2O was added. After 5 minutes incubation the solution was aspirated and cells were rinsed with water. Colonies were enumerated under a standard light microscope.

2.10 Histology

The right gastrocnemius muscle was removed post mortem for histological analysis. The muscle tissue was placed in 10% neutral buffered formalin (NBF) for analysis using light microscopy (LM). Saphenous blood vessels from both hind limbs were dissected away from the surrounding tissue and samples proximal to the area of maximum blast exposure taken for histological processing and analysis. Tissues were fixed in NBF for at least 72 hours prior to histochemical processing for LM. Muscle tissues were sectioned longitudinally to allow maximal extent of the injury to be assessed. Blood vessels were sectioned transversely to assess the pathology in a cross section through each vessel. Ten serial sections of each tissue were prepared on glass microscope slides with the first and last tissue sections in each series processed for H&E staining and the remaining sections retained for immunohistochemical staining protocols. Sections were stained with haematoxylin and eosin (H&E) and examined using an Axioscop microscope for pathology scoring. Representative images were captured using an Axiocam MC5 digital camera and Axiovision software. A

histologist blinded to the blast loading groups carried out the pathology scoring. Incidence of haemorrhage, inflammatory cell infiltration and oedema were subjectively recorded as either not present (score=0), mild (score=1), moderate (score=2), or severe (score=4). The maximum percentage of muscle fibre bundles displaying evidence of necrosis was subjectively estimated in each section.

2.11 ELISA

ELISA was used to look at plasma and tissue concentrations of markers of endothelial activation. Blood (2 ml) was collected into EDTA tubes and rolled for 5 min at room temperature before centrifugation at 13,000 rpm for 5 min. The plasma was then aliquoted into tubes and stored at -80°C until use. For tissue ELISA, one half of the left gastrocnemius was divided into three equal portions and snap frozen in liquid nitrogen and stored at -80°C until required. Tissue samples were homogenised and muscle protein extracted. All samples were assayed in duplicate using ELISA kits from Cusabio Ltd (China) as per manufacturer's instructions. The sandwich ELISA assay procedure used is summarised below, and a full version of the manufacturer's instructions for the vWF ELISA is included in the appendix. Table 4 shows which ELISAs were performed and the detection limits for the different markers.

Summary of Assay Procedure

1. Coat the wells of a PVC microtiter plate with the capture antibody at a concentration of 1-10µg/ml in carbonate/bicarbonate buffer (pH7.4). Seal the plate and incubate overnight at 4°C or 2h at room temperature.

2. Wash plate 3 times with PBS.

3. Block the remaining protein-binding sites in the coated wells by adding 200µl blocking buffer/PBS per well.

4. Cover the plate with an adhesive plastic and incubate for at least 1-2h at room temperature or, if more convenient, overnight at 4°C.

5. Add 100µl of appropriately diluted samples to each well. For accurate quantitative results, always compare signal of unknown samples against those of a standard curve. Standards (duplicates or triplicates) and blank must be run with each plate to ensure accuracy. Incubate for 90 min at 37°C.

6. Wash the plate twice with PBS.

7. Add 100µl of diluted detection antibody to each well.

8. Cover the plate with an adhesive plastic and incubate for 2h at room temperature.

9. Wash the plate 4 times with PBS.

10. Add 100µl of secondary antibody conjugated, diluted at the in blocking buffer immediately before use.

11. Cover the plate with an adhesive plastic and incubate for 1-2h at room temperature.

12. Wash the plate 5 times with PBS.

13. Dispense 100µl (or 50µl) of the substrate solution per well with a multichannel pipette

14. After sufficient colour development add 50-100µl of stop solution to the wells.

15. Record the absorbance at 450 nm on a plate reader within 30min of stopping the reaction.

Marker	Plasma ELISA	Tissue ELISA	Detection Range
vWF	X		0.312ng/mL- 20ng/mL
ICAM		X	0.625ng/mL- 40ng/mL
VCAM		X	78pg/mL-5000pg/mL
SDF-1	X	X	0.57ng/mL-10ng/mL
ET-1	X	X	6.25pg/mL-400pg/mL
Soluble Thrombomodulin	X		
P-selectin		X	31.25pg/mL-2000pg/mL
E-selectin	X	X	

Table 4. Table to summarise which markers were tested for by ELISA and their different detection ranges where available.

2.12 qRT-PCR

Levels of mRNA for markers of endothelial activation were determined using qRT-PCR. The left gastrocnemius was excised post-mortem and cut in half along its length. One half was divided into three 0.25cm^3 sections and placed into RNAlater® (Sigma-Aldrich) at 4°C overnight followed by freezing at -80°C the following day until required. RNAlater is a tissue storage reagent that rapidly permeates tissue to stabilise and protect cellular RNA in situ, eliminating the need to immediately process tissue specimens.

RNA was extracted from muscle using a hybrid Trizol/RNeasy kit (Qiagen) protocol as detailed (see appendix). Following extraction RNA quantity and quality was assessed using NanoDrop 1000 and Bioanalyser (BioRad). All RNA Integrity Numbers (RIN) were > 8.8. A RIN >5 is considered good total RNA quality and >8 perfect total RNA quality for obtaining meaningful gene expression data (Fleige and Pfaffl, 2006). Any contaminating DNA was removed using Turbo DNase as per manufacturer's instructions (Applied Biosystems) and RNA re-assessed after DNase treatment. All A260/280 ratios were between 1.9 and 2.0. An optical density 260nm/280nm ratio greater than 1.8 is usually considered an acceptable indicator of good RNA quality (Fleige and Pfaffl, 2006). The RNA was used in a reverse transcription reaction using SuperScript III (Invitrogen) to manufacturer's instructions with random hexamer primers. cDNA was diluted 1:5 with nuclease-free water for PCR and stored at -20°C during regular use and at -80°C for longer-term storage. Real-time PCR was carried out using Taqman-style probe technology with primers and probes designed, synthesised and tested by PrimerDesign Ltd (Southampton, UK). For each PCR reaction 5 µl cDNA was used with 15 µl PrecisionR mastermix in

duplicate with the following cycling conditions: 95°C 12 min, 40 cycles of 95°C 15 s, 60°C 1 min followed by a hold at 4°C. CNRQ (calibrated normalised relative quantities) were calculated from Cq values using qBasePlus (Biogazelle Ltd., Belgium). Samples were normalised to the three most stable reference genes as determined by a GeNorm experiment and calibrated to the average of all samples. The efficiency of the PCR reaction for each gene was determined using a standard curve comprised of pooled cDNA from at least 15 animals.

2.13 Statistics

Differences in CECs between groups were assessed with 2 way ANOVA test ($p<0.05$ and power = 0.8). Incidence of haemorrhage, oedema and inflammatory cell infiltration were subjectively recorded as detailed above. Maximum percentage of muscle fibre necrosis was estimated as detailed above. These scores were assessed using a Cuzicks trend test ($p<0.05$). Gene expression data (CNRQs) were logged transformed before being assessed with one-way ANOVA with Dunnett's post-test compared to Sham.

Chapter 3: Results

A total of 38 female New Zealand white rabbits were used in this study. All of them survived to the twelve-hour point at the end of the experiment. Throughout the time course of the experiment all animals maintained stable physiological parameters and did not demonstrate the apnoea, bradycardia and hypotension seen following a blast directed over the chest (Kirkman and Watts, 2011). A table detailing the blast exposure for each animal, the corresponding distance from the nozzle of the blast apparatus, which of 2 blast apparatus was used, the surgeon and operators of the blast apparatus can be found in the appendix (table 1).

3.1 Circulating Endothelial Cells

Circulating endothelial cells are present in very low numbers in healthy individuals. Their levels have never previously been measured in a model of blast injury. They have, however, been identified as a reliable marker of endothelial injury in a range of other conditions including cardiovascular disease (Makin et al., 2004, Lee et al., 2005, Mutin et al., 1999), ANCA associated small vessel vasculitis (Woywodt, 2002), Mediterranean Spotted Fever (George et al., 1993) and septic shock (Mutunga et al., 2001). Levels have also been shown to increase after stress and exercise (Boos et al., 2008). Therefore, raised levels of CECs in the blood can be interpreted as endothelial damage.

Levels of circulating endothelial cells were measured in the four different blast exposure groups (sham, low, medium and high). Blood samples were taken at

baseline, and at 1, 6 and 11 hours post blast injury. CECs were extracted by CD146 based immunomagnetic separation and fixed with methanol, then stained with UEA-1-FITC and enumerated with fluorescence microscopy at 553nm, as described in the methods section. Figure 14 shows some microscopic images of CECs on the gridded haemocytometer, demonstrating the criteria for identification. Eleven animals were required to develop the CEC enumeration technique in the rabbit, therefore only data from 27 animals (sham n=6, low n=6, medium n=6 high n=9) was used for the CEC analysis.

In the sham, low and medium blast groups there was no increase in CEC numbers over time, when compared with the baseline sample, nor was there a significant difference between those groups. However, in the high blast group the number of CECs in the blood at 6 hours was significantly higher than either at base line or at 1 hour post blast. The 6 hour CEC levels in the high blast group was also significantly higher than the sham group at the same time point (Figures 15 and 16). These findings indicate a greater amount of endothelial damage in the high blast group, with CECs reaching their highest level in the blood stream at 6 hours post blast injury.

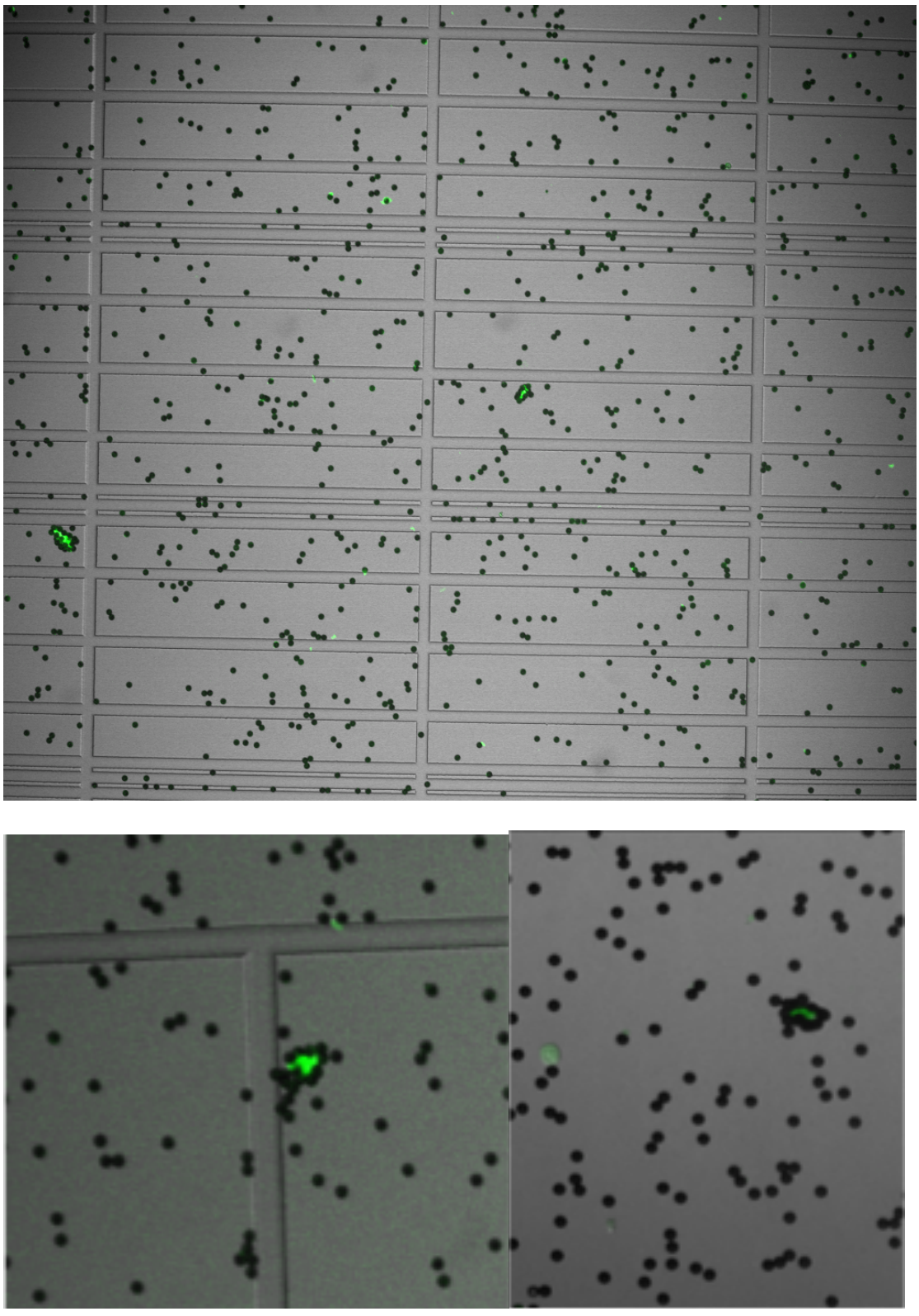


Figure 14. Examples of CECs stained with FITC-UEA-1 and with 5 or more 4 μm magnetic beads attached.

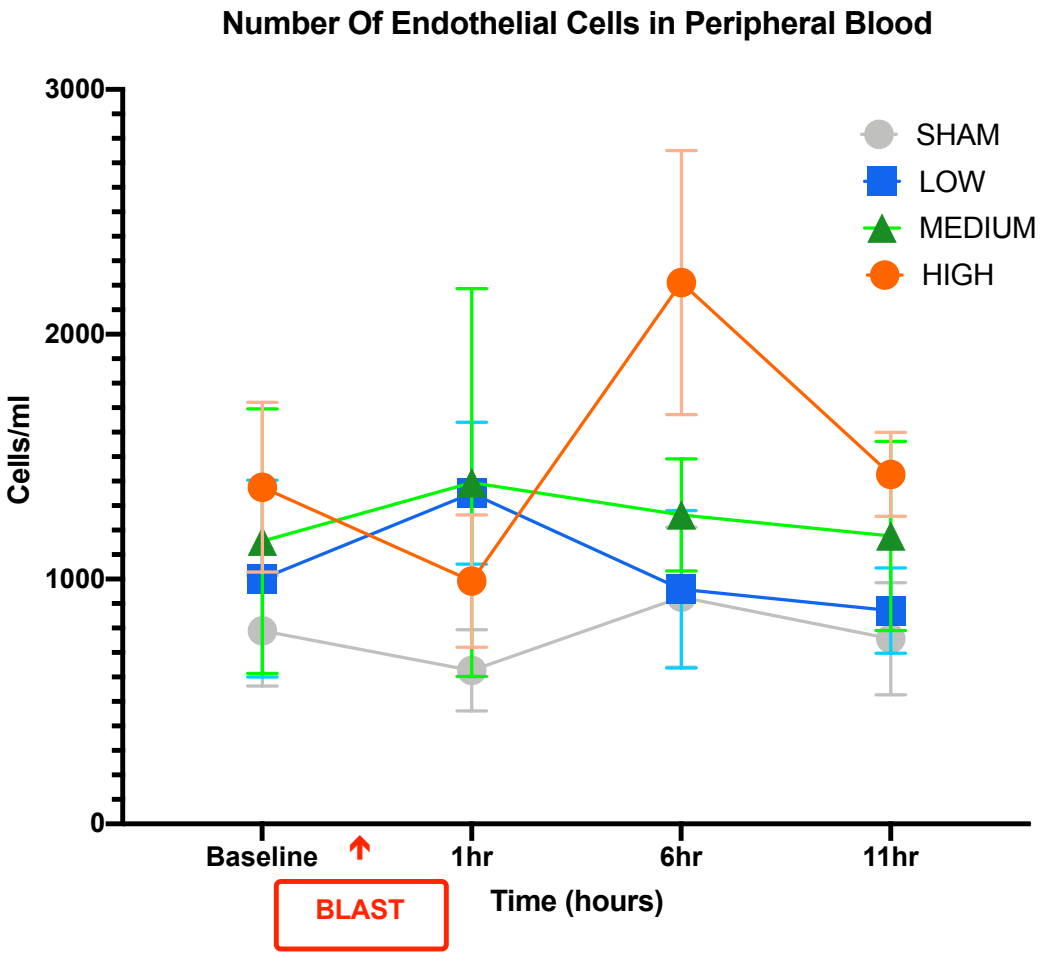


*Figure 15. A Graph to show the effect of time and of blast treatment group on the number of CECs per ml of peripheral blood. Rabbits were exposed to different levels of blast (Sham, Low, Medium, High) and blood drawn at 4 time points. Two-way ANOVA showed that the number of CECs varied significantly with blast treatment ($p<0.05$) and that High was significantly different from Sham overall ($p<0.05$). *=$p<0.05$ for High at 6h compared to High at 1h and Sham at 6 h by post-hoc Tukey test. Data shown are mean ± SEM for n=6(Sham, Low, Medium) or n=9 (High) rabbits.*

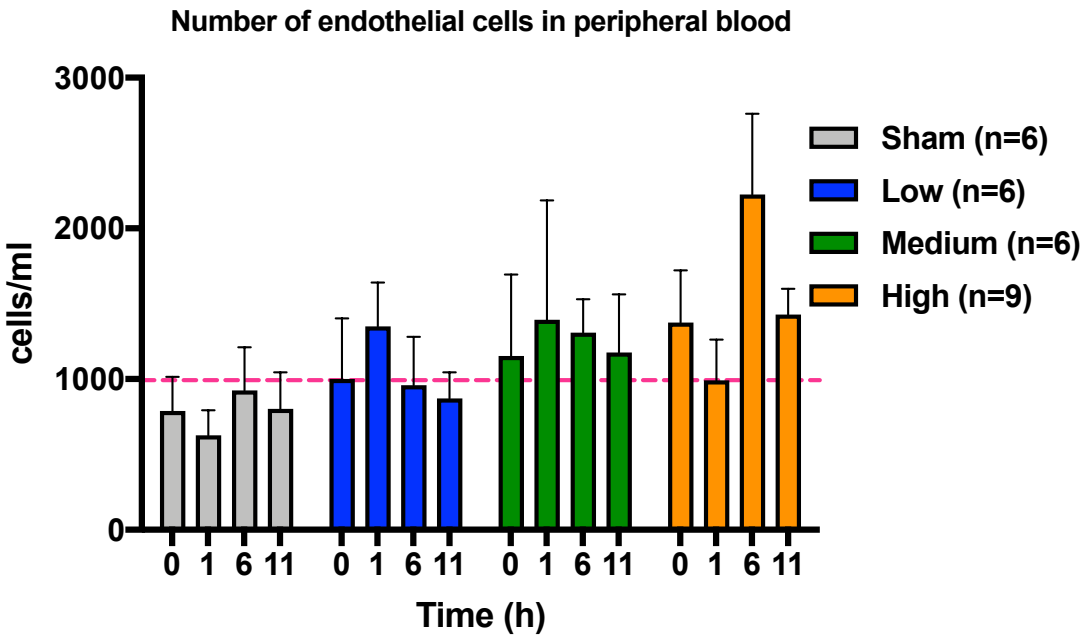


*Figure 16. Alternative representation of the data in figure 14 as a grouped bar graph to better show how the CEC numbers varied within each treatment group with time. Two-way ANOVA showed that the number of CECs varied significantly with blast treatment ($p<0.05$) and that High was significantly different from Sham overall ($p<0.05$). *=$p<0.05$ for High at 6h compared to High at 1h and Sham at 6 h by post-hoc Tukey test. Data shown are mean ± SEM for n=6(Sham, Low, Medium) or n=9 (High) rabbits.*

3.2 Endothelial Progenitor Cells

Attempts were made to grow colonies of endothelial progenitor cells using 1ml blood samples taken at 11 hours post blast injury. Mononuclear cells were identified, separated and placed in fibronectin coated plates with CFU-Hill growth medium. Unfortunately attempts to grow both early and late outgrowth EPC colonies were unsuccessful. After the specified incubation periods no colonies of EPCs consistent with the features in figures 6 and 7 were identifiable. Potential reasons for this failure are explored further in the discussion chapter.

3.3 Histology

Twelve hours following blast injury (or sham) the animals were euthanased, a post mortem examination was carried out, and tissue samples were harvested. The hind limb muscles which were subjected to the blast injury demonstrated pathological changes of varying degrees, at all three blast doses, on both a macroscopic and microscopic level. There was, however, no adverse pathology observed in hind limb muscles from the sham group. The forelimb of each animal was also examined for comparison and did not show pathological changes in any of the groups. Figure 18 shows examples of the macroscopic appearances of the hind limb muscle in high blast and sham animals. Haemorrhage and oedema are visible in the high blast animal, whereas the sham animal has appearances of normal muscle for comparison.

It was possible to perform histological examination of the tissue from all 38 animals as this technique was already well developed. Histological sections of muscle tissue and blood vessel were prepared and stained with Haemotoxylin and Eosin (H&E). Figures 18 and 19 provide examples of the pathological features observed in the

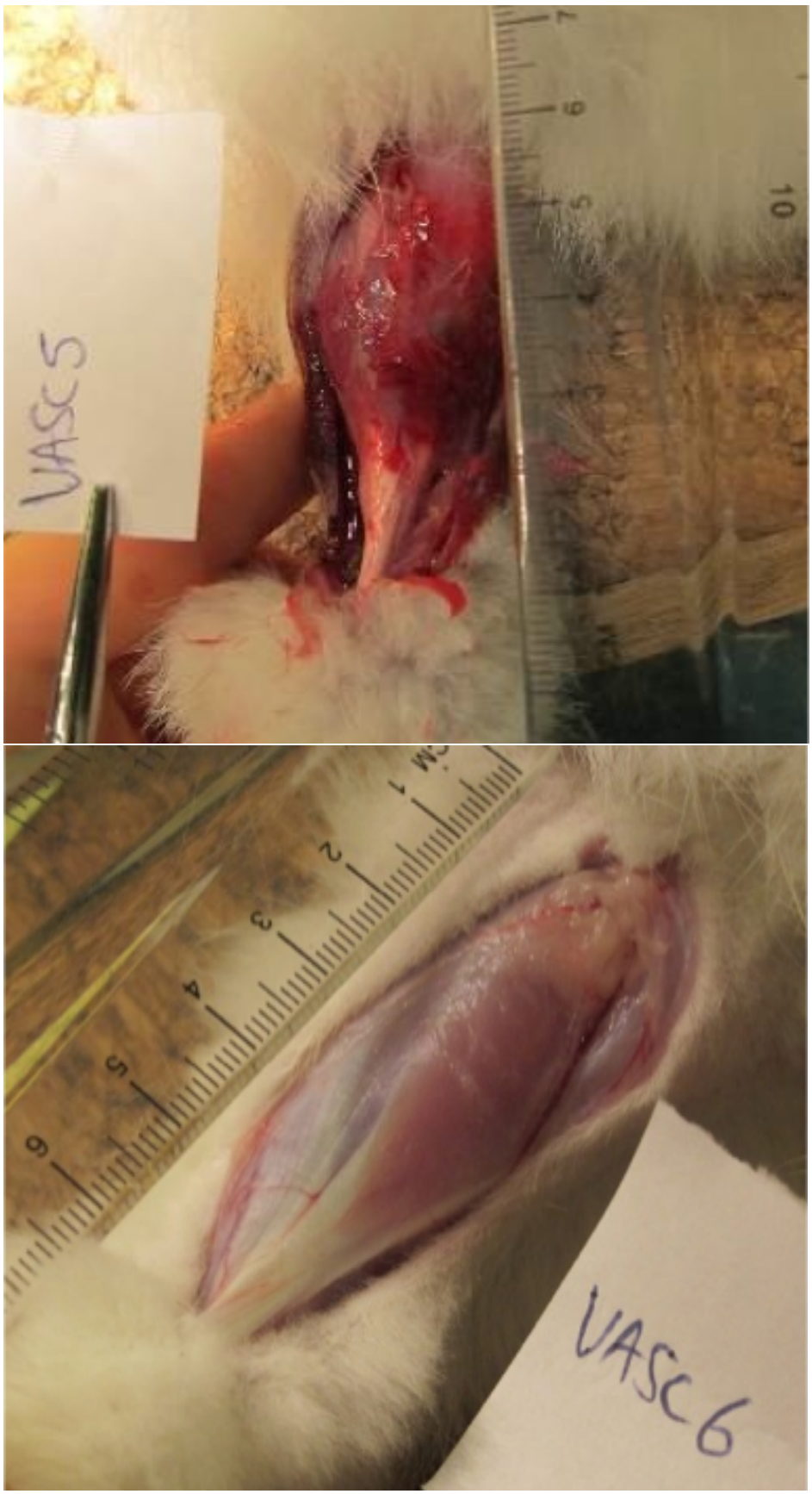


Figure 17. Macroscopic appearance of hindlimb muscles prior to harvesting at post mortem; in the high blast group (top) and in the sham group (bottom).

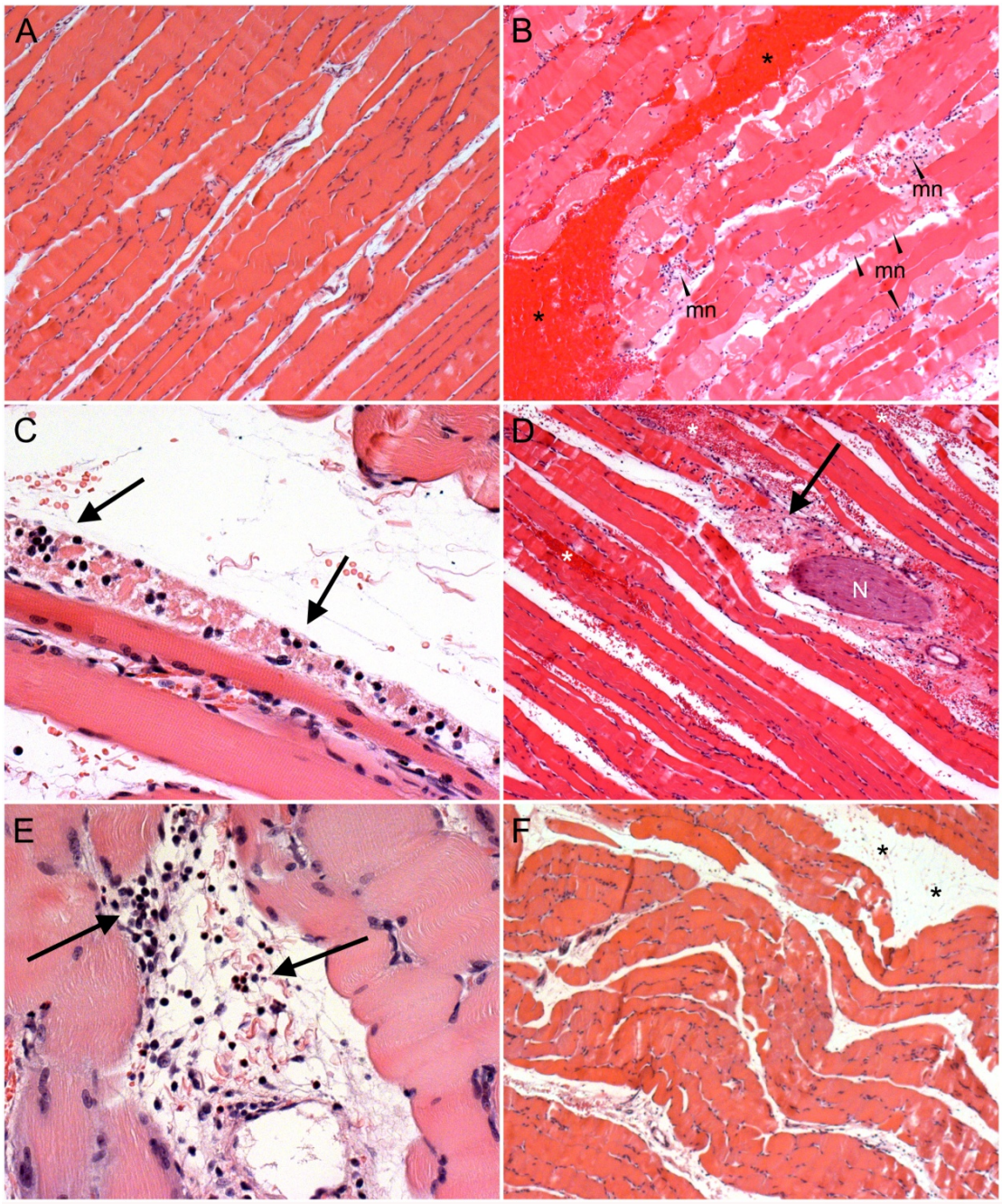


Figure 18. H&E stained muscle tissue. See corresponding legend on next page.

A. Muscle tissue from non-blasted (sham) animals displayed normal morphology with no evidence of background pathology.

B. Muscle tissues from many of the blast exposed animals displayed varying degrees of haemorrhage (*) and muscle fibre necrosis (mn).

C. Necrosis was frequently associated with concentrations of polymorphonuclear cells (heterophil) infiltrating the muscle tissues (↑).

D. Examples of inflammatory cell infiltration (↑) and haemorrhage (*) were also observed in adipose and connective tissues surrounding some nerve fibre bundles (N) within the area of injured muscle tissue.

E. Some areas of connective & adipose tissue within the muscle were also seen to contain mononuclear lymphocytes (↑).

F. Oedema was observed between muscle fibre bundles in some animals exposed to blast injury (*).

Images A, B, D & F scale=400µm, C scale=50µm, E scale=100µm

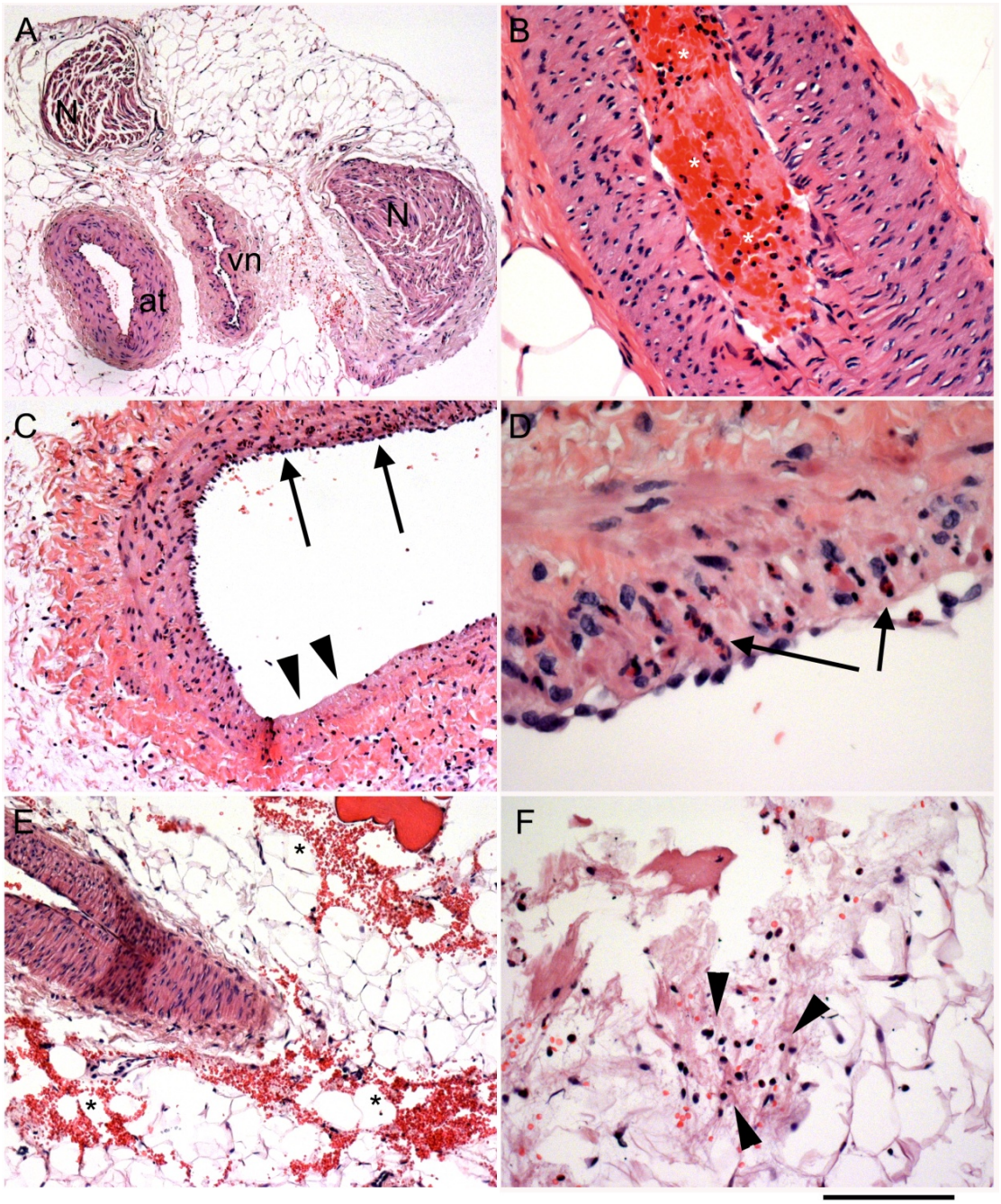


Figure 19. H&E stained vessels. See legend on following page.

A. Example of artery (at), vein (vn) and nerve (N) surrounded by connective and adipose tissue.

B. Polymorphonuclear cells (heterophils) were observed in the blood vessel lumen of some blood vessels (*).

C. One blood vessel from a single animal displayed blood vessel pathology including areas of vascular endothelial denudation (arrow heads) and heterophil adherence to the blood vessel lining.

D. High power LM examination revealed numerous heterophils present in the blood vessel wall (↑).

E. Haemorrhage into the surrounding connective and adipose tissue was seen in many of the animals (*).

F. Inflammatory cells were also a common finding in the adipose and connective tissue surrounding blood vessels (arrow heads).

Image scale A=400µm, B&F=100µm, C&E =200µm, D=50µm.

sections. Figure 18 shows the H&E stained muscle tissue sections and Figure 19 shows the H&E stained vascular tissue sections. In sections from each animal, oedema, haemorrhage and inflammatory cell infiltrate to the muscle tissue were observed. The incidence of each was subjectively scored as follows; 0 (not present), 1 (mild), 2 (moderate), 3 (moderate to severe), 4 (severe) by a histologist blinded to blast loading (table 2, figures 20 and 21). In the high blast group a larger proportion of the muscle sections were scored as severe for oedema, haemorrhage and inflammatory cell infiltrate than in any other group. There was a statistically significant trend of increasing scores with increasing blast load as shown below ($p<0.0001$ Cuzick's trend test).

The maximum percentage of muscle fibre bundles displaying evidence of myolysis was estimated in each section. Again, this demonstrated a significant trend of increasing myolysis with increasing blast load ($p<0.0001$ Cuzick's trend test) (see figure 22).

The histological sections show increasing degrees of tissue oedema and haemorrhage, and larger numbers of infiltrating neutrophils with increasing blast load. This means that there is also a correlation between increasing activation and/or damage to the endothelium with increasing blast load. Oedema occurs when the tight junctions of the endothelium have become leaky and fluid is able to pass through, and expression of adhesion molecules attracts neutrophils to roll on the endothelium, bind to it and migrate out of the circulation into the surrounding tissues. The greater percentage of destruction and myolysis of muscle fibres as the blast load increases demonstrates increasing damage to the surrounding muscle tissue as well.

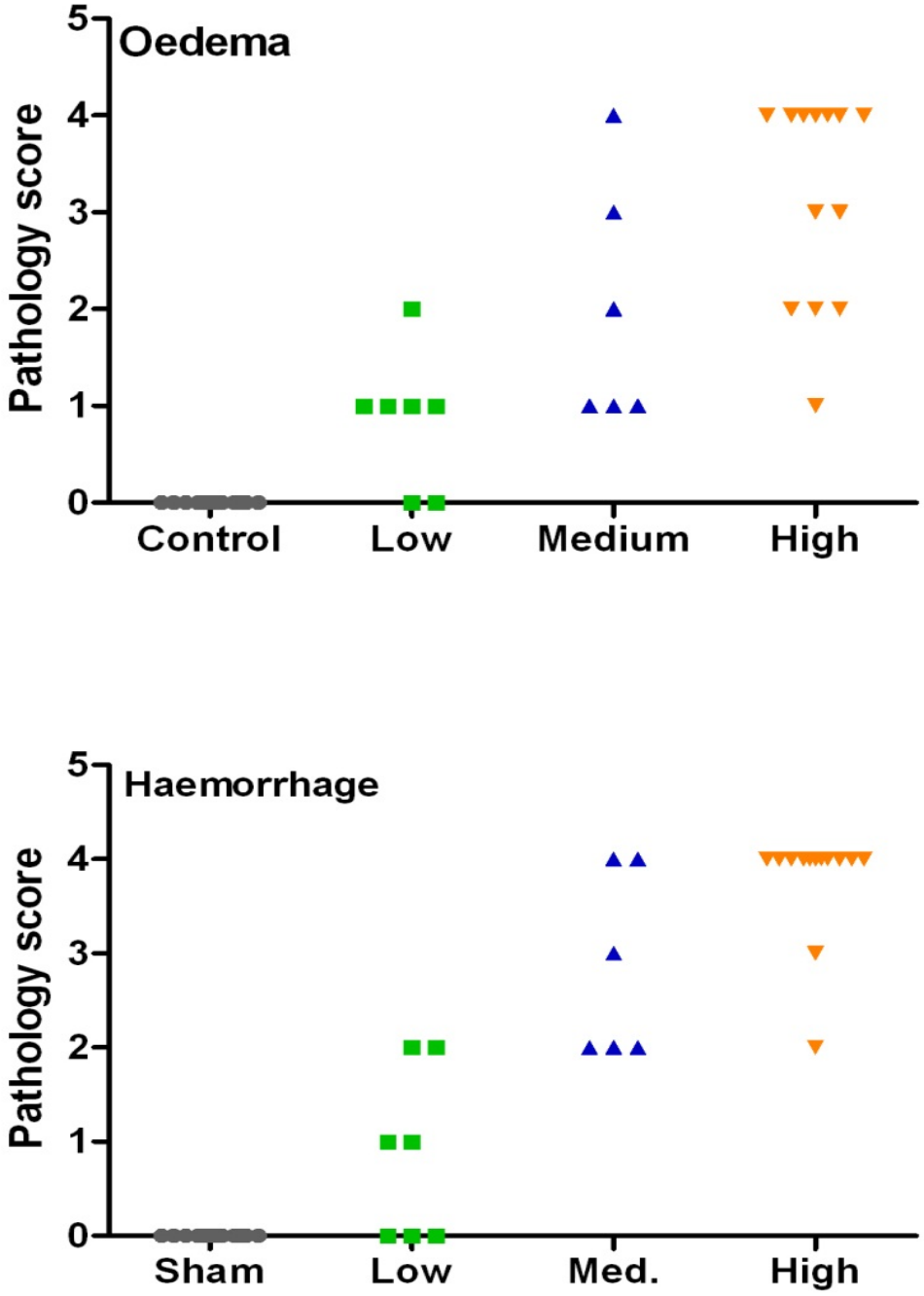


Figure 20. Pathology scoring for the severity of oedema and haemorrhage seen in muscle tissue following blast exposure. There was a statistically significant trend of increasing scores with increasing blast load ($p<0.0001$ Cuzick's trend test).

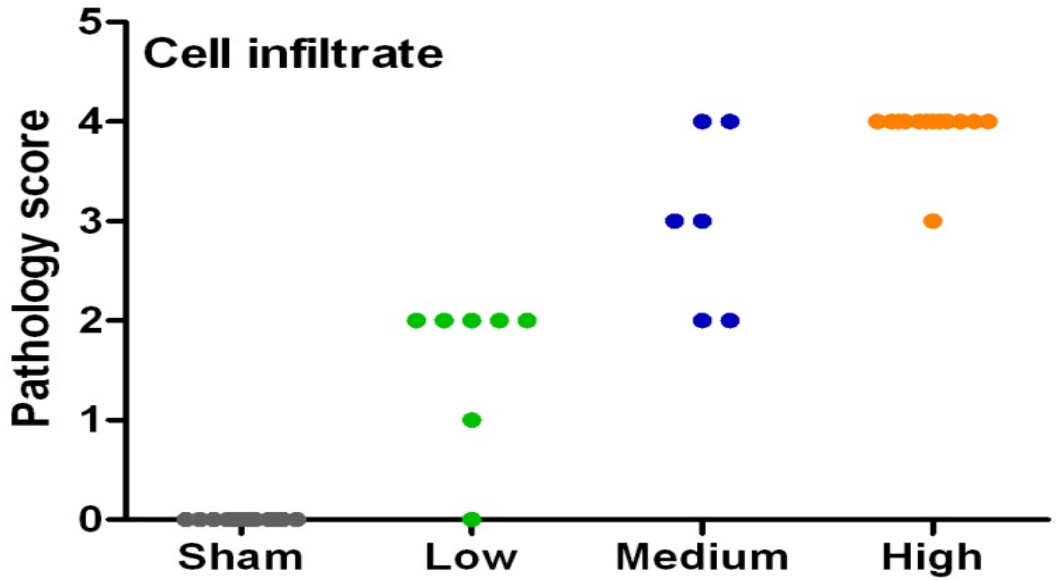


Figure 21. Pathology scoring for inflammatory cell infiltrate seen in muscle tissue following blast exposure. There was a statistically significant trend of increasing score with increasing blast load ($p<0.0001$ Cuzick's trend test).

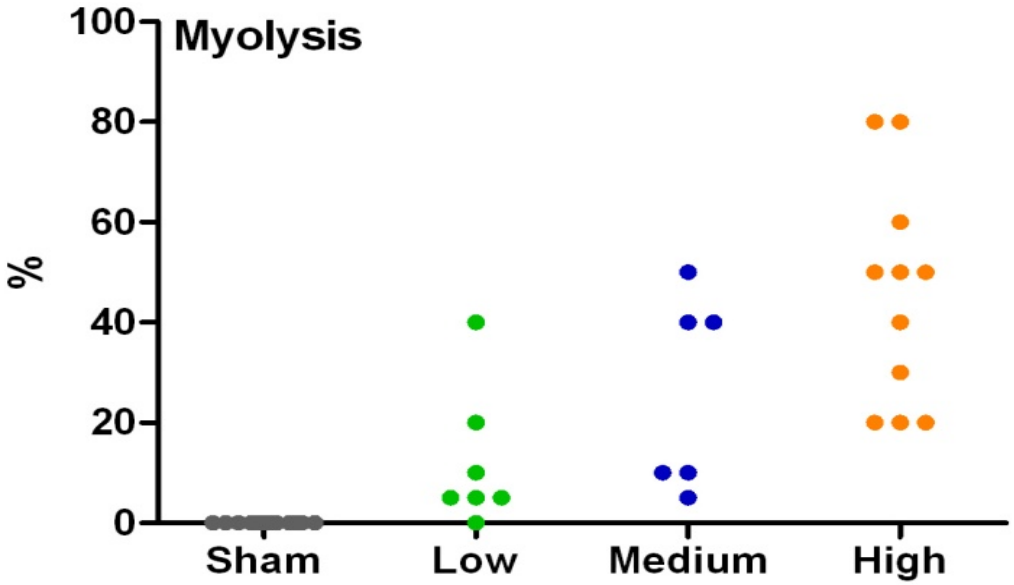


Figure 22. Subjective estimation of the maximum percentage of muscle fibres showing signs of myolysis in each blast group. There was a statistically significant trend of increasing percentage with increasing blast load ($p<0.0001$ Cuzick's trend test).

3.4 Markers of Endothelial Activation and Damage

Circulating levels of neutrophils were measured from the blood samples taken at each time point. They showed some changes over the time course of the study but levels were not significantly different between groups (figure 23).

Plasma and muscle tissue samples were taken in order to look for markers of endothelial activation. The plasma samples from the first 8 animals (4 high blast, 4 sham) were used in the first instance to run ELISAs for detection of vWF, ET-1, soluble thrombomodulin, e-selectin and SDF-1. It was not terribly successful and levels of plasma ET-1, soluble thrombomodulin, e-selectin and SDF-1 were all below the detection limit of the assays. Levels of vWF were detectable, and there appeared to be slightly higher amounts in the high blast animals compared to sham, but the difference did not reach significance (P=0.09)(Figure 24).

Tissue ELISAs for ET-1, SDF-1, e-selectin, P-selectin, ICAM and VCAM were then run on the muscle lysate samples from the same animals. There was no significant difference identified between high and sham groups for any of the markers (see figures 25 and 26). Further tissue ELISAs were carried out for ET-1 using the muscle lysate samples from all the blast groups. This showed that whilst there was no significant difference in ET-1 between the high blast group and the sham group, there was in fact a significant difference between the medium blast group and the sham group (see figure 27).

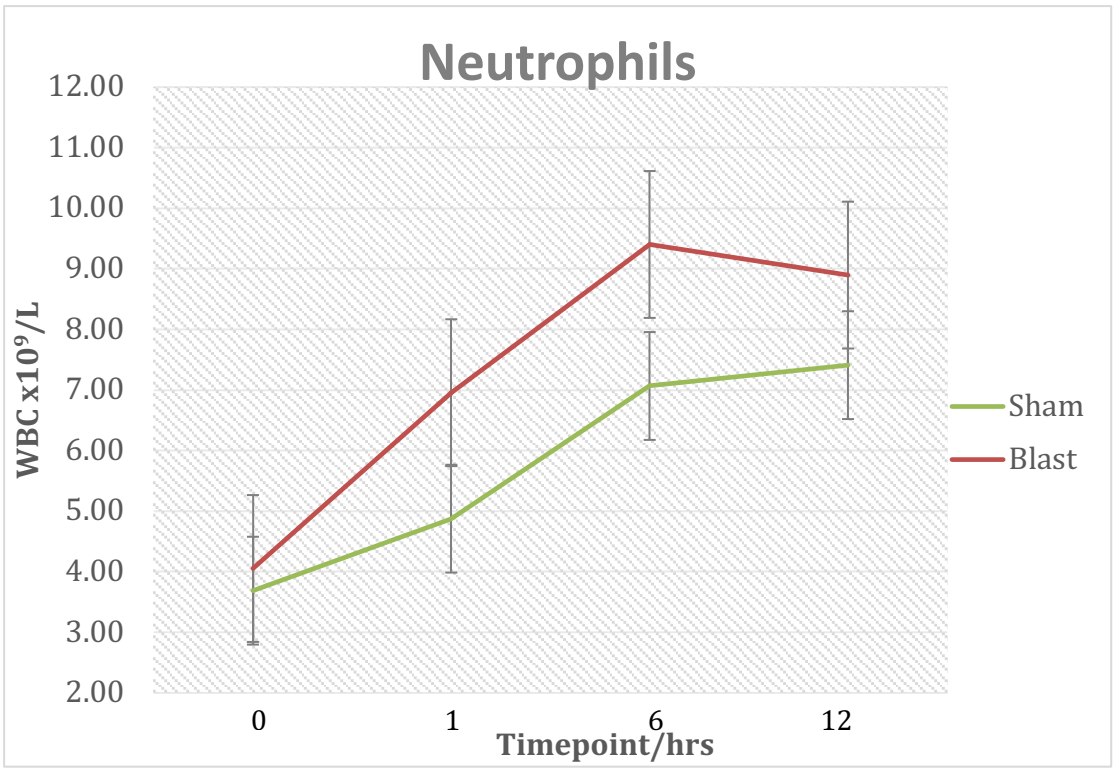


Figure 23. Levels of neutrophils measured over the time course of the study. No significant difference was identified between groups. Error bars show 95% CI.

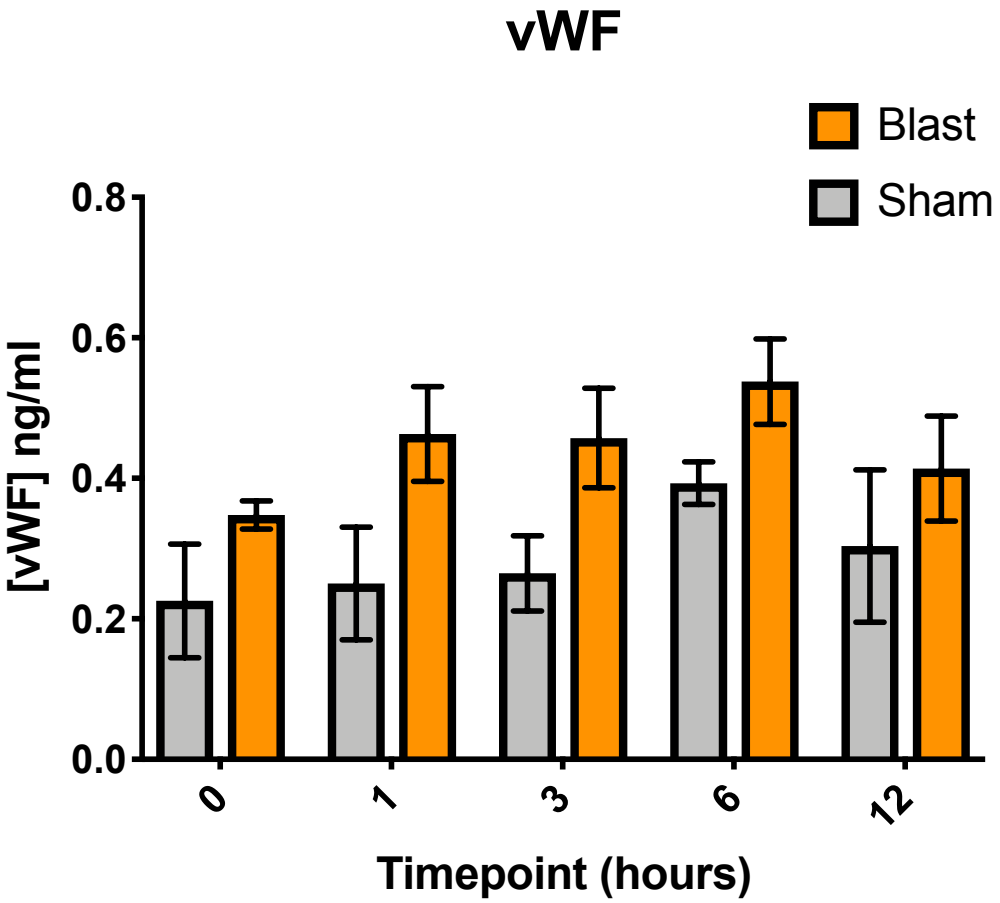


Figure 24. Levels of von Willebrand Factor (vWF) detected in plasma ELISA. No significant difference was detected (P=0.09) between blast and sham groups on 2 way anova. Error bars show 95% CI.

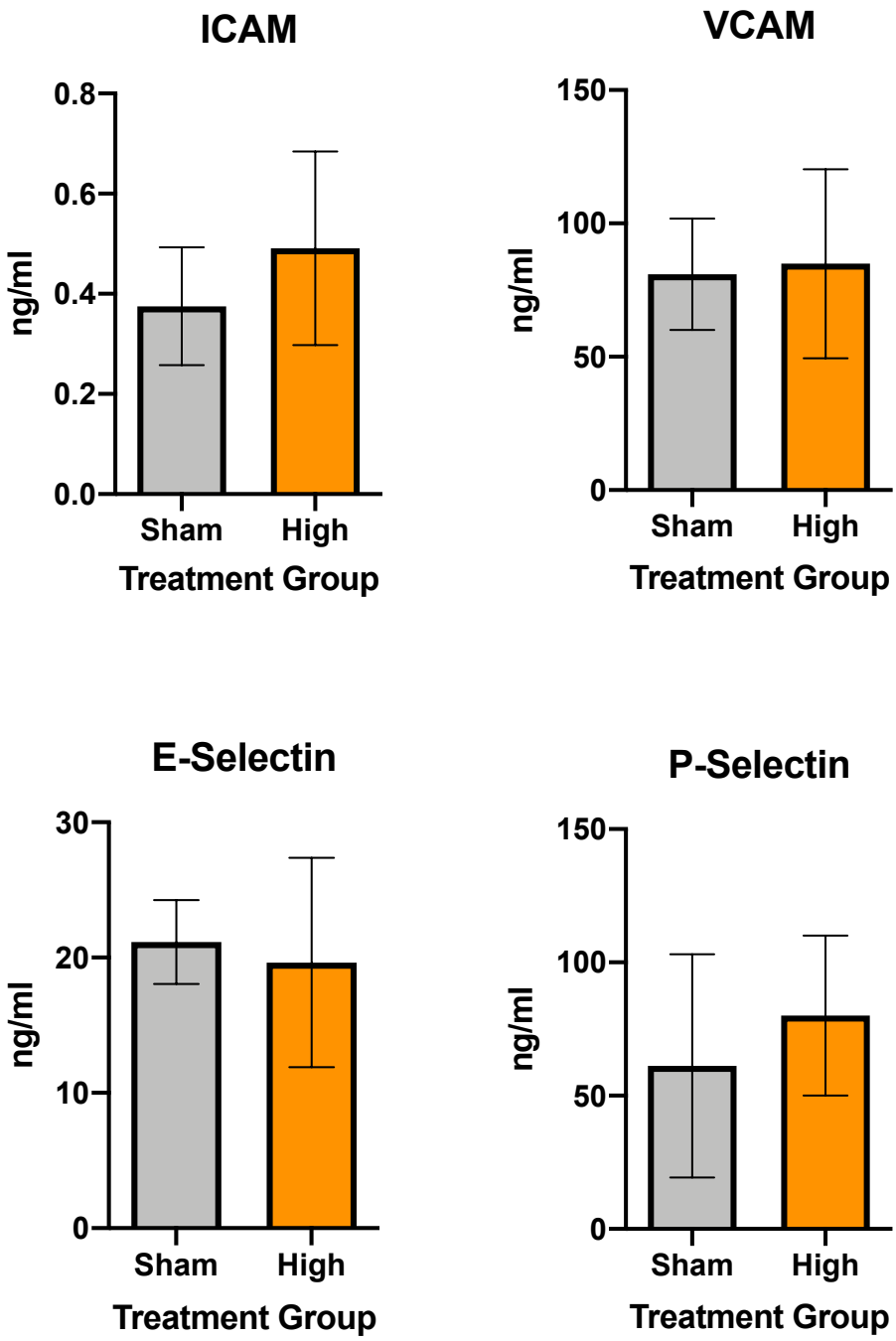


Figure 25. Levels of ICAM, VCAM, E-selectin and P-selectin detected on tissue ELISA. There were no significant differences between levels in the sham and the high blast group. Error bars show 95% CI.

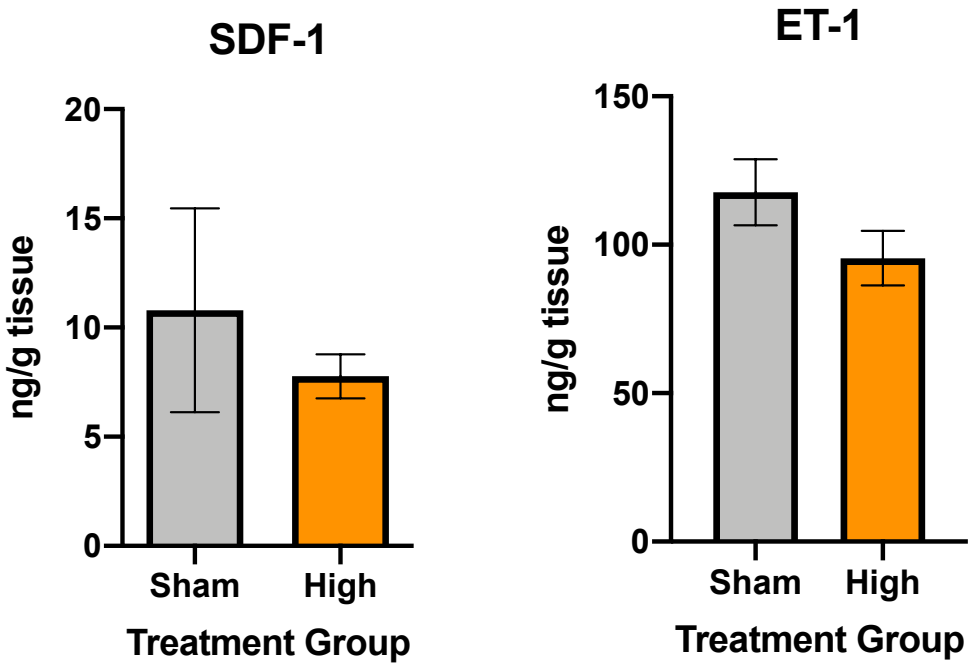


Figure 26. Levels of SDF-1 and ET-1 detected on tissue ELISA. There were no significant differences between levels in the sham and the high blast group. Error bars show 95% CI.

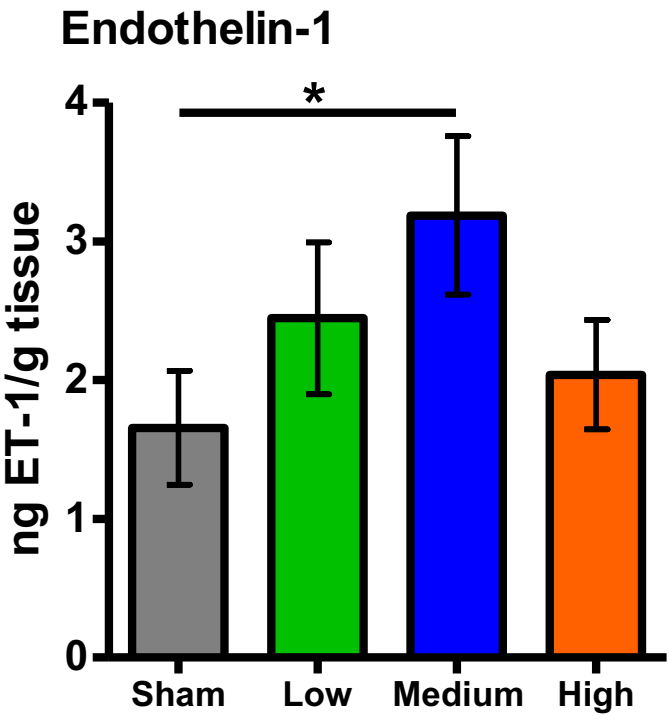


*Figure 27. Levels of endothelin-1 protein in muscle tissue indicating local blood flow changes in response to injury. * $p < 0.01$, one-way ANOVA with Dunett's post-test compared to sham. Error bars show 95% CI.*

As an alternative way to look at the markers of endothelial activation, the quantity of mRNA expressed in the muscle tissue for the markers was determined using qRT-PCR. RNA was extracted from the muscle tissue samples and reverse transcribed as described in the methods section.

Levels of mRNA expression for the inflammatory cytokines IL-6 and TNF-α were significantly raised in the high blast group when compared to sham indicating endothelial activation after blast (see Figure 28).

Expression of mRNA for the adhesion molecule E-selectin, which signals to neutrophils to bind to and migrate across the endothelium, was significantly raised in the high blast group, as was expression of thrombomodulin which is released into the circulation by damaged endothelium (see figure 29).

During the twelve-hour time course of this study levels of platelet-derived growth factor (PDGF) were significantly elevated in the high blast group, but there was no demonstrable difference in the expression of vascular endothelial growth factor (VEGF) between groups (see figure 30).

The hypoxia-induced transcription factor HIF-1α, which regulates the expression of VEGF, was significantly elevated in the high blast group, whereas PGC-1α, the transcription factor involved in the alternative pathway by which VEGF expression is regulated, was not raised (see figure 31).

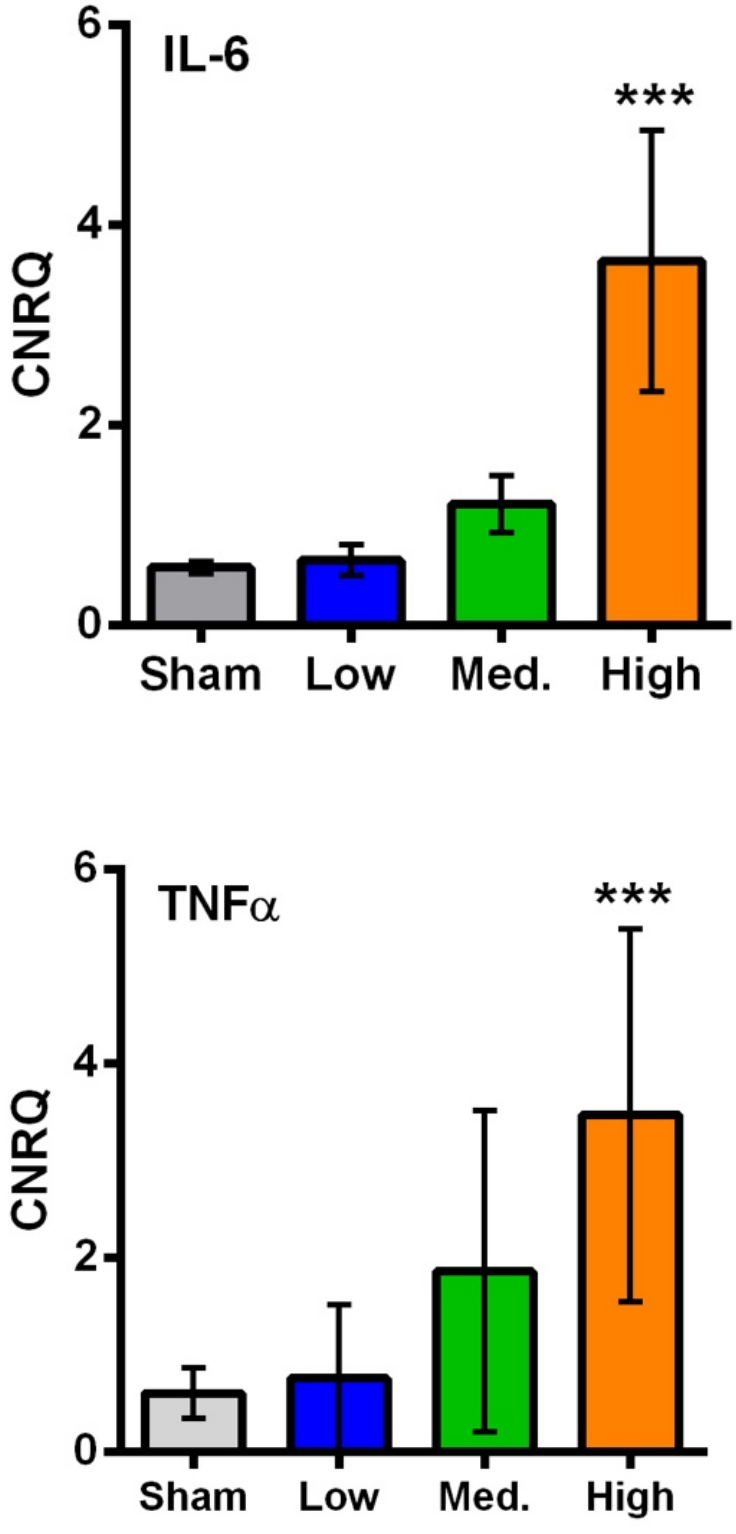


*Fig 28. Expression levels of IL-6 and TNFα within muscle tissue. CNRQ = calibrated normalised relative quantities as calculated from Cq values using the qBasePlus program. ** $p < 0.01$, one-way ANOVA with Dunett's post-test compared to sham. Error bars show 95% CI.*

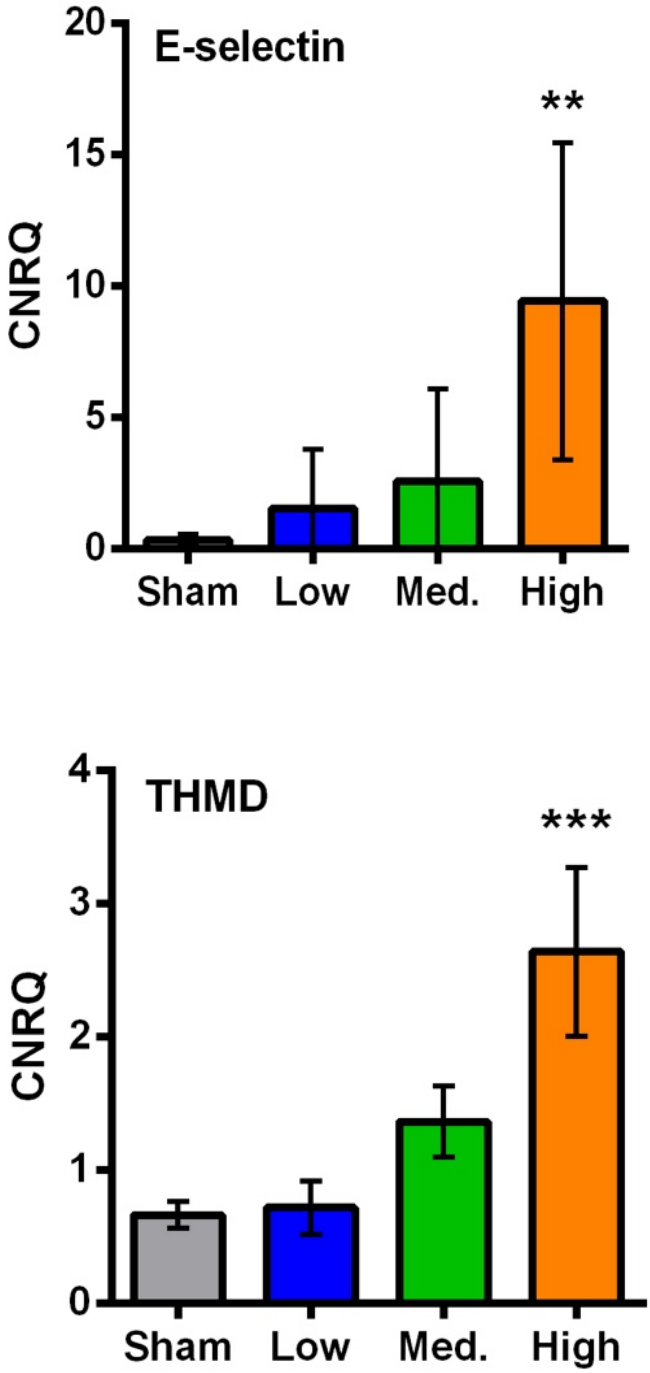


*Figure 29. Expression levels of e-selectin and thrombomodulin within muscle tissue. CNRQ = calibrated normalised relative quantities as calculated from Cq values using the qBasePlus program. *** $p < 0.01$, one-way ANOVA with Dunett's post-test compared to sham. Error bars show 95% CI.*

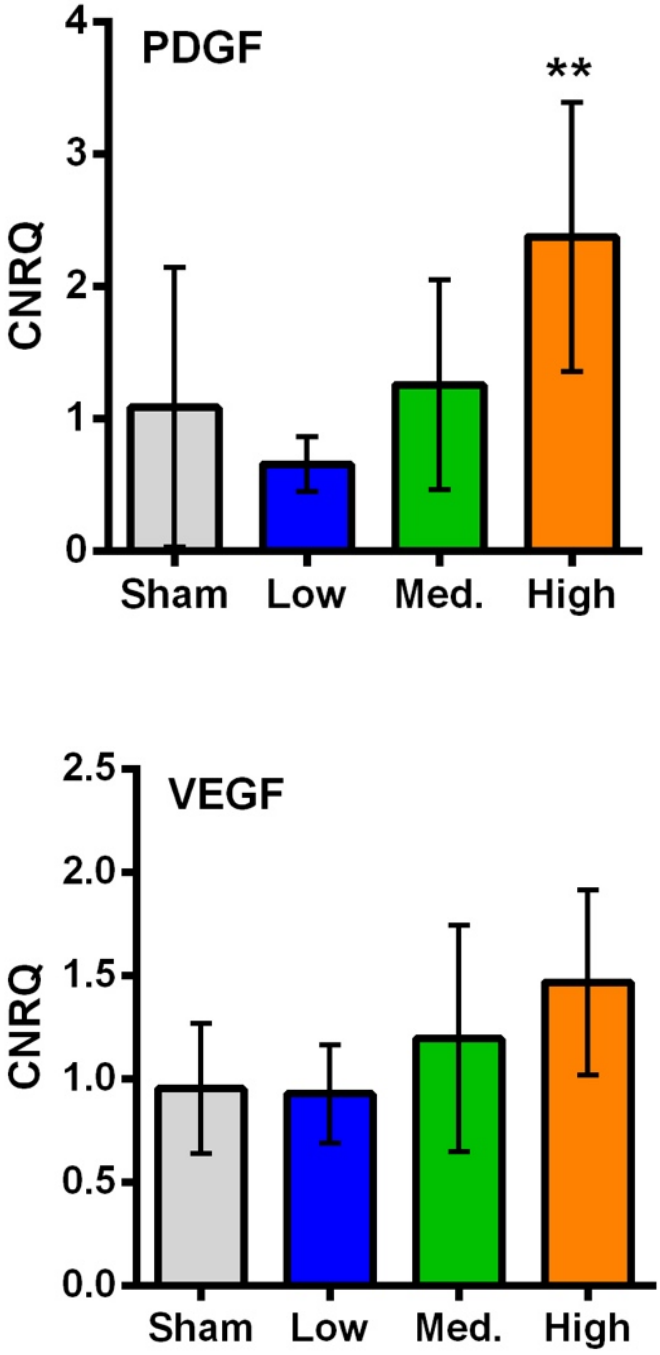


*Figure 30. Expression levels of VEGF and PDGF in tissue. **Indicates significant difference compared with sham. CNRQ = calibrated normalised relative quantities as calculated from Cq values using the qBasePlus program.*

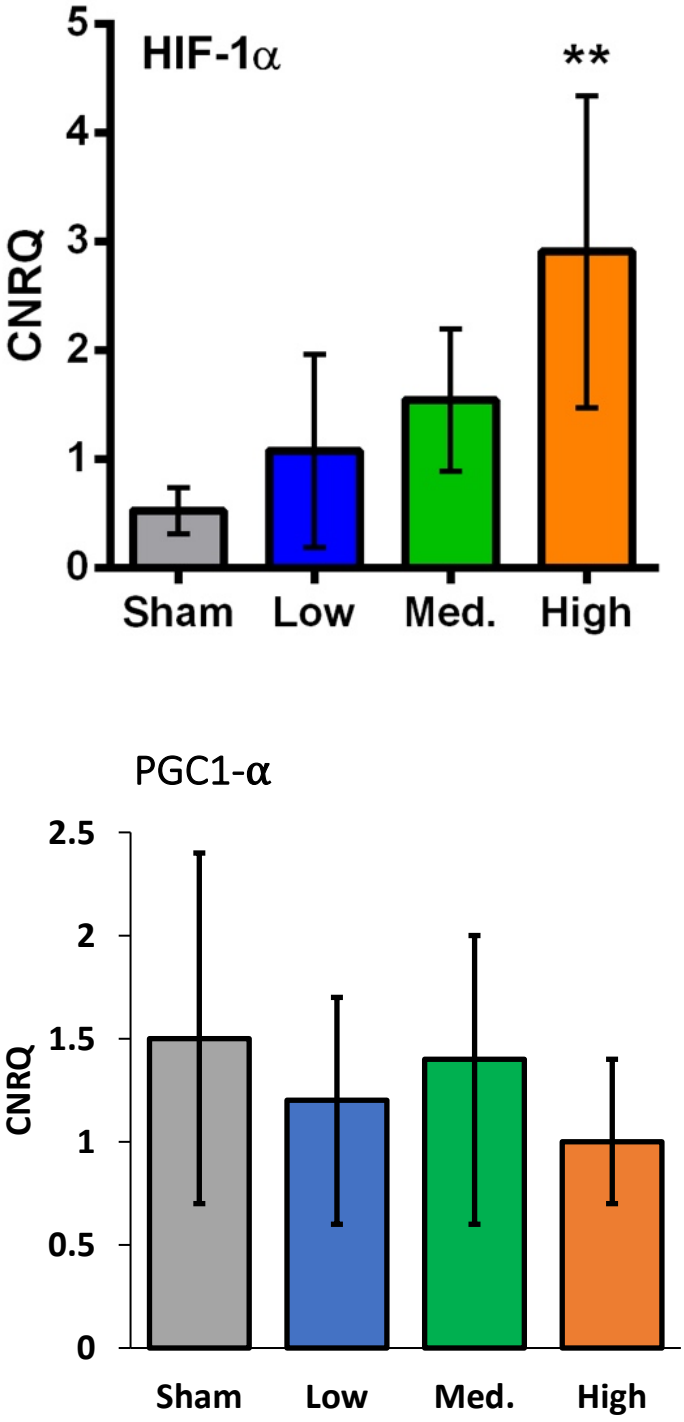


*Figure 31. Expression levels of HIF-1α and PGC-1α in tissue. **Indicates significant difference compared with sham. CNRQ = calibrated normalised relative quantities as calculated from Cq values using the qBasePlus program.*

Chapter 4: Discussion

The endothelium is a diverse structure with variable characteristics depending on its location within the body. It plays a vital role in the regulation of blood flow to the tissues and in preventing thrombus formation. Endothelial cells secrete and express a number of products involved in coagulation/anticoagulation, inflammation, metabolism, vasomotor control, and growth. The endothelium maintains an anti-inflammatory and antithrombotic phenotype under normal conditions, but this changes to a pro-inflammatory, prothrombotic, pro-proliferative, and vasoconstricting phenotype when activated or damaged by injurious stimuli. Activation and damage to endothelium can be assessed by a number of circulating markers as wells as local gene and protein expression.

This study is the first to look at how blast injury to the limbs affects the endothelium both at the site of injury and remotely. It has demonstrated that blast injury to the hind limbs of anaesthetized rabbits causes activation and damage to the endothelium. The magnitude of the effect is related to the level of blast loading, with low blast exposures resulting in endothelial activation, and with endothelial damage (evidenced by significant numbers of CECs) only identified after high blast exposure. Injury to the endothelium was also related to changes in the histological appearance of muscle tissue at and around the site of injury with alterations in endothelial permeability leading to oedema, inflammatory cell infiltrate, and necrosis.

4.1 Discussion of the Blast Animal Model

Blast can cause either a generalised, or a more localised injury, depending on both the size of the charge and the position of the body in relation to it, and whether or not it takes place within and enclosed space. Both human experience and experimental models have demonstrated this. As the aim of this study was to look at a localised extremity model of primary blast injury the compressed air blast wave generator was chosen as the most appropriate. It successfully delivered a localised shock wave to the tissues of the hind limbs of the rabbit in a reproducible fashion. This caused both macroscopic and microscopic changes to the affected limbs without causing more systemic effects of blast such as apnoea, bradycardia or blast lung. It was possible to administer different doses of blast by adjusting the distance between the subject and the blast nozzle. All these factors mean that the compressed air blast wave generator remained the best choice of blast model; avoiding complicating local injuries such as burns and fractures, as well as the systemic blast effects, and allowing a straight forward way of adjusting the blast dose.

The experimental blast data in this study can be translated into clinical context, but care needs to be taken to avoid incorrect interpretation. The measured pressures from the compressed air blast generator for each blast dose (see figure 10) can be equated to the output of real explosives, such as trinitrotoluene (TNT) in a similar way to the mouse model published by Lashoff–Sullivan (Lashoff–Sullivan et al, 2014). For spherical free air burst charges of TNT equivalent charge sizes and distances can be estimated from the equations of Kingary and Bulmash as implemented within the ConWep (Conventional Weapons Effects Program) tool (Kingary and Bulmash, 1984). In this study the high blast dose is equivalent to a 0.71kg charge at 1.3m, the

medium blast dose is equivalent to a 0.67kg charge at 1.5m and the low blast dose is equivalent to a 0.3kg charge at 1.6m. These figures should be considered as indicative predictions only, to put the pressure loadings into the context of explosive charges.

Blast overpressure can be related to an index of lethality as in the work published by Bowen et al (Bowen et al 1968). Extrapolation of overpressure data in the current study indicates that exposing an unprotected person to the "high blast" pressure loading used has a >0.99 probability of certain lethality at 24h post-blast. On the other hand the "low blast" exposure has a probability of lethality of about 0.10. These predictions, however, need to be put in context and it is very important to note that the lethality is based on a number of assumptions that need to be acknowledged to prevent misrepresentation of the data in the current study. The first assumption is that the whole body is exposed to the blast wave and all areas of the body receive the same loading. In reality the blast pressure loadings are much more variable, depending, for example, whether a soldier stands on a footplate IED resulting in blast loading up through the lower limbs, or whether a passenger is in an enclosed train carriage when a bomb explodes. The second assumption is that no protective equipment is in place such as helmet or body armour. A third assumption is that no medical attention or treatment is given to the casualty during that 24 hour period. The reality is that a person is likely to receive some form of medical care postinjury. Taken as a whole it is highly likely that the probability of lethality would be reduced, but it is not possible to determine the actual reduction, as that data is simply not available.

Another of the major limitations of this study came in the use of a rabbit model. The rabbit model was chosen in preference to a mouse or rat model as it offered larger

volumes of blood and tissue per animal. The mouse and rat would simply have not provided adequate samples for all of the tests that were required, and if separate animals were used for the separate tests, then it would have greatly increased the number of animals required. Thus, the rabbit model kept the number of animals required to a minimum. It was also significantly cheaper and there was less waste than with a larger animal model of blast. However, whilst a satisfactory volume of tissue could be harvested from the rabbit model, there was a limitation to the permissible volume of blood that could be drawn whilst the animal was under terminal anaesthesia. Ideally larger volumes of blood would have been better. This would have allowed for development of processing and enumeration techniques without using up the whole samples for those animals, and essentially meaning the data for more animals was available for analysis. This is discussed further in the separate sections below.

The other major drawback of the rabbit model was the fact that many of the commercially available antibodies for ELISA/ flow cytometry/ immunohistochemistry are raised in rabbits and therefore do not work on rabbit samples. It therefore limited both the techniques available to identify cells and markers, and the number of markers that could be tested for at all.

4.2 Discussion of CEC Results

Development of a successful isolation and enumeration protocol for the CECs used the samples from the first 11 animals and so CEC data from these animals could not be used in the analysis. This was based on the technique described by Woywodt (Woywodt et al, 2006), but using mouse-anti rabbit CD146 and pan mouse

dynabeads. Some adjustment was required to optimise the amount of antibody required to couple the beads. The end of each 12 hour post blast experimental period fell late in the evening (approaching midnight) and, therefore, it was not practical to enumerate the CECs straight away. A way of fixing the samples was required, so that the number of CECs did not degrade overnight. Addition of ice-cold methanol proved the most effective way of achieving this. It had to be checked that addition of methanol as fixative did not cause any non-CEC cell lines to become stained with UEA-1 and thus alter the cells being counted. It has been reported that enumeration of CECs using CD146-based immunomagnetic separation may include large platelet particles in the counting process (Woywodt et al., 2006). Whilst rigid identification criteria were adhered to (fluorescent cells 10 – 50 μm in size and with more than 5 beads attached) it is possible that a small proportion of the cells counted as CECs were in fact large platelets. More sophisticated methods of CEC enumeration such as flow cytometry were not feasible in the rabbit due to the lack of available antibodies. Some adjustment was required to get the optimum amount of UEA-1 stain for visualizing the cells most clearly. As accurate counting as possible was achieved by changing to a gridded haemocytometer with a fixed volume, and by limiting the personnel counting the cells to two. Inter-observer variability was assessed by both observers double counting a number of the same samples to ensure there was not a significant difference between the two counts.

The overall numbers of CECs reported in this study are somewhat higher than in other studies, including the baseline values. This may be for a variety of reasons. Firstly, this study was carried out in rabbits, and rabbits are a species prone to stress, which is a reported cause of raised CEC levels (Boos et al 2008). Normal CEC levels in

rabbits have not previously been characterised, and it is possible that their baseline levels may be higher than those of humans. Secondly, a surgically placed indwelling catheter was used for blood sampling. The mechanical force of catheter insertion has previously been shown to detach endothelial cells from the basement membrane (Erdbruegger et al., 2010). This is likely to give a higher baseline CEC level, despite waiting 30 minutes post insertion and not using the first 1 ml of blood withdrawn. Ideally a longer post catheterisation period would be allowed to minimise the effect of this.

As previously described, CEC detachment from the endothelium involves multiple factors including mechanical injury, alteration of endothelial cellular adhesion molecules, defective binding to anchoring matrix proteins and cellular apoptosis or necrosis (Goon et al., 2006). In this study the levels of CECs in the blood peaked at 6 hours post-injury in the animals subjected to high blast doses, then returned towards baseline levels by 11 hours. If solely mechanical forces due to the blast wave were the cause of endothelial cell detachment, a more immediate rise in CEC levels (with a peak at 1 hour rather than 6 hours post-injury) would have been expected. It is, therefore, more likely that the detachment of the CECs from their basement membrane was due to activation of biochemical pathways. What is not known is whether the CECs were viable cells, apoptotic or necrotic.

However, the presence of raised levels of CECs demonstrates a severity of endothelial injury likely to have clinical consequences. Activated endothelium adopts a pro-inflammatory phenotype, and, even after detachment, evidence suggests that CECs could be pro-inflammatory (Woywodt et al., 2002). For example, if the cells were

necrotic, they may release cellular substances such as HMGB-1 and DNA, and form endothelial derived microparticles, which have been shown to further exacerbate endothelial dysfunction (Brodsky et al., 2004). The proinflammatory state may have local effects at a cellular level, affect regional blood flow to tissues, and have more widespread systemic effects, which all have implications when considering tissue reperfusion in the blast injured casualty.

4.3 Discussion of EPC Results

Attempts to grow EPC colonies in this study were unsuccessful, however, given the other findings in this study, it is likely that there were EPCs present in the circulation. In an ideal situation it would have been possible to enumerate them directly, but, just like CEC enumeration, a lack of compatible antibodies for the rabbit prevented the use of flow cytometry.

There were several factors that could have contributed to the failure of EPC colony growth. Firstly, only a small volume of blood (1ml) was available for each sample. This is a lot less than the sample volumes used by other groups, who have successfully grown EPC colonies using 20-30mls of human blood (Hill et al., 2003). Rabbits have a much smaller blood volume than humans, and therefore it was not possible to take such large samples. Some of the blood taken was also required for other tests and could not be solely used for EPC growth. Given the blood volumes of greater than 20mls used previously for successful growth of EPC colonies, it was perhaps unrealistic to think that results could be obtained from just 1ml of blood.

With such a small volume the Ficoll separation was very difficult indeed, and the volume of mononuclear cells separated for plating was tiny.

The other factor that may have significantly affected outcome was the growth media used. CFU Hill Growth medium and ECFC complete medium were those developed for optimal human EPC growth, and were not designed for culturing rabbit EPCs. They may have lacked some vital growth factor essential to rabbit cells that we are unaware of. It is unfortunate that commercial growth media specific to rabbit EPCs are not available. If EPCs were to be looked at again in a model of blast endothelial injury, a larger animal model may be required, or at least, if a rabbit model was used again, dedicating the entire permissible volume of blood sampled to EPC colony growth. It may also be necessary to develop a growth media more suitable for rabbit EPCs.

4.4 Discussion of Histology Results

A model of isolated blast injury to the limb that looks at the local pathological changes to the tissue has not previously been reported in the literature. The histopathological changes in this study were assessed using standard preparation techniques by a single histopathologist blinded to blast loading, so there is not any inter-observer variability. Whilst the scoring was subjective, it did demonstrate a significant correlation between blast dose and the extent of tissue injury. The findings correspond well with the markers and CEC results, and what would be expected to happen following endothelial activation and damage; loss of endothelial integrity allows fluid and inflammatory cells into the tissue, and it is likely that local hypoxia coupled with inflammation leads to muscle cell death. With between 20% and 80%

muscle fibre necrosis seen in the high blast animals it no longer seems surprising that the success of revascularising blast injured limbs has been limited.

A more severe model of blast injury to the lower limb of rats has been used to study the systemic effects caused, in particular to the lungs, but did not look at either endothelium or the local tissue damage (Ning et al., 2012). Whereas the current study did not see any effect on distant organs, Ning's model of blast limb trauma induced lung injury with alveolar congestion, haemorrhage, breakdown of alveolar architecture, alveolar wall thickening and cell infiltration including neutrophils. They did not look at different blast doses. Another study by Gorbunov et al observed histological changes in a rat model of blast induced pulmonary trauma. They noted that the extent of the experimental blast lung injury was in proportion to the amplitude of shock wave overpressure, which is similar to our findings regarding the severity of tissue damage in the lower limb. There was an inflammatory neutrophilia in the shock wave exposed animals, with microvascular sequestration of poly morpho-nuclear neutrophils (PMNs) and monocytes, and transmigration of them into the alveoli at the sites of the haemorrhagic lung lesions. Gorbunov noted that this was synchronized with pro-inflammatory alterations in microvascular endothelium such as a decrease in endothelial integrity provided by the VE-cadherin adhesion junctions, and increased expression of ICAM-1 that is essential for the increase in the microvascular permeability and transmigration of leukocytes (Gorbunov et al., 2006, Gorbunov et al., 2007, Gorbunov et al., 2005).

It had been hoped to use some of the tissue sections for immunohistochemical staining. The poor availability of antibodies suitable for use in rabbit tissue again

severely limited the choice of markers that could be tested for. Some Tunel staining for detecting apoptotic DNA fragmentation was attempted in order to look for evidence of endothelial cell apoptosis in the vessel wall. However, it was not possible to get the assay to work and the work was abandoned.

4.5 Discussion of Markers Results

Markers of endothelial activation and damage were assessed by both plasma and tissue ELISA, and by qRT-PCR to quantify mRNA expression in muscle tissue.

The plasma ELISAs were largely unsuccessful as samples failed to reach the detection limit for the assays. Whilst this does not mean there were no markers present, it does mean that there is currently no test sensitive enough to detect them. Standard ELISAs have a variable detection range. In the kits we used from Cusabio this ranged from 6.25pg/mL-400pg/mL for ET-1 to 0.312ng/mL-20ng/mL for vWF. Plasma ELISA for vWF did show higher levels of vWF present in the plasma of the high blast animals at all time points when compared with sham, but the difference failed to reach significance.

Tissue ELISAs performed with the muscle lysate detected markers but also did not demonstrate a significant difference in marker levels between blast and sham groups in the early samples. Potentially refinement of sampling techniques and dilutions could reveal better results, as could including more of the samples within each group, however, time and resource limitations within this study meant that performing further ELISAs on more samples was not possible.

However, this study has been able to demonstrate endothelial activation by an increase in expression levels of pro-inflammatory cytokines, with levels of IL-6, TNF-α and E-selectin significantly raised in animals receiving the high blast dose when compared to sham. This corresponds with other studies of endothelial activation and injury. Elevated levels of IL-6 were demonstatred by Gorbunov in a rat model of blast lung injury (Gorbunov et al., 2005), and by Lee in a human study, looking at endothelial injury in patients with acute coronary syndromes where it was found that levels of IL-6 were raised on admission, and at 48 hours, (Lee et al., 2005). There was a correlation between elevated levels of IL-6 (and also CECs) in those patients who went on to develop further major cardiovascular events.

The expression of cellular adhesion molecules by activated endothelium is well documented in a number of disease states, with associated attraction and binding of leukocytes. In particular elevated levels of e-selectin have been reported in burns, cardio-vascular disease and ischaemia-reperfusion injury. The expression of e-selectin is induced by TNF-α and enhanced by hypoxia, but has been shown not to be induced by hypoxia alone in the absence of TNF-α (Zund et al., 1996a, Zund et al., 1996b). In turn e-selectin then greatly increases the expression of ICAM-1 and slightly increases the expression of VCAM-1 (Oh et al., 2007). Importantly, e-selectin is also a chemotactic factor for EPCs and is able to induce a dose-dependent migration of EPCs into ischaemic tissue. It may also be a potent mediator of angiogenesis like VEGF and SDF-1 as it is able to augment matrigel tube formation of EPCs in vitro. E-selectin also enhances incorporation of EPCs into the endothelial cell layer and increases secretion of IL-8 from EPCs to further mediate angiogenesis (Oh et al., 2007). Our results show a significant increase in e-selectin levels in animals

subjected to a high blast dose compared with sham. This not only corresponds with the rise seen in TNF-α and supports the hypothesis that blast does cause activation of the endothelium, but also raises the suggestion that tissue injury created by a blast mechanism is inextricably linked to ischaemia on a microvascular level, even before ischaemia caused by major vascular injury or tourniquet use is taken into account. This has implications for treatment of blast-injured limbs. The study by Oh et al found that local injection of soluble e-selectin potentiated the therapeutic efficacy of EPC transplantation to increase vasculogenesis in ischemic limbs (Oh et al., 2007). Conversely, the expression of e-selectin has been shown to be reduced by treatment with statins in patients with burns (Akcay et al., 2005). It was postulated that this might have a beneficial effect by reducing inflammatory cell migration and oedema in the tissues. Other studies have shown that EPC mobilisation and functionality has been improved by administration of statins, leading to improved cardiac outcomes (Antonio et al., 2014, Vasa et al., 2001). If statins reduce e-selectin levels, but improve EPC mobilisation and function, then it must be via a different mechanism.

Thrombomodulin is manufactured by endothelial cells and plays an important role in maintaining a thromboresistant endothelial surface. Thrombomodulin binds to thrombin to form a thrombin-thrombomodulin (TM-THMB) complex. This complex is directly anticoagulant and also has activity via the accelerated production of activated protein C (aPC). In the context of trauma and hypoperfusion aPC has additionally been implicated as a fibrinolytic (Brohi et al, 2007). Thrombomodulin is released into the circulation by damaged endothelium and has proven a reliable marker of endothelial injury (Boehme et al., 1996). Our study found that expression levels for thrombomodulin are elevated following blast injury; highest in the group

with the greatest vascular injury, the high blast group. Inflammatory cytokines, such as TNF-α, are generally considered as downregulators of thrombomodulin (Seguin et al, 2008), but in the current study, the group with the greatest TNF-α expression also had the highest thrombomodulin expression, thus highlighting the complex nature of responses seen *in vivo* compared with *in vitro*. Potentially, the presence of Heat Shock Proteins, known to be released after trauma, could have caused the increased thrombomodulin expression (Conway et al, 1994). PDGF in the high blast group was also significantly increased, and this too may have resulted in the increased thrombomodulin expression in the homogenized muscle tissue in this group (Lo et al, 2009).

Elevated levels of VEGF have previously been demonstrated in patients following vascular trauma due to insults such as coronary artery bypass grafting (CABG) and burns (Gill et al., 2001). They describe a rapid rise in VEGF levels, peaking at 6-12 hours and returning to baseline in 48-72 hours, and proposed that the associated mobilization of endothelial progenitor cells expressing VEGF receptor 2 contributed to repair of the damaged endothelium. We were unable to demonstrate a significant rise in VEGF levels in this study. VEGF in the high blast group was higher than in the sham group, but did not reach statistical significance. There were however significantly elevated levels of both PDGF and HIF-1α in the high blast group. HIF-1α is a hypoxia-induced transcription factor that provides one of the pathways via which VEGF is regulated. Our tissue samples were taken at 12 hours so it is possible that we missed the peak of VEGF expression, or indeed that it occurs later.

HIF-1α is has been reported to change metabolism from oxidative to glycolytic and to stimulate the expression of glycolytic genes (Semenza, 2009). In the context of the current study this raises the possibility that blast injured tissue is more likely to rely on anaerobic respiration. The associated metabolic acidosis may exacerbate the local effects of tissue hypoperfusion due to haemorrhage or tourniquet use in the blast injured casualty, and may have implications for resuscitation strategies.

Chapter 5: Conclusions and Future Directions

In summary, this study has been able to demonstrate that blast injury to the hind limbs of rabbits does cause activation of and damage to the vascular endothelium. Both local and systemic biomarkers of endothelial injury are raised, significantly so in the high blast group. A dose-dependent response is implied by the significant trend of increasing pathology scores with increasing blast load. Therefore, it is possible to accept the hypothesis proposed at the beginning of the study.

Further work needs to be done to fully understand the manner in which blast interacts with the endothelium. Ideally a larger model of blast extremity injury would be developed, in an animal for which all of the desirable antibodies and reagents were available. Then larger volumes of blood could be drawn and potentially EPC colonies could be grown, or flow cytometry to enumerate CECs and EPCs could be attempted. It would be useful to look in more detail at the blast doses which cause endothelial activation, and those which tend more towards with damage and potentially characterise a dose-response curve for levels of blast and time from injury.

It would be interesting to investigate the mode of endothelial detachment from the basement layer, and identify whether the damaged endothelium is apoptotic or necrotic. Another aspect that this study did not even touch upon was whether the endothelial glycocalyx layer becomes degraded in blast injury, and if so at what doses. Potentially the glycocalyx is protective in the first instance and its degradation or disruption by shockwave may be the forerunner to endothelial cell detachment.

Changes in endothelial dependent vasodilation (which is mediated by the release of NO) could be investigated for different levels of blast using flow mediated dilatation. This could provide valuable information about the ability of blast injured blood vessels to increase local blood supply by vasodilation. An inability to regulate tissue hypoxia by vasodilatation has huge implications for the casualty injured by an explosion. The damaging effects of haemorrhage and resulting shock could be exacerbated by blast injury leading to a vasoconstricted state, and vice versa so that potentially even low levels of blast become damaging, and impact upon overall recovery and functionality. In particular it may have severe consequences for revascularizing blast injured limbs with major vessel injury, and whilst only speculative at present this picture fits with the low success rate of limb salvage in blast injury.

Another future direction would be in the development of treatments which mitigate the effects of the blast injury. Whilst such treatments are only hypothetical at present drugs that reduce inflammation, or dampen the inflammatory processes associated with endothelial activation, and promote endothelial repair would be of interest. For example, statins may have a role to play, as may anti-inflammatory drugs – both steroidal and non-steroidal. Drugs that cause NO-independent vasodilation may be of use where limb ischaemia is the main problem rather than systemic blast injury with hypotension. There is a vast array of work still to be done to fully understand the cellular effects of blast injury and how we can reverse or treat these to improve survival and limb salvage in the blast injured casualty.

Publications and Presentations Arising from this work

Publication

Spear*, A.M., Davies*, E.M., Taylor, C., Whiting, R., Kirkman, E., Midwinter, M., Watts, S.A. *Joint first authors. 2015. Blast wave exposure to the extremities causes endothelial activation and damage. *Shock* 44 (5), 470-478

Oral Presentations

The dose-dependent response of vascular endothelium to blast injury.

Davies, E., Spear, A., Taylor, C., Kirkman, E., Midwinter, M., Watts, S.

Society of Academic and Research Surgery Annual Scientific Meeting 2013, London.

The dose-dependent response of vascular endothelium to blast injury.

Davies, E., Spear, A., Taylor, C., Kirkman, E., Midwinter, M., Watts, S.

Colt Foundation Military Research Symposium 2012, London.

Identification of circulating endothelial cells (CECs) and utilisation of CECs as a marker to determine endothelial damage following blast injury.

Davies, E., Spear, A., Kirkman, E., Midwinter, M., Watts, S.

Association of Trauma and Military Surgery Conference 2012, Liverpool.

Posters

Blast Injury and its effects on the endothelium.

Davies. E, Spear. A, Whiting. R, Macildowie. S, Taylor. C, Midwinter. M, Kirkman. E, Watts. S.

Trauma and Blast Inflammation Symposium 2014, Birmingham.

Blast Injury and its effects on the endothelium.

Spear. A, Davies. E, Whiting. R, Macildowie. S, Taylor. C, Midwinter. M, Kirkman. E, Watts. S.

International Union of Physiological Sciences ASM 2013.

Blast Injury and its effects on the Endothelium.

Davies, E., Spear, A., Taylor, C., Kirkman, E., Midwinter, M., Watts, S.

Military Health System Research Symposium 2012, Florida, USA.

Identification of circulating endothelial cells (CECs) and utilisation of CECs as a marker to determine endothelial damage following blast injury.

Davies, E., Spear, A., Kirkman, E., Midwinter, M., Watts, S.

Association of Surgeons of Great Britain and Ireland ASM 2012, Liverpool.

Appendix

Animal	Blast Dose	Nozzle Distance	Machine	Surgeon	Blast Operator	Blast position/ exposures
1	High	1.5cm	1	ED	EK/JR	Lateral 4R 1L
2	Sham	n/a	n/a	ED	n/a	Lateral 5R 5L
3	High	1.5cm	1	ED	EK/ED	Lateral 5R Prone 5L
4	Sham	n/a	n/a	SW	n/a	Prone 5R 5L
5	High	1.5cm	1	ED	EK/ED	Prone 5R 5L
6	Sham	n/a	n/a	SW	n/a	Prone 5R 5L
7	High	1.5cm	1	ED	ED	Prone 5R 5L
8	Sham	n/a	n/a	SW	n/a	Prone 5R 5L
9	High	1.5cm	1	ED	EK/ED	Prone 5R 5L
10	Sham	n/a	n/a	SW	n/a	Prone 5R 5L
11	High	1.5cm	1	ED	ED	Prone 5R 5L
12	High	1.264cm	2	SW	EK	Prone 5R 5L
13	Medium	2.5cm	1	ED	ED	Prone 5R 5L
14	Low	4.0cm	2	SW	EK	Prone 5R 5L
15	Medium	2.5cm	1	ED	ED	Prone 5R 5L
16	Medium	2.0cm	2	SW	EK	Prone 5R 5L
17	Sham	n/a	n/a	ED	n/a	Prone 5R 5L
18	Low	4.0cm	2	SW	ED/SW	Prone 5R 5L
19	Low	5.0cm	1	ED	ED/SW	Prone 5R 5L
20	Sham	n/a	n/a	SW	n/a	Prone 5R 5L
21	High	1.5cm	1	ED	ED/SW	Prone 5R 5L

22	Medium	2.0cm	2	SW	EK/CD	Prone 5R 5L
23	High	1.5cm	1	ED	ED/SW	Prone 5R 5L
24	Sham	n/a	n/a	SW	ED/SW	Prone 5R 5L
25	High	1.5cm	1	ED	ED/CD	Prone 5R 5L
26	Low	4.0cm	2	SW	SW/EK	Prone 5R 5L
27	Medium	2.5cm	1	ED	EK/ED	Prone 5R 5L
28	Sham	n/a	n/a	SW	n/a	Prone 5R 5L
29	Sham	n/a	n/a	ED	n/a	Prone 5R 5L
30	Low	5.0cm	1	SW	ED/SW	Prone 5R 5L
31	Low	5.0cm	1	ED	ED/SW	Prone 5R 5L
32	Medium	2.0cm	2	SW	EK/CD	Prone 5R 5L
33	High	1.5cm	1	ED	ED/EK	Prone 5R 5L
34	Low	4.0cm	2	SW	ED/SW	Prone 5R 5L
35	Sham	n/a	n/a	ED	n/a	Prone 5R 5L
36	High	1.5cm	1	SW	ED/SW	Prone 5R 5L
37	Sham	n/a	n/a	ED	n/a	Prone 5R 5L
38	High	1.5cm	1	SW	ED/SW	Prone 5R 5L

Table 3. Specifics relating to the blast dose administered to each animal

Spec	Fore Muscle			Hind Muscle				Vessel		
	Oed-ema	Haemo-rrhage	Infil-trate	Oed ema	Haemo-rrhage	Infil-trate	Myo-lysis	Oed-ema	Haemo-rrhage	Infil-trate
High										
1	0	0	0	2	4	4	20	0	1	1
3	0	0	0	3	4	4	20	0	4	4
5	0	0	0	2	3	3	45	0	2	3
7	0	0	0	4	4	4	80	0	1	1
9	0	0	0	4	4	4	80	1	2	1
11	0	0	0	4	4	4	40	0	1	1
12	0	0	0	3	4	4	20	1	2	2
21	0	0	0	4	4	4	60	0	1	0
23	0	0	0	2	4	4	30	0	3	1
25	0	0	0	4	2	4	55	2	2	2
33	0	0	0	4	4	4	45	1	1	1
36	0	0	0	1	4	4	30	0	0	0
38	0	0	0	4	4	4	50	0	0	0
Med										
13	0	0	0	1	2	2	5	0	1	0
15	0	0	0	4	4	4	50	0	2	1
16	0	0	0	1	2	2	10	0	1	0
22	0	0	0	3	4	4	40	0	0	0
27	0	0	0	1	2	3	10	-	-	-
32	0	0	0	2	3	3	40	0	1	0

Low										
14	0	0	0	1	2	2	5	0	1	0
18	0	0	0	0	0	0	0	0	3	0
19	0	0	0	0	0	2	5	0	2	1
26	0	0	0	1	2	1	5	0	1	0
30	0	0	0	1	1	2	10	0	0	0
31	0	0	0	1	1	2	40	0	2	0
34	0	0	0	2	0	2	20	0	2	0
Shm										
2	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	2	0
8	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	1	0
28	0	0	0	0	0	0	0	0	0	0
29	0	0	0	0	0	0	0	0	1	0
35	0	0	0	0	0	0	0	0	2	0
37	0	0	0	0	0	0	0	0	0	0

Table 4. Pathology scoring for all experimental animals

Rabbit von willebrand factor (VWF) ELISA Kit Catalog Number. CSB-E08525Rb

For the quantitative determination of rabbit von willebrand factor (VWF) concentrations in serum, plasma.

This package insert must be read in its entirety before using this product.

If You Have Problems
Technical Service Contact information

Phone: 86-27-87582341 Fax: 86-27-87196150 Email: tech@cusabio.com Web: www.cusabio.com

In order to obtain higher efficiency service, please ready to supply the lot number of the kit to us (found on the outside of the box).

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. Antibody specific for VWF has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any VWF present is bound by the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for VWF is added to the wells. After washing, avidin conjugated Horseradish Peroxidase (HRP) is added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of VWF bound in the initial step. The color development is stopped and the intensity of the color is measured.

DETECTION RANGE

0.312 ng/ml-20 ng/ml.

SENSITIVITY

The minimum detectable dose of rabbit VWF is typically less than 0.078 ng/ml. The sensitivity of this assay, or Lower Limit of Detection (LLD) was defined as the lowest protein concentration that could be differentiated from zero. It was determined the mean O.D value of 20 replicates of the zero standard added by their three standard deviations.

SPECIFICITY

This assay has high sensitivity and excellent specificity for detection of rabbit VWF. No significant cross-reactivity or interference between rabbit VWF and analogues was observed.

Note: Limited by current skills and knowledge, it is impossible for us to complete the cross-reactivity detection between rabbit VWF and all the analogues, therefore, cross reaction may still exist.

PRECISION

Intra-assay Precision (Precision within an assay): CV%<8%

Three samples of known concentration were tested twenty times on one plate to assess.

Inter-assay Precision (Precision between assays): CV%<10%

Three samples of known concentration were tested in twenty assays to assess.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, dilute the

  samples with Sample Diluent and repeat the assay.

- Any variation in Sample Diluent, operator, pipetting technique, washing

  technique, incubation time or temperature, and kit age can cause variation

  in binding.

- This assay is designed to eliminate interference by soluble receptors,

  binding proteins, and other factors present in biological samples. Until all factors have been tested in the Immunoassay, the possibility of interference cannot be excluded.

MATERIALS PROVIDED

Reagents	Quantity
Assay plate (12 x 8 coated Microwells)	1(96 wells)
Standard (Freeze dried)	2
Biotin-antibody (100 x concentrate)	1 x 120 µl
HRP-avidin (100 x concentrate)	1 x 120 µl
Biotin-antibody Diluent	1 x 15 ml
HRP-avidin Diluent	1 x 15 ml
Sample Diluent	1 x 50 ml
Wash Buffer (25 x concentrate)	1 x 20 ml
TMB Substrate	1 x 10 ml
Stop Solution	1 x 10 ml
Adhesive Strip (For 96 wells)	4
Instruction manual	1

STORAGE

<table>
<tr><td>Unopened kit</td><td colspan="2">Store at 2 - 8°C. Do not use the kit beyond the expiration date</td></tr>
<tr><td rowspan="8">Opened kit</td><td>Coated assay plate</td><td>May be stored for up to 1 month at 2 - 8°C. Try to keep it in a sealed aluminum foil bag, and avoid the damp.</td></tr>
<tr><td>Standard</td><td rowspan="3">May be stored for up to 1 month at 2 - 8°C. If don’t make recent use, better keep it store at -20°C.</td></tr>
<tr><td>Biotin-antibody</td></tr>
<tr><td>HRP-avidin</td></tr>
<tr><td>Biotin-antibody Diluent</td><td rowspan="5">May be stored for up to 1 month at 2 - 8°C.</td></tr>
<tr><td>HRP-avidin Diluent</td></tr>
<tr><td>Sample Diluent</td></tr>
<tr><td>Wash Buffer</td></tr>
<tr><td>TMB Substrate</td></tr>
<tr><td>Stop Solution</td><td></td></tr>
</table>

*Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- ● Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- ● An incubator which can provide stable incubation conditions up to 37°C±0.5°C.
- ● Squirt bottle, manifold dispenser, or automated microplate washer.
- ● Absorbent paper for blotting the microtiter plate.
- ● 100ml and 500ml graduated cylinders.
- ● Deionized or distilled water.
- ● Pipettes and pipette tips.
- ● Test tubes for dilution.

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

SAMPLE COLLECTION AND STORAGE

● Serum Use a serum separator tube (SST) and allow samples to clot for

two hours at room temperature or overnight at 4°C before centrifugation for 15 minutes at 1000 ×g. Remove serum and assay immediately or aliquot and store samples at -20°C or -80°C. Avoid repeated freeze-thaw cycles.

● Plasma Collect plasma using EDTA, or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g, 2 - 8°C within 30 minutes of collection. Assay immediately or aliquot and store samples at -20°C or -80°C. Avoid repeated freeze-thaw cycles. Centrifuge the sample again after thawing before the assay.

Note:

1. CUSABIO is only responsible for the kit itself, but not for the samples consumed during the assay. The user should calculate the possible amount of the samples used in the whole test. Please reserve sufficient samples in advance.
2. Samples to be used within 5 days may be stored at 2-8°C, otherwise samples must be stored at -20°C (≤1month) or -80°C (≤2month) to avoid loss of bioactivity and contamination.
3. Grossly hemolyzed samples are not suitable for use in this assay.
4. If the samples are not indicated in the manual, a preliminary experiment to determine the validity of the kit is necessary.
5. Please predict the concentration before assaying. If values for these are not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.
6. Tissue or cell extraction samples prepared by chemical lysis buffer may cause unexpected ELISA results due to the impacts of certain chemicals.
7. Owing to the possibility of mismatching between antigen from other resource and antibody used in our kits (e.g., antibody targets conformational epitope rather than linear epitope), some native or recombinant proteins from other manufacturers may not be recognized by our products.
8. Influenced by the factors including cell viability, cell number and also sampling time, samples from cell culture supernatant may not be detected by the kit.
9. Fresh samples without long time storage are recommended for the test. Otherwise, protein degradation and denaturalization may occur in those samples and finally lead to wrong results.

REAGENT PREPARATION

Note:

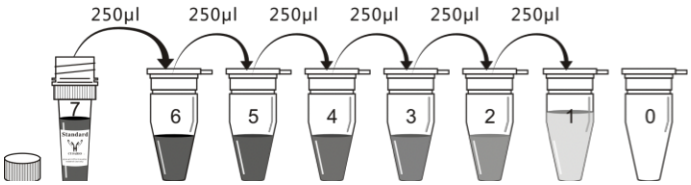
- Kindly use graduated containers to prepare the reagent. Please don't prepare the reagent directly in the Diluent vials provided in the kit.
- Bring all reagents to room temperature (18-25°C) before use for 30min.
- Prepare fresh standard for each assay. Use within 4 hours and discard after use.
- Making serial dilution in the wells directly is not permitted.
- Please carefully reconstitute Standards according to the instruction, and avoid foaming and mix gently until the crystals have completely dissolved. To minimize imprecision caused by pipetting, use small volumes and ensure that pipettors are calibrated. It is recommended to suck more than 10μl for once pipetting.
- Distilled water is recommended to be used to make the preparation for reagents or samples. Contaminated water or container for reagent preparation will influence the detection result.

1. Biotin-antibody (1x) - Centrifuge the vial before opening. Biotin-antibody requires a 100-fold dilution. A suggested 100-fold dilution is 10 μl of Biotin-antibody + 990 μl of Biotin-antibody Diluent.
2. HRP-avidin (1x) - Centrifuge the vial before opening.
   HRP-avidin requires a 100-fold dilution. A suggested 100-fold dilution is 10 μl of HRP-avidin + 990 μl of HRP-avidin Diluent.
3. Wash Buffer (1x) - If crystals have formed in the concentrate, warm up to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 ml of Wash Buffer Concentrate (25 x) into deionized or distilled water to prepare 500 ml of Wash Buffer (1 x).

8

4. Standard
Centrifuge the standard vial at 6000-10000rpm for 30s.
Reconstitute the Standard with 1.0 ml of Sample Diluent. Do not substitute other diluents. This reconstitution produces a stock solution of 20 ng/ml. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 250 μl of Sample Diluent into each tube (S0-S6). Use the stock solution to produce a 2-fold dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Standard serves as the high standard (20 ng/ml). Sample Diluent serves as the zero standard (0 ng/ml).



Tube	S7	S6	S5	S4	S3	S2	S1	S0
ng/ml	20	10	5	2.5	1.25	0.625	0.312	0

ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. Centrifuge the sample again after thawing before the assay. It is recommended that all samples and standards be assayed in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells and the desiccant back into the pouch and seal the ziploc, store unused wells at 4°C.
3. Add 100µl of standard and sample per well. Cover with the adhesive strip provided. Incubate for 2 hours at 37°C. A plate layout is provided to record standards and samples assayed.
4. Remove the liquid of each well, don't wash.
5. Add 100µl of Biotin-antibody (1x) to each well. Cover with a new adhesive strip. Incubate for 1 hour at 37°C. (Biotin-antibody (1x) may appear cloudy. Warm up to room temperature and mix gently until solution appears uniform.)
6. Aspirate each well and wash, repeating the process two times for a total of three washes. Wash by filling each well with Wash Buffer (200µl) using a squirt bottle, multi-channel pipette, manifold dispenser, or autowasher, and let it stand for 2 minutes, complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining wash Buffer by aspirating ordecanting. Invert the plate and blot it against clean paper towels.
7. Add 100µl of HRP-avidin (1x) to each well. Cover the microtiter plate with a new adhesive strip. Incubate for 1 hour at 37°C.
8. Repeat the aspiration/wash process for five times as in step 6.
9. Add 90µl of TMB Substrate to each well. Incubate for 15-30 minutes at 37°C. Protect from light.
10. Add 50µl of Stop Solution to each well, gently tap the plate to ensure thorough mixing.
11. Determine the optical density of each well within 5 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. Subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Samples may require dilution. Please refer to Sample Preparation section.

Note:

1. The final experimental results will be closely related to validity of the products, operation skills of the end users and the experimental environments.
2. Samples or reagents addition: Please use the freshly prepared Standard.

   Please carefully add samples to wells and mix gently to avoid foaming. Do not touch the well wall as possible. For each step in the procedure, total dispensing time for addition of reagents or samples to the assay plate should not exceed 10 minutes. This will ensure equal elapsed time for each pipetting step, without interruption. Duplication of all standards and specimens, although not required, is recommended. To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.

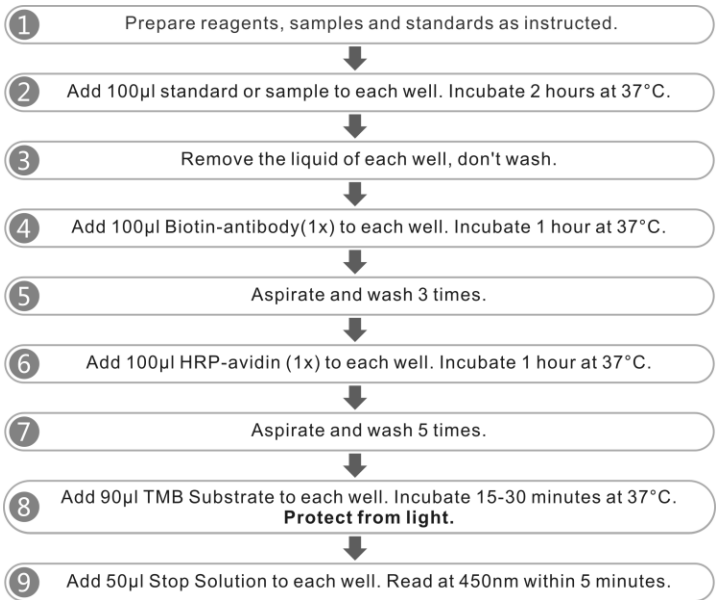
3. Incubation: To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods between incubation steps. Once reagents have been added to the well strips, DO NOT let the strips DRY at any time during the assay. Incubation time and temperature must be observed.

4. Washing: The wash procedure is critical. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Solution by aspirating or decanting and remove any drop of water and fingerprint on the bottom of the plate. Insufficient washing will result in poor precision and falsely elevated absorbance reading. When using an automated plate washer, adding a 30 second soak period following the addition of wash buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.

5. Controlling of reaction time: Observe the change of color after adding TMB Substrate (e.g. observation once every 10 minutes), TMB Substrate should change from colorless or light blue to gradations of blue. If the color is too deep, add Stop Solution in advance to avoid excessively strong reaction which will result in inaccurate absorbance reading.
6. TMB Substrate is easily contaminated. TMB Substrate should remain colorless or light blue until added to the plate. Please protect it from light.
7. Stop Solution should be added to the plate in the same order as the TMB Substrate. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the TMB Substrate.

ASSAY PROCEDURE SUMMARY

*Samples may require dilution. Please refer to Sample Preparation section.



CALCULATION OF RESULTS

Using the professional soft "Curve Expert 1.3" to make a standard curve is recommended, which can be downloaded from our web.

Average the duplicate readings for each standard and sample and subtract the average zero standard optical density.
Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the x-axis against the concentration on the y-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the VWF concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

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