Spectrin Breakdown Products in the Investigation of Blast Induced Traumatic Brain Injury

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"Spectrin Breakdown Products in the Investigation of Blast Induced Traumatic Brain Injury"

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"The purpose of fighting is to win. There is no possible
victory in defense. The sword is more important than the shield,
and skill is more important than either. The final weapon is the brain.
All else is supplemental."

John Steinbeck

Abstract

Blast injury has become the 'signature injury' of modern conflict, and there is increasing concern that within this injured population mild to severe Traumatic Brain Injury (TBI) is becoming more prevalent. TBI is difficult to diagnose using conventional diagnostic methods.

One component of a blast, the blast wave, has been associated with TBI. This has led to a suspicion of a subset of blast-exposed personnel who appear uninjured, but then develop TBI. Consequently, there has been much research into the use biomarkers for diagnosis and prognosis of TBI.

αII-Spectrin is a 240kDa cytoskeletal protein found in the cell membrane of neurons. Spectrin is irreversibly cleaved by the action of the proteases Calpain and Caspase, producing 145kDa and 120kDa Spectrin Breakdown Product (SBDP) respectively.

The aim of this study was to see if a blast wave to the head would produce SBDPs of interest and if they could be found in the Cerebrospinal Fluid (CSF) and plasma.

Terminally anaesthetised rats were subjected to a blast wave to the head; an equally sized control group was used. Blood was periodically sampled and at eight hours post-injury the brains, CSF and plasma were collected. Western Blot Gel Electrophoresis was used to identify SBDP in brain homogenate, CSF and plasma.

SBDP 145 and 120 were identified in brain homogenate and in plasma in blast and control groups, but there were no significant differences between groups. This suggests that blast exposure *per se* does not specifically lead to elevations in SBDP. It was not possible to reliably assess SBDPs in CSF due to blood contamination.

This study did not provide evidence that SBDPs are a reliable indicator of any TBI caused by a blast wave to the head in the acute phase, although they may have a role in polytrauma.

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Chapter 1 – Introduction

1.1 Background

Throughout the ages, the nature of war has evolved. With the development of new doctrine and new tactics, there has been a contiguous technological development of weapons and weapon systems. At the same time there have also been advances in personal protective equipment.

Just as the weapons and protection have changed, so has the nature of the injuries sustained by both combat troops and civilian populations.

Through both World Wars, warfare was characterised by opposing fronts of combatants. During these events the types of injuries sustained ranged from penetrating injuries caused by gunshots and fragments, to massive trauma sustained from the use of high explosives. The month of September in 1940 saw the commencement of the Blitz, the strategic bombing of the United Kingdom by Germany. As a result of the huge numbers of casualties from high explosives, Professor Zuckerman (1941) was among the first to try and investigate these injuries and, in the case of blast related injuries, to categorise them according to their nature.

The advent of the Vietnam War saw a changing face of warfare which was fought on a much more intimate level, where there were large numbers of small scale battles, but without the pitched offensive lines seen in the great wars. This resulted in another change in the pattern of the injuries seen (Okie 2005, Ivey et al 2012) with an increase in penetrating injuries.

Since 2001 there have been two medium scale offensives in Afghanistan and Iraq and these have seen the effective use of a tactic known as asymmetric warfare. This is not a new concept and has been in use as long as war itself. It originally referred to war in which two or more factions' military power differed in size. In the modern context, asymmetric warfare has come to mean opposing sides that may differ in size or degree of technological development. The 'weaker' side may then use techniques to offset its own relative weakness by exploiting a weakness in its opponent. The conflict in Northern Ireland saw successful development and deployment of Improvised Explosive Devices (IEDs) where 36%

of fatalities in one three year period were caused by explosives (Moffat 1976). This is a tactic which has been utilised with continuing success in Iraq and Afghanistan.

IEDs are devices which are constructed from locally available components and, at their most basic, comprise a power pack, a detonator, an explosive charge and some form of initiating trigger. They can be cunningly concealed and are often initiated when trodden on or disturbed. As a result the victim is often located very close to the seat of the explosion.

The resulting blast varies in power depending upon the quantity and type of explosive fill used in the device, and results in a wide range of injuries, though victims near the point of initiation generally receive major injuries. The explosive fill in IEDs varies from commercial or military grade explosives (for example, the use of unexploded ordnance), or it can be a 'homemade' concoction.

As operations in both Iraq and Afghanistan have developed, there has been a shift in the pattern of injuries sustained, partly due to the increased use of personal protective equipment such as body armour, and an increase in the use of IEDs such that blast injuries have become much more common. Indeed, blast injury has come to be called the 'signature' injury of these conflicts (Warden 2006, Hoge et al 2008, Moss et al 2009).

An increasing area of concern within this population of injured personnel is that of blast-induced Traumatic Brain Injury. This is currently a poorly understood phenomenon. Explosions are complex events, comprising elements such as shock wave, blast winds and gross acceleration forces and while it is recognised that personnel are suffering injury, it is not yet known which component or components of an explosive event produces the injury, or how it can be reliably tested for.

1.1.1 Blast Physics and Blast Injury Categorisation

1.1.1.1 Blast Physics

Explosive compounds contain an enormous amount of potential energy, contained within chemical bonds. Detonation of an explosive involves its near instantaneous exothermic chemical conversion into gas with the release of heat and kinetic energy.

The conversion from solid to gas state is comprised of two distinct phases. First there is the production of a Shock Wave which travels in three dimensions away from the point of the detonation. This is followed by an expansion of the gases producing the Blast Wind.

The speed, or detonation velocity of the shock wave can be extremely high, for example the detonation velocity of Trinitrotoluene (TNT) can be as high as 6,900m/s (by comparison the speed of sound is 343m/s)(Cooper 1996). The shock wave is characterised by a rapid increase in pressure, often followed by a period of negative pressure, as shown in the stylised blast wave form (Figure 1). The duration of the shock wave will depend upon the amount and type of explosive used, but is generally in the order of 1-5 milliseconds for conventional weapons (Wiener & Barrett 1986). The pressure waveform is known as the Friedlander wave, where the maximal pressure is known as the peak overpressure. This wave has a limited life as it decays proportional to the third power of the distance from the seat of the explosion (Wiener & Barrett 1986, Mellor 1992).

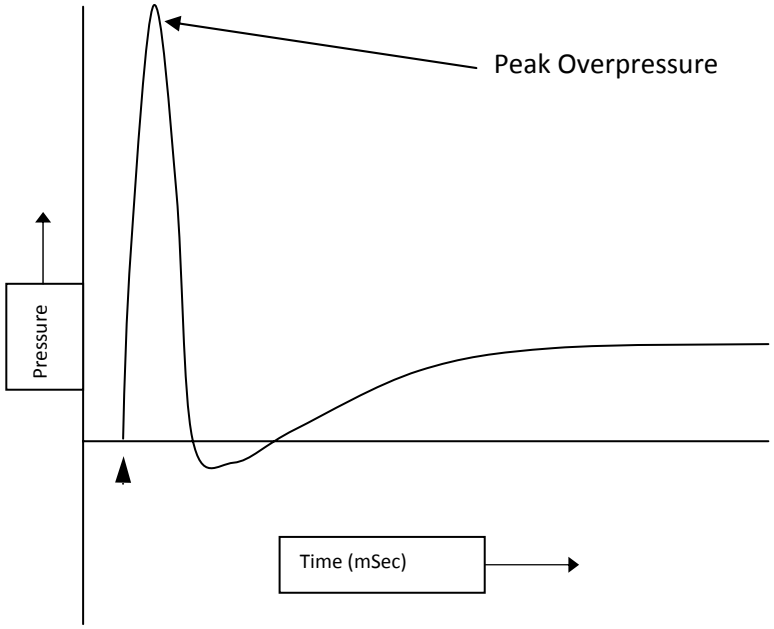

Figure 1: Idealised blast wave form (black arrowhead is detonation point) showing immediate increase in pressure, followed by a relative negative pressure period.

As the shock wave propagates, the gases produced by the detonation continue to expand producing the blast wind, which may travel at speeds of 940miles/hr (Wiener & Barrett 1986). The explosion is also associated with the release of heat, light and sound; and, where the explosive is contained within a solid structure, fragments and debris will also be propelled.

1.1.1.2 Injury Categorisation

The continuing use of high explosives during the Blitz of the Second World War, and the injuries that they inflicted, led to the development of a category of injury types that can be induced by a blast (Zuckerman 1941). This classification is still in used today and has changed very little since its inception. There are five main categories as follows:

a) Primary Blast Injury

Primary Blast Injury (PBI) is the direct effect of a blast shock wave upon the body and is unique to injuries caused by explosions. The shock wave travels through the body as stress waves (high amplitude/short duration) and shear waves (lower amplitude/longer duration). Subsequent injury will result from the effect of each of these waves, or a combination of the two upon tissues.

There are three main potential cellular injury mechanisms caused by the propagation of the blast shock wave, stretching, shearing and spalling. Stretching occurs by the shock wave 'dragging' tissues along its path, thus stretching them. Shearing causes movement of tissues, but where these tissues are of different densities, or are joined against a fixed point, they move at different rates, resulting in tearing. Spalling injury occurs as a result of a disruption of the surface of a tissue and occurs when a blast shock wave travels through tissues of differing density. The reflection of the wave creates cavitation and turbulence, propelling the denser material into the less dense. This is the type of injury that occurs at, for example, the fluid/gas interface within alveoli (Born 2005). Gas filled spaces can also be prone to implosion. This is the rapid contraction of gas-containing space, and an example here would be the middle ear, separated from the external environment by the tympanic membrane (Born, 2005).

It can be readily seen therefore that gas containing organs such as the lungs, ears and gastrointestinal tract will be particularly vulnerable to PBI due to the soft tissues' close proximity with gas filled spaces, although air-filled spaces surrounded by bone such as the paranasal sinuses have also been reported as being affected by blast (Turegano-Fuentes 2008).

b) Secondary Blast Injury

Secondary blast injury results from the fragments which originate from the site of initiation. They may comprise fragments of the device itself (either the casing of the device, or fragments such as nails and ball-bearings added to it to maximise damage), or fragments from the immediate environment where the device detonated (stones and dirt for example). These fragments will travel at immense speed, depending on their mass and origin, reaching velocities of approximately 1370m/sec (Wiener & Barrett1986). Upon contact with the body, whether they penetrate or are blocked by body armour, they deposit energy in their immediate surroundings. The degree of damage will depend upon variables such as the mass of the fragment and the amount of energy it carries. Fragments with relatively low energy will have poor penetrating ability while those with a high energy load will cause deep penetration.

Where penetration of the body by a high energy fragment occurs, the fragments may leave a small entry hole, but cause massive damage internally due to tissue cavitation. Tissue cavitation is the formation of a temporary cavity caused by the kinetic energy of a slowing projectile forcing tissue radially away from the fragment path (Hollerman et al 1990).

Tissue cavitation is highly variable depending as it does on variable factors such as fragment mass, speed and energy. The situation may be further complicated if the fragment further divides when inside the body. As such, apparently minor entry wounds should be treated with extreme caution as they may be misleading.

There have also been reports of allogenic implantation of bone or body fragments from suicide bombers or victims which have become embedded in the casualty (Leibner et al 2004).

c) Tertiary Blast Injury

Tertiary blast injury occurs where the blast wind that follows the peak overpressure propels the body to impact with objects. It may result in blunt trauma, although it has been suggested it may be part of a mechanism, following bone disruption by the blast shock wave, by which traumatic amputations take place, (Hull 1992, Hull et all 1994, Hull & Cooper 1996, Horrocks 2001).

d) Quaternary Blast Injury

This category of injury covers the miscellany of injury that occurs as a result of an explosion. It may include burns from the device itself or from secondary fires. It also includes crush injuries from structural collapse and, where the device contains a chemical fill, the clinical effects of this type of chemical.

e) Quinary Blast Injury

Continuing advancement in research and knowledge of blast injury has led to the use of a fifth category of blast injury, though its definition and application still appears to be under some debate. In some instances, the quinary group has been used to describe injury that occurs as a result of the environmental hazard left after the detonation (Moore & Jaffee 2010). Alternatively, quinary blast injury has been used to describe a generalised hyper-inflammatory state seen in some casualties characterised by hyperpyrexia, sweating and low central venous pressure (Kluger et al 2007) although the cause remains unclear. This category has also been used to describe the psychological effects and their sequelae that may occur after exposure to a blast.

In addition, to these effects there has been a suggestion that explosive devices, particularly those with metal casings, generate electromagnetic disturbances (Ling et al 2009) that cause brief radio-frequency pulses. The physiological effects of these are still the subject of debate (Hicks et al 2010).

In summary, at the present time, there is no universally accepted definition of quinary blast injury and it is used interchangeably to include exposure to hazards such a radiation, bacteria and hazardous debris thrown up by the explosion (Ritenour et al 2010), to a systemic inflammatory response (Mackenzie & Tunnicliffe 2010).

1.1.2 Blast in a Military Context

The military forces of all nations are supported by a medical chain that is able to provide point of wounding care, and then support the casualty back through an evacuation chain to definitive care at a field hospital or equivalent. Seriously wounded personnel may then be further evacuated to a second or home nation for further care. The goal is to provide the best level of medical care that the current operational situation allows.

Over the years, not only have the facilities evolved; the continuing numbers of casualties have refined both medical and surgical protocols. Because of this the emergency and critical care provided has continued to improve so that injured personnel are now surviving what would previously have been fatal injuries. One source states that the current standards of medical care have resulted in the highest survival rate in modern history where less than 1 in 10 patients die as a result of their injuries (Ling et al 2009). By comparison, 1 in 4 blast injured soldiers in Northern Ireland were killed (Mellor 1992).

Just as there have been advances in the medical care of blast related and other ballistic injuries, so there have also been advances in methods of transport and logistics. There can now be very much earlier medical evacuation from the point of wounding to a hospital facility where life-saving surgical care can be provided, further improving survival rates.

In addition, there have been technological advances in the nature and efficacy of soldiers' personal protective equipment that protects them from potentially fatal injuries. An example of this in the UK forces is Combat Body Armour. The latest iteration provides an unparalleled degree of ballistic protection against both fragments and bullets, while being more comfortable and having a lower physiological burden on the wearer. Other developments include the Combat Helmet, Ballistic Eye Protection, and even ballistic underwear that protects the groin from fragment injury.

Despite providing excellent protection from both bullets and fragments, all these forms of personal protection do little to attenuate the energy of the blast wave which produces Primary Blast Injury (Mellor 1992).

While body armour is now providing excellent protection from potentially fatal secondary and tertiary injuries to the chest and abdomen, it has been cited as one of the reasons for increasing incidence of head injury in soldiers in current conflicts (Okie 2005) as previously they would not have survived beyond the point of wounding.

This appears to be confirmed in a military scenario where Iraqi prisoners of war treated by a US Army Forward Surgical Team suffered a significantly greater number of thoracic and abdominal injuries compared to US soldiers who wore body armour (Patel et al 2004).

The ongoing conflict in Afghanistan has meant that the threat from IEDs is probably now greater than it has ever been, and despite huge advances in ways of both detecting and defeating the devices, there are many service personnel who have been, and are continuing to be, exposed to blast events.

For example, during one six month period at the medical facility in Camp Bastion in 2006, of 106 battle-injured casualties, 65% were treated for blast injuries (Tai et al 2008), and over a three year period in Iraq of 1151 casualties, 82% were injured by explosions, (Champion et al 2010). The combined figure of combat casualties as a result of blast for Iraq and Afghanistan has been reported as 60% in one report (Ling et al 2009). These figures are similar to another report where 55.5% of injured personnel were exposed to a nearby IED blast on more than two occasions (Hoge et al 2008).

Blast injury is sadly not confined to the military population. There are numerous instances over the years where civilian populations have been exposed to blast in various circumstances (Stein 2005, Leibovici et al 1996,) and still fresh in the memory, the bombing of Madrid in 2004 (Turegano-Fuentes et al 2008) and London in 2005 (Lockey et al 2005, Ryan & Montgomery 2005). A large proportion of the casualties are exposed to blast as a result of a terrorist action where explosive devices have been placed in busy public areas with the aim of causing the maximum amount of injury. The scenario results in a different subset of injured people as, in a civilian attack, none of the individuals will be wearing protective equipment. In addition, terrorists will often add fragments in the form of nails, screws or ball-bearings in order to increase the number of fragmentation injuries (Leibovici et al 1996, Born 2005).

An area of increasing concern within the military medical community, (and their civilian counterparts in the case of terrorist incidents) is the area of traumatic brain injury. A comparison of the current conflicts versus Vietnam, Korea and World War II shows a trend towards an increasing incidence of head injuries versus a decrease in thoracic injuries (Owens et al 2008, Belmont et al 2010). The incidence of head injury among wounded soldiers in current conflicts is 22%, whereas it was only 12-14% of combat casualties in the Vietnam conflict (Okie 2005). This difference may be the result of the previously mentioned factors of body armour permitting survival from fragmentation injuries, and increased use of explosive devices.

It has been recognised that after exposure to a blast event a number of personnel are going on to develop neurological impairment with symptoms such as fatigue, memory loss, irritability and sleep disturbance featuring in many cases. While some of the personnel had been severely injured in the blast, it is of note that some appeared to have been exposed the blast yet were apparently uninjured.

1.2 Traumatic Brain Injury

Traumatic Brain Injury (TBI) of non-military origin is a common occurrence in the civilian population where it has been estimated that there are greater than two million people affected in the United States of America (Cardali & Maugeri 2006). In England, approximately 110,000 people were admitted to hospital with head injury in 2001 (Tennant 2005).

TBI can occur after an acute insult to the brain and is a complex injury which varies greatly in severity. Closed TBI is defined as TBI with no penetration to the cranial vault. Severe closed TBI may cause moderate to severe neurological or other dysfunction whereas mild closed TBI may be clinically silent or cause a myriad of cognitive dysfunctions such as fatigue, headaches or depression which are difficult to assess by conventional imaging modalities such as CT or MRI scanning.

One of the difficulties of establishing the presence of TBI is that it has no concise definition, though the US Department of Defense now has an accepted definition that covers the multitude of symptoms as well as their causes (Health Affairs Memorandum 07-030):

"*Traumatic brain injury (TBI) is a traumatically induced structural injury and/or physiological disruption of brain function as a result of an external force that is indicated by new onset or worsening of at least one of the following clinical signs, immediately following the event:*

1. *Any period of loss, or a decreased level, of consciousness.*
2. *Any loss of memory for events immediately before or after the injury.*
3. *Any alteration in mental state at the time of the injury (confusion, disorientation, slowed thinking, etc.)*
4. *Neurological deficits (weakness, loss of balance, change in vision, praxis, paresis/plegia, sensory loss, aphasia, etc.) that may or may not be transient.*

5. *Intracranial lesion.*

External forces may include any of the following events: the head being struck by an object, the head striking an object. The brain undergoing an acceleration/deceleration movement without direct external trauma to the head, a foreign body penetrating the brain, forces generated from events such as a blast or explosion, or other force yet to be determined."

Clearly, although brief, this definition is extremely wide-ranging in its application, and goes someway to showing just how complex TBI can be, in that it can be mild to severe, transient to permanent and with many different symptoms.

As stated in the definition, one of the external causes thought to induce TBI is 'force generated from events such as a blast or explosion'.

It has become increasingly apparent that TBI features significantly in victims of blast exposure (Okie 2005, Taber et al 2006, Hoge et al 2008), and blast induced TBI is now becoming recognised as a distinct syndrome compared to penetrating and concussive TBI (Ling et al 2009). Blast induced TBI may vary in nature from severe neurological dysfunction to really quite subtle cognitive abnormalities, and while severe TBI may be diagnosed relatively easily based on patient examination, blast induced TBI can be difficult to identify as it may manifest in a number of ways; it is not always identifiable by imaging, and it may or may not be transient. There are also concerns that repeated trauma may have a cumulative effect (Bey & Ostick 2009). To add to the complexity, other injuries sustained elsewhere in the body may mask signs of TBI. In addition, as part of point of wounding first aid and stabilisation, the use of Morphine Auto-Injectors may further complicate the assessment of a patient.

1.2.1 The Pathophysiology of Blast Induced TBI

As has been intimated in the previous section, TBI is a difficult syndrome to diagnose. In order to facilitate our learning about TBI it is necessary to understand something of its origin, before trying to establish objective and quantifiable means of measuring it in order that it may then be treated.

There have been several proposed mechanisms of blast induced TBI, encompassing systemic, local and cerebral responses, but as with any entire-body system, physiology is

rarely as simple as direct cause and effect. There may be a number of physical mechanisms in action, and behind these may be a number of physiological processes occurring, possibly as a result of other injury, that may affect the development of the TBI. Some of the important mechanisms will now be discussed in more detail:

1.2.1.1. Primary Mechanisms:

These are mechanisms which exert a direct effect on the Central Nervous System tissues:

a) Primary blast wave:

There is scant clinical evidence thus far that a primary blast wave can directly induce TBI (Taber et al 2006) although there is evidence that a primary blast waves can cause cell damage with cortical cell loss, gliosis, haemorrhage and necrosis (Long et al 2009). It has been proposed that there may be direct transcanial propagation effect (Bhattacharjee 2008). The mechanisms for neural cell injury by a primary blast wave are not clear, as similar lesions can be seen following whole body blast exposure (Cernak et al 2001).

While the involvement of primary blast in the development of TBI seems plausible, it is not understood which characteristics of the blast wave are important for injury development and direct involvement of the primary blast wave cannot be ruled out.

b) Increased Intracranial Pressure (ICP):

It has been recognised that after exposure to a blast pressure wave, there follow short (in the order of milliseconds) increases in ICP that are of sufficient magnitude to cause cellular damage (Chavko et al 2007). It has been speculated that this increase in ICP is caused by the propagation of pressure waves in the vasculature, via the great vessels, from the body to the Central Nervous System (Cernak et al 2001). This has been termed the 'Blood Hammer' effect, and is analogous to the 'Water Hammer' effect seen in hydraulics (Damşa et al 1976). There has also been a suggestion that the hydraulic effect may be from pressure waves through the spinal Cerebrospinal Fluid, via the foramen magnum (Hicks et al 2010), and this is supported by one report where there was spinal cord injury without vertebral

fracture, possibly caused by cord concussion secondary to CSF pressure waves (Turegano-Fuentes et al 2008).

In some instances, there can be a delayed increase in ICP that is thought to be due to a delayed onset cerebral vasospasm (Armonda et al 2006, Ling et al 2009).

c) Air Emboli:

It has been proposed that air microemboli are forced into the bloodstream as a result of the blast wave effect on the lungs. The emboli then travel via the circulation to the CNS where they effectively cause an infarction, leading to cell death (Clemedson 1956, Okie 2005). This is now thought to be a rare cause of CNS damage following blast, although it has occasionally been documented as causing death (Freund et al 1980, Wiener & Barrett 1986). It remains a difficult syndrome to quantify as air emboli are hard to find in tissue at a histopathological level unless they are specifically looked for (Mellor 1992).

d) Skull Deformation:

While it would appear logical that the bone of the skull creates a secure vault for the brain to reside in, experimental modelling using a simulated head has demonstrated that even at non-lethal blast overpressures, there was deformation of the skull such that the brain was subjected to mechanical loads equivalent to impact derived TBI (Moss et al 2009).

1.2.1.2 Secondary Mechanisms:

Here there are processes occurring elsewhere in the body that either exert their own effect on the CNS or exacerbate processes which have occurred as a result of primary injury mechanisms:

a) Hypoxia:

It has long been recognised that one of the major organs affected by blast is the lungs, where the blast overpressure can result in so- called 'Blast Lung' (Pizov et al 1999, Horrocks 2001). When lungs are damaged as a result of blast, there is extensive haemorrhage, contusion, fluid infiltration and breakdown of alveolar integrity. The effect of this is to decrease alveolar gas exchange resulting in a

ventilation-perfusion mismatch and the development of a relative hypoxaemia. The cells of the CNS are extremely sensitive to lowered oxygen tension and extended hypoxaemia will initiate cell death. Although case reviews have shown that the incidence of blast lung is relatively low in the casualties seen at the medical facilities in Iraq and Afghanistan, between 7.3 and 11% respectively (Smith 2011), when it does occur the hypoxia is a sequel that significantly worsens the clinical outcome in these cases.

b) Haemorrhage

A common condition seen in blast victims is haemorrhage (Cooper et al 1983), indeed 90% of all patients with combat injuries seen at a US Marine Corps Forward Resuscitation Facility in Operation Iraqi Freedom had penetrating injury (and thus some degree of haemorrhage)(Chambers et al 2005).

As haemorrhage becomes profound (≥20-30% of blood volume), a vagally mediated bradycardia and reduction in peripheral vascular resistance occurs Barcroft & Edholm 1944), resulting in a fall in arterial blood pressure, i.e. hypotension (Sawdon et al 2002). Hypotension following profound haemorrhage has been recognised as a poor prognostic indicator in cases of head injury. One study reported that hypotension occurred in 34.6% of severe head injury patients and was associated with 150% increase in mortality (Chesnut et al 1993). Inadequate cerebral blood flow has also been recognised as an important contributor to mortality and morbidity after TBI (DeWitt & Prough 2009) and this will clearly be the situation in cases of severe haemorrhage.

c) Physiological Responses to Blast:

Mild to moderate blast exposure to the thoracic region results in a reflex apnoea, hypotension and bradycardia (Guy et al 1998, Long et al 2009), via a vagally mediated reflex (Ohnishi et al 2001). These responses can lead to decreased blood flow to the brain, thus reducing oxygen delivery. Relative systemic hypoxaemia, that may be present as a result of concomitant lung injury, further decreases cerebral oxygen tensions thus exacerbating damage to the CNS tissue. It is well

recognised that in cases of severe TBI, both hypoxia and hypotension are associated with a worse outcome (Chesnut et al 1993).

TBI also affects the ability of the blood vessels within the brain to vasodilate or constrict (known as autoregulation) and so regulation of cerebral blood flow in the face of blood pressure changes elsewhere in the body may be compromised (DeWitt & Prough 2009).

d) Reactive Oxygen Species:

Work has shown that oxygen free radicals are released following traumatic injury (Cernak et al 2001) and ischaemic events (Chan 1996) in the brain, and that these free radicals cause damage to cell membranes by a process of peroxidation (Kontos & Povlishock 1986). It is possible that an imbalance in the endogenous antioxidant pathways permits the radicals to cause the damage, (Cernak et al 2001).

It is unlikely that military TBI will be as a result of a single damaging mechanism; rather it will be a function of subtle interactions between those mechanisms described above, and possibly others; that will result in a clinically significant injury.

1.2.2 Injury Types and Introduction to Diagnosis

Clearly, no two explosive incidents are likely to be the same. There are numerous factors which determine the type and degree of injury. At times, it can be difficult to identify those symptoms which are due to the blast-induced TBI and those symptoms which are due to other injuries. At the present time there is no definitive means of diagnosis of TBI, rather there are a number of indirect measures of assessment and an enhanced diagnosis of blast induced TBI is clearly a requirement. Concomitant injuries have been suggested as a marker for the presence of TBI, but the majority of assessment is based on either imaging of the neurological system, or assessment of its function by means of neurological testing. The next section describes some methods of assessing for the presence of blast induced TBI.

1.2.2.1 Tympanic Membrane Perforation

One injury that was thought to be useful in TBI diagnosis is tympanic membrane perforation. It was hypothesised that exposure to a blast overpressure sufficiently strong to induce tympanic perforation, will in itself be sufficient to induce TBI. The incidence of perforation varies from reports of approximately 50% in one study (Mellor 1992), to 35.2% in a cohort of blast-injured US soldiers who suffered at least unilateral perforation. In this case 35.7% of the same cohort also suffered loss of consciousness, with a significant association between the conditions (Xydakis et al 2007). In the train bombings that occurred in Madrid in 2004, 46.8% of all casualties suffered tympanic perforation (Turegano-Fuentes et al 2008) although in this case the perforations were not linked to the possibility of neurotrauma. More recent research suggests that tympanic membrane perforation may not be as useful as a diagnostic tool as was first thought (Harrison et al 2009), and that while the presence of perforation may raise suspicion of TBI, its absence cannot rule out TBI's presence.

1.2.2.2 Conventional Imaging Techniques:

Conventional imaging modalities are defined as those that are both readily available within the hospital environment, and are accepted and licensed for use.

a) Magnetic Resonance Imaging (MRI)

MRI works by aligning the magnetisation of atoms within the body, then using radio-frequency fields to rotate that alignment. The rotating magnetic field can be detected by the scanner and used to produce an image of the region of interest within the body. Serial scans can be used to produce three dimensional images. There are two main advantages of MRI over conventional radiography and Computed Tomography (CT). The first is that the images produced show good differentiation between different soft tissues as well as between soft and hard tissues. This makes it suitable for the imaging of structures such as the brain, muscles and the heart. The second main advantage is that the scanner does not produce ionising radiation, making it safer for patient and operator. There are two distinct disadvantages of MRI, especially in the early stages of patient care. Firstly,

MRI generates intense magnetic fields and thus may not be suitable unless patients have first received survey plain radiography to rule out the presence of ferrous fragments. Secondly, it is more time consuming and the patient is less visually accessible to medical staff. Also, they must relatively stable prior to imaging as intervention is difficult.

MRI has been used to successfully identify Diffuse Axonal Injury in patients with normal CT scans but with current neurological derangement (Paterakis et al 2000), although in this case there was some selection bias in the patient cohort, in that only those with a significant neurological impairment were included. However, MRI is not necessarily the best imaging modality for assessing subtle neurological damage; indeed, MRI has shown structural normality in between 43% to 68% of patients with mild TBI (Hofman et al 2001, Hughes et al 2004).

b) Computed Tomography (CT)

Also called X-ray Computed Tomography and Computerised Axial Tomography (CAT), this technique uses a computer to generate a three dimension image from two dimensional x-ray beams around a single axis of rotation. CT is particularly well suited to imaging of hard structures which are able to differentially block the path of the x-ray beam, although it can be useful to identify haemorrhagic lesions within the brain. It remains, however, less sensitive to subtle neurological damage when compared to MRI (Paterakis et al 2000).

CT involves a moderate to high exposure to ionising radiation, but has the advantage over MRI in that it is quick, relatively cheap and does not require specialist non-magnetic ventilators, monitors etc. Both CT and MRI have the disadvantage that repeat exposure, especially in the short term may be contraindicated for patient safety (Wang et al 2005).

1.2.2.3 Non-Conventional or Novel Imaging Techniques:

These are imaging modalities which are still comparatively new and may be used either in research or at specialist neurological centres. The techniques can be broadly divided into those which look at the structural integrity of tissues and organs, and those which are able to assess the functioning of those same tissues.

1) Structural

a) Diffusion Tensor Imaging (DTI)

This is a relatively new MRI-based technique which is able to assess the restricted diffusion movement of water molecules as they travel in a particular plane. In addition, DTI is able to measure the diffusion movement in a number of different directions. This allows an image to be constructed showing water movement along axonal length. Only one study (Arfanakis et al 2002), has been performed which looks at mild TBI (mTBI) in the first 24 hours post-injury. The theory is that alterations to the structural integrity of neurons in Diffuse Axonal Injury (DAI) will decrease diffusion along axon length and relatively increase the diffusion in a perpendicular plane to them. In this study all patients with mTBI had decreased diffusion in areas affected by DAI compared with unaffected areas in the contralateral hemisphere. These areas had often improved by one month post-injury.

Some research has been conducted into the use of DTI specifically for blast induced TBI (Brody 2010), where DTI identified 20 out of 63 blast-related TBI patients with abnormalities indicative of traumatic axonal injury which were not detectable on conventional MRI or CT scan.

b) Magnetization Transfer Imaging (MTI)

This is another technique that uses magnetization interaction, in this case between protons in free water and protons in water bound to macromolecules. The application of a radiofrequency pulse allows the difference between free water and macromolecules to be seen, and the presence or absence of macromolecules (i.e. tissues) to be determined (Wolff & Balaban 1994). Studies have found it to have correlation between TBI and clinical outcome, and may be able to be used to quantify some types of mTBI (Sinson et al 2001).

c) Magnetic Resonance Spectroscopy (MRS)

This technique utilises Magnetic Resonance as with DTI and MTI, however, it is able to specifically measure neurochemicals which provide a measure of the metabolic status of the brain, rather than its structural integrity. For example, N-

acetylaspartate (NAA) is a chemical which is present in normal functional neurons, and decreases in its concentration are consistent with neuronal destruction (Cvoro et al 2009). In a similar fashion, Choline is a marker of inflammation (Brenner et al 1993). Studies have shown that MRS imaging can provide a reliable index of injury severity and can be linked to disease outcome, where NAA and Choline were found to correlate closely with Glasgow Coma Score at time of injury and three months later (Marino et al 2007).

2) Functional

a) Functional MRI (fMRI)

fMRI works on the basis that increases in neuronal activity results in increased local cerebral blood flow. As oxygenated blood becomes more available there is a concurrent decrease in levels of deoxyhaemoglobin (dHb). The reduction of dHb (in itself paramagnetic and thus a natural MR contrast agent) gives a regional MRI signal indicating greater neural activity, and this can be related to spatial images of the brain provided by MRI allowing point sourcing. This method of action is termed the Blood Oxygen-Level Dependent (BOLD) fMRI (Belanger et al 2007).

b) Positron Emission Tomography (PET)

Positron Emission Tomography is another technique that is able to measure regional cerebral metabolism. In this case, areas of the brain that have increased activity, will have increased regional cerebral blood flow (rCBF) in order to provide sufficient metabolic products such as glucose. In PET, glucose (or water) is labelled with a short-half life radionuclide and is injected into the body, and its path traced. In TBI, there is an initial state of increased glucose utilisation (hyperglycolysis) (Bergsneider et al 1997), followed by an extended period of metabolic depression (Belanger et al 2007), this can be followed by recovery.

c) Single Photon Emission Computed Tomography (SPECT)

This is a similar imaging technique to PET, although it is used to measure the patient's brain while at rest, while PET can be used for imaging during tasking (such as speech recognition or acoustic stimulation). Another difference between SPECT and PET is that in order to establish abnormality the region of interest (ROI), as

determined by other imaging techniques, must be compared with an apparently unaffected area of the brain. Given the sometimes diffuse nature of neural injury, this can be problematic with regards interpretation of abnormalities seen.

1.2.2.4 Neurological Assessment

It is possible to conduct tests of the neurological system which can be used as an indirect assessment of neural function. There are several tests available and some can be conducted without patient compliance (i.e. they are an observational test only) such as the Glasgow Coma Scale. Others are tests of Cognitive Process and as such require some degree of patient participation. Cognition is defined as "the mental act or process by which knowledge is acquired, including perception, intuition and reasoning (Collins Concise Dictionary 3rd Edn.). Cognitive function tests are not tests of intelligence, rather of process integrity. By testing factors such as memory, association and pattern recognition, an assessment can be made of the degree of neurological dysfunction. Dysfunction in this context may refer to level of consciousness (e.g. Glasgow Coma Scale) or inferred damage by the inability or decreased capacity to perform tasks.

a) Glasgow Coma Scale (GCS)

This scoring system was published in 1974 (Teasdale & Jennett 1974) and involves an assessment of the patients' ability to perform an eye, verbal and motor response, and was developed to assess both the depth and duration of impaired consciousness.

b) Military Acute Concussion Evaluation (MACE)

This has become a recognised method of cognitive assessment used to screen personnel deemed to be at risk of developing TBI, mainly those who have experienced either a loss of consciousness or an episode of being dazed (i.e. concussion). This questionnaire based test can be administered by suitably trained personnel and assesses such parameters as orientation (awareness of location, time and date etc), immediate memory, concentration and memory recall. As the likelihood of TBI being present increases, the score achieved on the test decreases. Although a cut-off value is not yet available, scores below a certain level indicate possible TBI (French et al 2008, McCrea et al 2009).

c) Immediate Post-Concussion Assessment and Cognitive Testing (ImPACT)

This test is a computer based test of concussion symptoms and neurocognitive function. The test battery evaluates factors such as attention, verbal recognition memory, visual working memory, reaction time, numerical sequencing ability and learning (Covassin et al 2009).

d) King-Devick Test

The King-Devick test is a rapid test that uses the speed of rapid number naming, taking only approximately two minutes to complete. It has so far been used mainly in sports related concussion, and has been proposed as a rapid screening tool for the presence of neurological impairment prior to further workup (Galleta et al 2011).

Thus there are several tests which may be useful for the indication of mTBI in a blast injured casualty, but most have the same disadvantage in that the patient must be conscious and able to participate in the test. Cognitive testing may therefore be useful in a minimally injured individual in order to screen them for mTBI, but it cannot be used where there is severe injury elsewhere in the body and mTBI may remain unseen at the clinical presentation. Hence, alternative diagnostic methods need to be explored and evaluated, one such method is the use of biomarkers.

1.3 Biomarkers – An Introduction

It is clear from the previous section that there are several means of trying to diagnose TBI, but that none of them are fully reliable, or can be obtained soon after injury during the likely therapeutic window of opportunity (Wang et al 2005). It would be beneficial to be able to not only consistently detect TBI, but also to quantify it so that the severity could be determined. In order to do this, much research has been conducted into biomarkers which are specific to brain injury. A biomarker is a substance which can be used to identify the presence or progression of a specific biological state or process, either natural or pathological. Biomarkers can indicate altered enzymatic activity, changes in gene or protein expression, altered protein or lipid metabolites, or a combination of these, (Dash et al 2010).

For a biomarker to be of use it must be determined how the levels of that biomarker change according to injury severity, and also how they change over a time period following insult. The biomarkers of interest would ideally be able to be measured in peripheral fluids such as plasma or serum, although historically it has been difficult to determine small amounts of markers diluted in the circulating volume. Cerebrospinal fluid (CSF) is likely to contain a greater proportion of markers of neural injury than the blood but has two distinct disadvantages in that the proteome of the brain and its adnexa is extremely complex, containing at least 20,000 proteins (Wang et al 2005), and that there is an associated risk in CSF collection while peripheral blood is easy to collect.

1.3.1 Potential Biomarkers of Brain Injury

Much work has been done in recent years in looking at target biomarkers for various disease states, and there are numerous possibilities. Here some of the promising candidates for the measurement of TBI will be described:

a) S-100B

S-100B is a calcium-binding protein that is found at high concentration within astroglial cells of brain tissue. It is known to cross the blood-brain barrier in demonstrable levels following mild head trauma (Ingebrigsten et al 1997), and it has been suggested as a means of predicting the outcome of head injury (Rosén et al 1998, Townend et al 2002, Mehta 2010). Following further investigation it was found that S-100B may not be as useful as first thought as a predictor for cerebral ischaemic injury as it has extra-cerebral sources (Missler et al 2002), including ischaemic myocardial tissue (Mazzini et al 2005). It has been suggested that its use may be restricted to clinical cases of closed head injury with little or no systemic injury, in order to reduce confounding sources of the protein (Mehta 2010). Clearly, this will not be suitable in the military scenario where systemic injury is common.

b) Glial Fibrillary Acidic Protein (GFAP):

This is a neural specific protein found in astrocytes, and is released following TBI where it may have a use as a determinant of severity (Vos et al 2004), and it is not released following polytrauma without cerebral involvement (Pelinka et al 2004).

c) Neuron Specific Enolase (NSE):

NSE is an isoenzyme of Enolase, a glycolytic enzyme, which was originally thought to be specific to neurons (Dash et al, 2010), but has subsequently been found in neuroendocrine cells, oligodendrocytes, thrombocytes and erythrocytes. Although it can be detected in peripheral blood within 6 hours of injury, it has a half life of 24 hours, limiting its use for monitoring (Dash et al 2010). At serum levels of >21.7μg/L, NSE strongly correlates with mortality and poor outcome, (Vos et al 2004) but has poor sensitivity/specificity (55%:77.9% respectively) of predicting neuro-psychological outcome compared to S-100B (65%:88%) (Herrmann et al 2001). This is in conflict with suggestions that measurement of NSE on the day of injury may be a useful predictor of three month outcome (using the Glasgow Outcome Score) compared with S-100B (Mehta 2010). As a sole marker, elevated NSE has also been associated with small cell lung cancer, neuroendocrine bladder tumours, ischaemic stroke and neuroblastomas (Dash et al 2010). These are unlikely to be significant in the military population profile, but they serve to demonstrate the non-specific nature of NSE.

d) Microtubule Associated Protein (MAP):

MAPs are proteins which interact with the microtubules associated with cytoskeletal structure, and MAP1 is particularly found in the axons and dendrites of nerve cells. They are cleaved by the same group of proteases that act upon Spectrin (see Spectrin Breakdown Products below), and MAP breakdown products can be found in TBI in rats (Gabbita et al 2005), so they may prove useful in the determination of TBI in humans.

Table 1 below shows a selection of the numerous other markers which have been suggested as being potentially useful in the diagnosis of TBI.

Marker	References
Structural Protein markers	
Myelin Basic Protein	Berger et al 2005
Fatty Acid Binding Proteins	Pelsers et al 2004
Phosphorylated Neurofilament H	Anderson et al 2008
Microtubule Associated Protein-tau (C-tau)	Gabbita et al 2005
Proline	Louin et al 2007
Ubiquitin C-Terminal Hydrolase-L1 (UCH-L1)	Liu et al 2010
Inflammatory Markers	
Interleukin-1	Chiaretti et al 2005
Tumour Necrosis Factor	Crespo et al 2007
Interleukin-6	Chiaretti et al 2008
Auto-antibodies	Wang et al 2005
Lipid Metabolites	
F2-Isoprostane	Varma et al 2003

Table 1: – Summary of biomarkers suggested for potential usefulness in the diagnosis and clinical prognosis of TBI.

The markers mentioned above have all been implicated in neural injury but none appear to be entirely specific for mTBI. Thus they will not be considered further in this thesis.

1.3.2 Spectrin Breakdown Products (SBDP)

Spectrin is a cytoskeletal protein, important for the maintenance of cell shape and stability, that has been described in the cell membrane structures of red blood cells and neural tissue. Brain spectrin is a distinct form of spectrin (Goodman & Zagon 1986) that is found in two subtypes, 240/235 and 240/235E (Riederer et al 1986). The 240 subunit (α unit) is bound to the 235 subunit (β unit) and this is forms a $(\alpha\beta)_2$ tetrameric cytoskeletal protein that is found particularly in the cytoskeleton of neurons (Zagon et al 1984).

Following trauma, cell death can take place in one of two main pathways, either necrosis or apoptosis (Figure 2). Necrosis occurs where there is trauma to the cell, for example by disruption to the blood supply or exposure to toxins, which leads to an uncontrolled cell disintegration with an associated inflammatory response to both the cell contents and released inflammatory mediators. Apoptosis is a process of programmed cell death in which specific biochemical pathways are initiated in order to kill the cell. The process is

tightly controlled and as such is not associated with the inflammatory response seen in the necrosis pathway.

Apoptosis can be further subdivided into an intrinsic and an extrinsic pathway depending on the source of the inducing factor. The extrinsic pathway is initiated by external signals acting on cell membrane receptors. The intrinsic pathway is initiated internally and causes increased permeability of the mitochondrial membrane with a resultant release of cell death initiators. Both pathways then involve the activation of enzymes with the ultimate result of cell death.

Spectrin is among the structures that are cleaved during the cell death pathways, and releases Spectrin Breakdown Products (Ringger et al 2004a). The SBDP are classified according to their size in kilo-Daltons, and SBDP 150, 145 and 120 are particularly of interest. Although the enzymes which produce these breakdown products are similar, their proportional production will largely depend upon the cell death pathway that is occurring within the tissue, either necrosis (SBDP 150/145), or apoptosis (SBDP 120) (Weiss et al 2009).

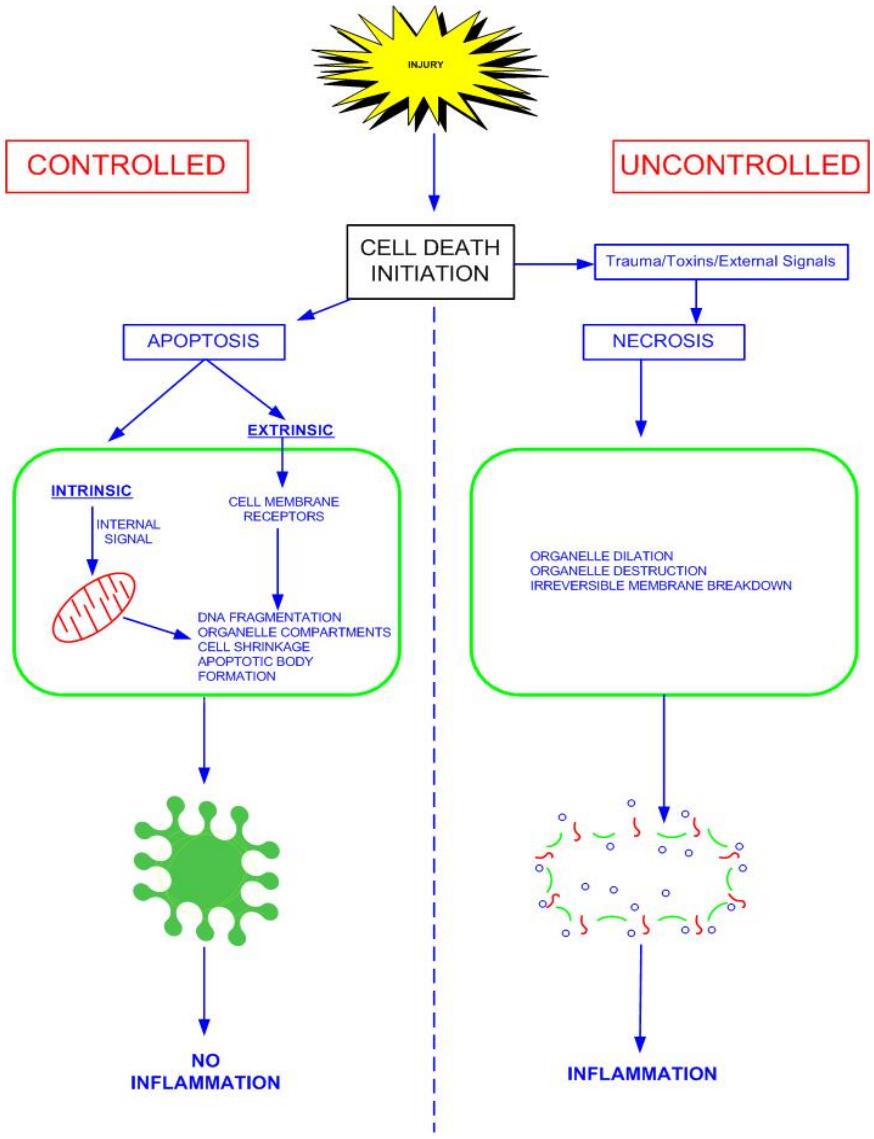

Figure 2: Diagram Illustrating Characteristics of the Apoptosis and Necrosis pathways.

a) Necrosis pathway:

The principle enzyme of interest in this pathway is the protease Calpain, a calcium activated enzyme which cleaves both spectrin into the 150kDa fragment size, and the 150 kDa fragment into 145 kDa (see Figure 3). Calpain can also be activated by the conjugation of the NMDA receptor.

b) Apoptosis Pathway:

As previously mentioned, the apoptosis pathway involves two potential routes, the extrinsic and the intrinsic (see Figure 3). The extrinsic pathway is initiated by the conjugation of the Fas receptor with the Fas ligand, while the intrinsic pathway is initiated by the reception of stress signals such as lowered metabolism, inflammatory mediators or oxidative stress by the mitochondria. Both pathways have a common factor in the activation of the cysteine-dependent aspartate-directed protease, Caspase-3. This enzyme cleaves spectrin into the 150 kDa fragment and then further cleaves the 150 kDa fragment into the 120 kDa.

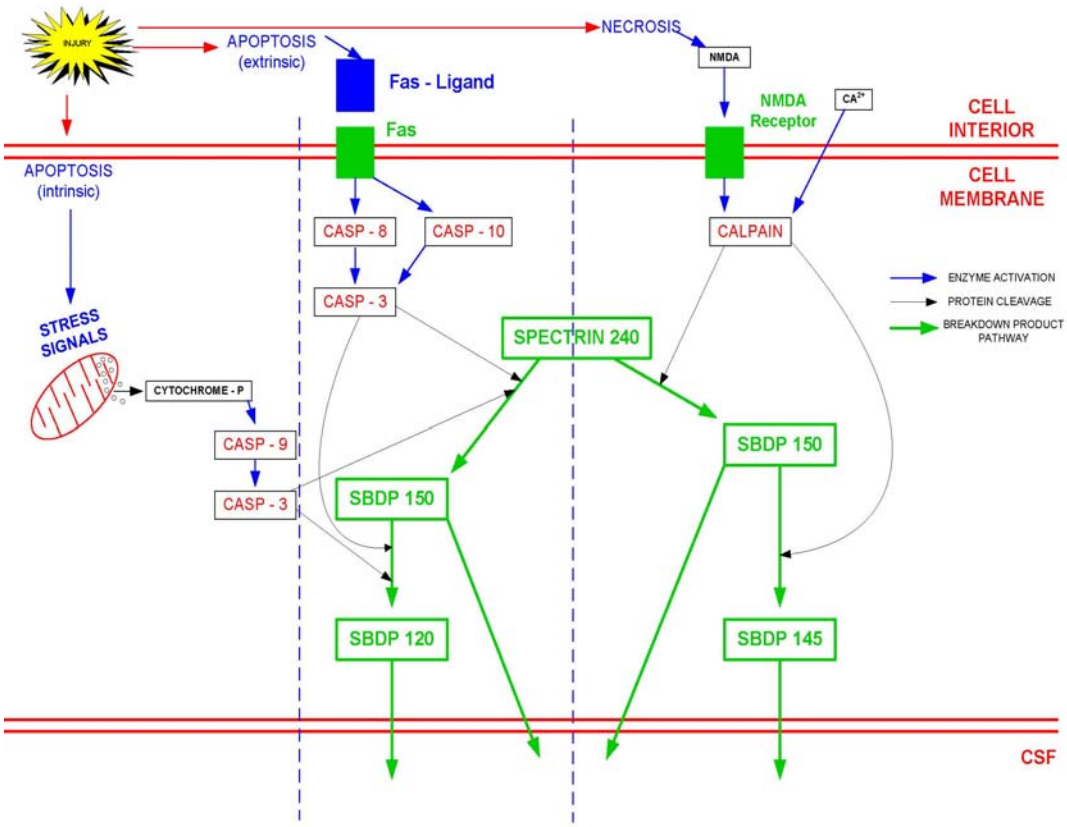

Figure 3: Diagram Illustrating Spectrin Breakdown Product Cleavage Pathways

Both proteolytic enzymes cleave spectrin into 150kDa fragments, but these have unique end-terminal regions (Pike et al 2004). The release of SBDP has been shown in canine

(Weiss et al 2009) and rat models of brain injury (Aikman et al 2006). SBDP have also been found in salmon that have incurred impact trauma to the head (Miracle et al 2009).

There has been investigation into the presence of SBDP in human (Cardali & Maugeri 2006) and canine (Weiss et al 2009) cases of TBI which concluded that SBDP are reliable indicators of severe TBI, and furthermore, that the temporal degradation profile of the markers may be an indicator of outcome (Cardali & Maugeri 2006). Although there is evidence that SBDP are present after TBI caused by mechanisms such as cortical impact (Pike et al 2001) or ischaemia (Pike et al 2004), there is currently no information on how they change in response to a primary blast waves. Previous work conducted at Dstl Porton Down, in collaboration with a group at the University of Florida (now Banyan Biomarkers) showed evidence of early (within 4 hours of blast exposure) elevations in SBDP 145 in brain tissue. Unfortunately no results were obtained for CSF or plasma, the assays were not quantitatively assessed against a standard, and difficulties were reported with the assay. Dstl has now developed an assay for SBDP in CSF which will allow investigation of the effects of blast on these biomarkers.

1.4 Aim of Study

It has been estimated that despite over two hundred clinical trials for the treatment of TBI, there are still no fully effective therapies for it, (Wang et al 2005). The inability to diagnose the TBI has been recognised as a major hurdle in the development of effective therapy, and the development of a specific biomarker would assist greatly (Wang et al 2005). The gold standard for biomarker use would be a marker which is easily and reliably measurable in serum, and secondarily in CSF, and ideally whose pattern of change could lead to the formulation of an informed prognosis.

In the military environment where personnel are often, and sometimes repeatedly, exposed to blast waves, a reliable marker of blast-induced TBI, if such an injury pattern exists, would be invaluable. It could be used as a screening tool for personnel exposed to large blast overpressures but who appear uninjured, thus allowing intervention at an early stage, if appropriate, prior to return to duties. In multiply-injured casualties a TBI specific assay would permit neuroprotective therapy at the same time as life-saving treatment was being initiated.

The aim of this study is therefore to establish whether exposure to a significant primary blast wave causes brain damage to a sufficient level to be detected by changes in SBDP concentration in CSF and plasma.

Chapter 2 – Research Methodology

2.1 Animal Selection and Husbandry

For this study adult male Wistar rats (Porton strain) were used with a mean weight (+/- SEM) of 251 +/-4g. These animals were kept in a 12 hour light/dark cycle with a dawn/dusk phase, with ad libitum access to food and water.

2.1.1 Group Allocation

Animals were allocated randomly to receive either blast or no blast (sham) on the day of the procedure. Those in the blast group were subjected to a controlled blast to the head, as described below. Those in the control (sham) group were treated the same during anaesthetic induction and cannulation surgery as the blast group but were not subjected to any form of blast wave. The identification of the individual animals can be found in Appendix 1.

2.2 Surgical Preparation

After weighing, anaesthesia was induced in an anaesthetic induction chamber using an initial 2% concentration of Isofluorane (IsoFlo® Abbott Animal Health) in 2ltr/min Oxygen, increasing after approximately a minute to 5% until the animal was unresponsive and the righting response was lost. Following induction, anaesthesia was maintained at approximately 2% Isofluorane in 1ltr/min of medical air by means of a nose cone. Maintenance was adjusted as necessary to maintain a surgical plane of anaesthesia with the minimum Isofluorane concentration to ensure there was no withdrawal from a toe pinch. A rectal temperature probe (Harvard Apparatus, Holliston, MA) was inserted and core body temperature maintained at approximately 38.5^{o}C using an electronic heating mat (Harvard Apparatus, Holliston, MA). If rectal temperature fell below 38^{o}C an external heat source was used by means of a heat lamp. A pulse oximeter (Starr Life Sciences, Oakmont, PA) was attached to the hind foot of each animal for arterial O_2 saturation and heart rate. The tail artery was then surgically cannulated (Vygon 2.5fr Umbilical Catheter) to the level of the abdominal aorta. Following cannulation, anaesthesia was maintained using Isofluorane in medical air for the duration of the experiment via a co-axial nose mask/scavenging system. Continual blood pressure monitoring was commenced using a

strain gauge manometer (Sensonor 840, SensoNor, Norway) and blood pressure and derived heart rate were captured using a computerised data acquisition system (MacLab, ADInstruments, UK; Chart v4.2.3, AD Instruments UK).

After a 30 minute stabilisation period post cannulation surgery, blood samples of approximately 0.2ml were taken anaerobically via the tail artery for blood gas analysis (pH, PaO_2, $PaCO_2$ and Base Excess) and haematocrit (Gem Premier 3000 Blood Gas Analyzer, Instrumentation Laboratory, Warrington, UK at the following time points (Table 2):

Sample	Time (min)
01	15 (pre-blast)
02	15 (post-blast)
03	30 (post-blast)
04	60 (post-blast)
05	120 (post-blast)
06	240 (post-blast)
07	360 (post-blast)
08	480 (post-blast)

Table 2 – Blood Sampling Time Points

2.3 Blast Apparatus

2.3.1 Apparatus and Calibration

A bench-top blast wave generator designed and constructed by Dstl Porton Down, (see Figure 4) and previously described (Jaffin et al 1987, Guy et al 1998) was used to provide a repeatable shock wave . Compressed air was used to prime the generator behind a 0.55mm thickness aluminium bursting disc to a pressure of approximately 10MPa (1450 psi). On rupture of the disc, the blast wave left the generator via a 20mm diameter nozzle, aimed at the target 15mm below the bottom of the tube.

Blast wave characterisation was achieved using a piezoelectric pressure transducer mounted in the surface of a plastic block. The transducer signal was amplified by a digital

oscilloscope, (Nicolet, Nicolet Technologies Ltd, UK). A minimum of three separate blast exposures were carried out which allowed determination of mean peak overpressure (700 kPa/101 psi).

2.3.2 Blast Exposure

Animals allocated to the blast group were subjected to a controlled blast wave from the blast nozzle. The anaesthetised animals were placed in right lateral recumbency with the centre of the blast nozzle directed at the lateral canthus of the left eye.

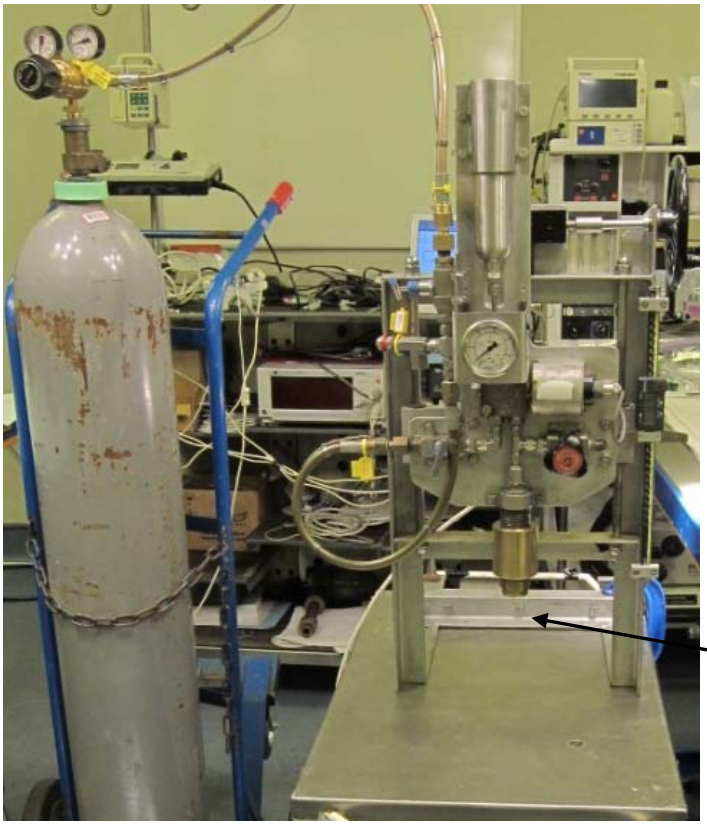

Figure 4: Photograph of Bench-top Blast Wave Generator

2.4 Experiment Termination

Just prior to the termination of the experiment CSF was collected by suboccipital puncture into the cisterna magna using a 27g Butterfly Infusion set (Abbott, UK).In this manner between 100 and 150µl of CSF was collected. Euthanasia was performed using 1ml of a 200mg/ml solution of Sodium Pentobarbital (Pentobarbital for Euthanasia 20% w/v

Solution for Injection , Ayrtons Saunders Ltd) administered via the tail artery cannula. Blood pressure monitoring was maintained until death was confirmed.

2.5 Sample Processing

Immediately after death, the brains of all 10 animals were externalised, the cerebellum and brainstem removed, and the two cerebral hemispheres separated prior to being cut coronally into four equal sections. The sections were frozen in liquid Nitrogen and stored at -80^{0}C for future analysis.

CSF samples were placed in plain Eppendorf tubes and centrifuged at 3000rpm for 10 minutes at 4^{0}C to spin out any cells in the CSF as well as any potential blood contamination. The supernatant was removed and placed in plain Eppendorf tubes, frozen in liquid Nitrogen and stored at -80^{0}C until analysis.

0.2ml of whole blood was placed in a Sodium Citrate Eppendorf and centrifuged at 3000rpm for 10 minutes at 4^{0}C. The supernatant was then carefully removed to avoid disturbing the cellular pellet, and the resultant plasma placed in a plain Eppendorf tube. This was frozen in liquid Nitrogen and stored at -80^{0}C until analysis.

2.6 Assay Methodology

After retrieval from storage the brain tissue was homogenised to allow separation of the breakdown proteins. The brain homogenate, the CSF and the plasma were then examined for SBDP using Western Blot Gel Electrophoresis. The breakdown products in the plasma were first concentrated using protein enrichment.

2.6.1 Brain Tissue Homogenisation

Approximately 50µg of tissue was homogenised in 250µl of lysis buffer (assay agent contents can be found in Appendix 2) with 1.4mm ceramic spheres and Lysing Matrix D (MP Biomedicals, Cambridge, UK) for 1min at 30Hrtz and then chilled on ice for 1min. Bead beating was repeated for a total of two homogenisation steps. Samples were centrifuged at 13,000g for 15mins and the sample supernatant was stored at -80^{o}C until analysis.

2.6.2 Plasma Protein Enrichment

Plasma samples were optimised using ProteoMiner Small Capacity Kit, (Bio-Rad Laboratories Inc, Hemel Hempstead, UK) in accordance with manufacturers specifications, prior to biomarker assay. This process utilises an extensive library of hexapeptide ligands to bind to specific sequences of the proteins in plasma. Highly abundant proteins saturate their ligands and the excess are eluted from the sample. Low abundance proteins remain bound to their own ligands. The effect is to remove excess high abundance protein in plasma (such as albumin or immunoglobulins), thus effectively concentrating low abundance proteins and reducing the signal-to-noise ratio for electrophoresis and blotting.

2.6.3 Biomarker Assay

2.6.3.1 Gel Electrophoresis

0.05g of Dithiothreitol (DTT) sample running buffer (Sigma-Aldrich, Gillingham, UK)) was added to 500µl of prepared 2x(XT) buffer (Bio-Rad Laboratories Inc, Hemel Hempstead, UK).

To 25µl of Phosphate Buffered Saline (PBS) 25µl of the sample being investigated was added and mixed using an electronic stirrer. To these diluted samples, 50µl of the dilute DTT sample running buffer was added. The top of the Eppendorf tube was pierced, and the proteins denatured by boiling at 100^{o}C for 5min.

Controls were made using recombinant SBDP145/120 (Life Technologies, UK)

A CriterionTM XT Bis-Tris Precast 4-12% 12-sample well Gel (Bio-Rad Laboratories Inc, Hemel Hempstead, UK) was rinsed with double-distilled water (ddH2O), and placed in the electrophoresis tank, and this was filled with MOPS running buffer stock solution (see Appendix 2). The samples were removed from the heater and spun down again at up to 8000rpm.

6µl of Precision Plus ProteinTM WesternCTM Standard (Bio-Rad Laboratories Inc, Hemel Hempstead, UK) was added to the control ladder wells on each side of the gel. 20µl of the samples or controls were added to the wells and the gel was run at 200V constant and 165mA for 50min.

2.6.3.2 Western Blotting

A Polyvinylidene Fluoride (PVDF) membrane (Bio-Rad Laboratories Inc, Hemel Hempstead, UK) was cut to the approximate size of the gel, and was then soaked in 100% methanol for 5min.

A piece of Extra Thick Blot filter paper (Bio-Rad Laboratories Inc, Hemel Hempstead, UK) was placed on top of the gel which was placed in the cathode buffer. The PVDF membrane was removed from the 100% methanol and placed in the anode tray, with another Extra Thick filter on top. The PVDF membrane, gel and filter papers were then left to soak for 30min at room temperature.

The samples were transferred from the gel to the PVDF membrane using semi-dry Western Blotting using 20V constant at 120mA for 60min.

2.6.3.3 Immunoblotting

The PVDF membrane was blocked using 60ml SuperBlock blocking buffer (ThermoScientific, Southend-on-Sea, UK) for 60min. The membrane was then blocked again using 60ml 0.05% Tween 20 PBS (PBST) containing 2% Goat serum and 1:1000 Goat anti-Mouse (GAM) antibody (ABD SeroTec, Kidlington, UK) for 60min.

12µl of primary antibody (mouse anti-spectrin) (BioMol, UK) was diluted to 1:5000 in 60ml PBST. This was then added to the membrane and incubated at 4^{o}C overnight.

The membrane was washed three times in 100ml of PBST before being incubated for 60min with 60ml of the secondary antibody, GAM-biotin (ABD SeroTec, Kidlington, UK) at a 1:20000 dilution (60ml PBST + 3µl GAM-biotin.

The membrane was then washed for 10min in PBST before adding 60ml of visualisation media, (60ml PBST + 6µl strep Tactin HRP (Bio-Rad Laboratories Inc, Hemel Hempstead, UK) + 15µl Neutravidin (ThermoScientific, Southend-on-Sea, UK)), and incubating at room temperature for 60min.

After washing in PBST for 10min, 6ml of Luminol enhancer (ThermoScientific, Southend-on-Sea, UK) and 6ml peroxide were added and the membrane incubated at room temperature for 5min. Images were then taken using Bio-Rad VersadocTM MP Imaging System.

2.7 Statistical Analysis

All data are presented as mean±SEM unless indicated otherwise. Data were assessed for normality (normal plot) and non-normality (Shapiro-Wilk test) and where necessary a transformation applied to normalize the data. Cardiovascular, blood gas and chemistry data were compared using two-way analysis of variance (ANOVA) with repeated measures over time. Single time-point analyses were made using an unpaired Student's t-test for between group comparisons. Within group assessment before and after an intervention (plasma levels of biomarkers before and after blast exposure) were compared using a paired t-test or, where fold changes were assessed, using a single sample t test. $P<0.05$ (two tailed) was considered statistically significant in all cases.

2.8 Ethical Considerations

All work was conducted in accordance with the Animals (Scientific Procedures) Act 1986 and followed approval after a full Ethical Review Procedure held at Dstl, Porton Down.

Chapter 3 – Results

Baseline (pre-blast) data for both groups of animals are shown in Table 3:

	Blast	Sham blast	Blast vs Sham
Number of animals	6	6	
Body weight (g)	243.4±2.1	232.6±3.1	P = 0.0216
MBP (mmHg)	78.6±5.1	87.2±3.6	P = 0.2008
HR (b/min)	416±10	410±10	P = 0.6748
PaO_2 (kPa)	15.70.6	14.3±1.4	P = 0.3949
$PaCO_2$ (kPa)	6.5±0.1	6.4±0.2	P = 0.4825
Art pH	7.392±0.010	7.410±0.007	P = 0.1646
ABE (mM)	4.0±0.8	4.7±0.04	P = 0.4434
Body temperature (°C)	38.4±0.2	38.2±0.1	P = 0.1966
Haematocrit (%)	35.8±1.0	35.8±0.9	P = 0.9806

Table 3: Baseline values recorded before exposure to blast or sham blast in two groups of animals. MBP, mean arterial blood pressure; HR, heart rate; PaO_2 and $PaCO_2$, arterial tensions of oxygen and carbon dioxide respectively; Art pH, arterial pH; ABE, actual base excess of arterial blood. Mean±SEM.

The only parameter that showed a significant difference between groups was body weight. However, the difference was small and unlikely to be of physiological significance.

3.1 Physiological effects of blast exposure

Blast exposure led to a transient fall in mean arterial blood pressure in 3/6 animals, and little change in the remaining 3/6 animals. An example hypotensive response to blast is shown in Figure 5.

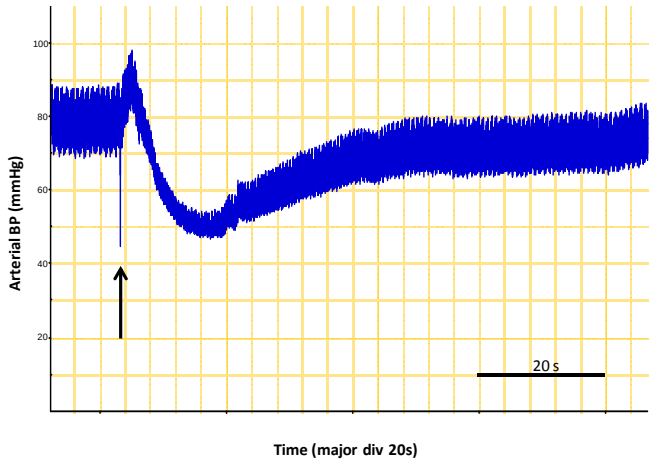

Figure 5: Cardiovascular response to a single exposure to a blast wave (indicated by the arrow) focused on the lateral aspect of the head in terminally anaesthetised rats showing a depressor response typical of 3/6 animals.

The group mean fall in blood pressure of 11.2±5.6 mmHg in response to blast did not attain statistical significance (P=0.101, Figure **5** 6). By contrast, mean arterial blood pressure was unchanged in response to sham blast (change immediately after blast 0.8±5.6 mmHg, P=0.8029). There was no significant change in heart rate after blast or sham blast (Figure 6).

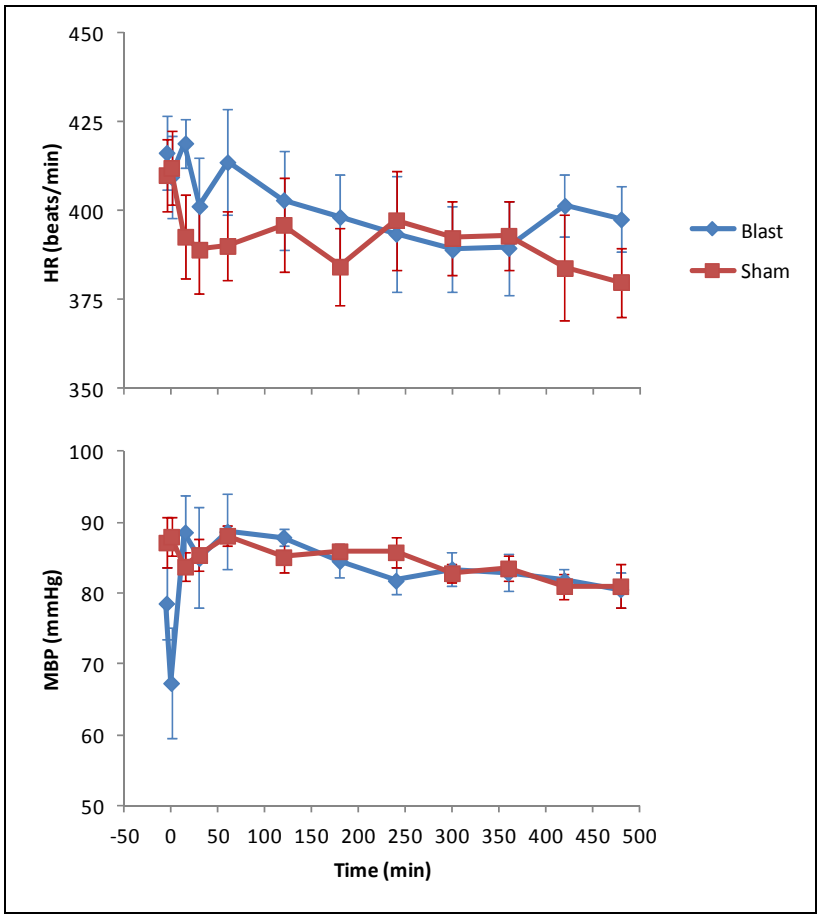

Figure 6: Effects of blast or sham blast exposure on heart rate (HR) and mean arterial blood pressure (MBP) in two groups of animals. Blast (or control) exposure was at time 0 h. Mean±SEM.

Serial measurements of both heart rate and mean arterial blood pressure between 15 min and 8 h after blast exposure revealed no significant difference between groups (P=0.4411 and P=0.1253).

Blast exposure had little physiological overall effect on the other physiological parameters. There were no significant differences between blast and control groups in either series for any of PaO_2, $PaCO_2$, or arterial base excess (Figure 7) (P = 0.6523, 0.1721, 0.3922 respectively) or body temperature (P = 0.0991, data not shown). There was a significant

decline in arterial haematocrit over the course of the study ($P < 0.0001$), and a difference between groups which *post hoc* analysis identified as a difference at 2h after blast exposure. However, this difference between groups was small (0.8%) and unlikely to be of physiological significance. The overall decline in haematocrit over the time course of the study may have been due to the volume of saline used to maintain cannula patency.

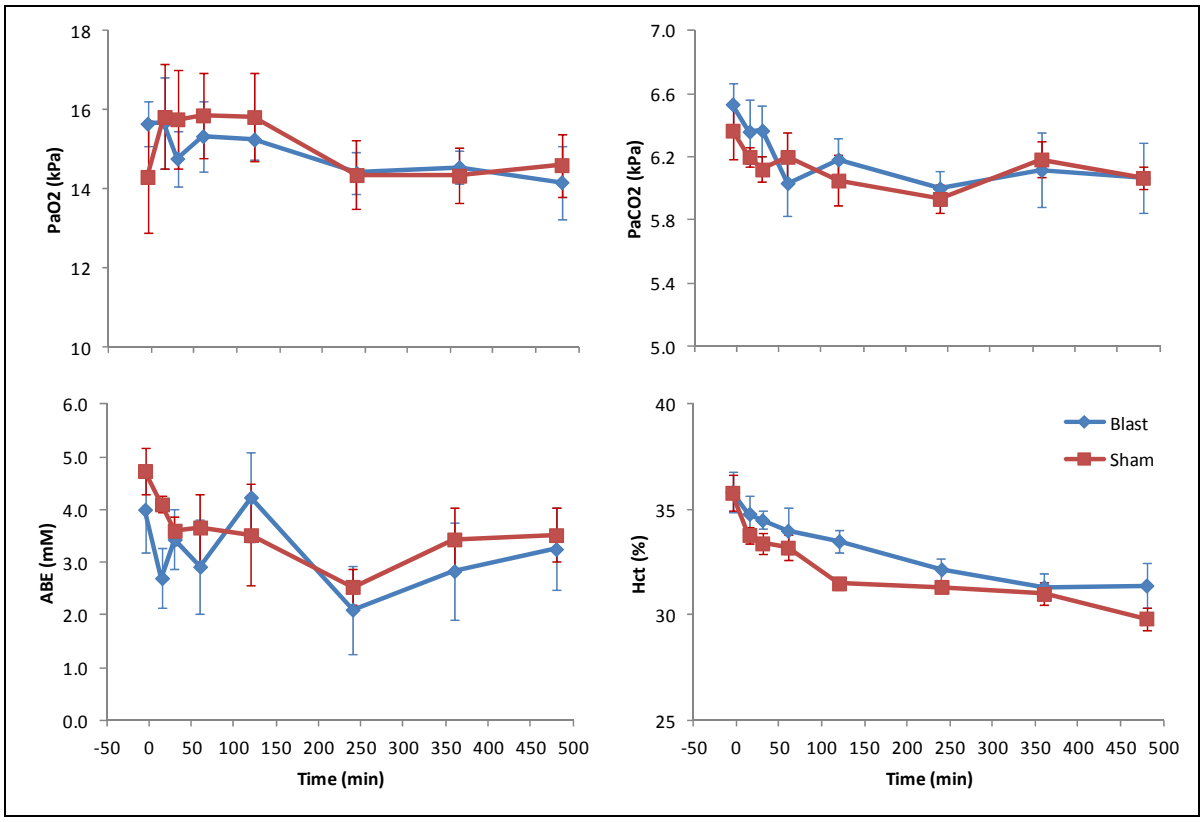

Figure 7: Effects of blast exposure (Blast) or control exposure (Sham) on arterial oxygen tension (PaO_2), carbon dioxide tension ($PaCO_2$) arterial base excess (ABE) and haematocrit (Hct) in two groups of terminally anaesthetised rats. Blast (or control) exposure was at time 0 h. Mean±SEM.

3.2 αII-Spectrin breakdown products in brain tissue

αII-Spectrin breakdown products SBDP120 and SBDP145 were identified in brain tissue lysate from both blast and non-blast exposed animals. A typical western blot from this study is shown in Figure 8.

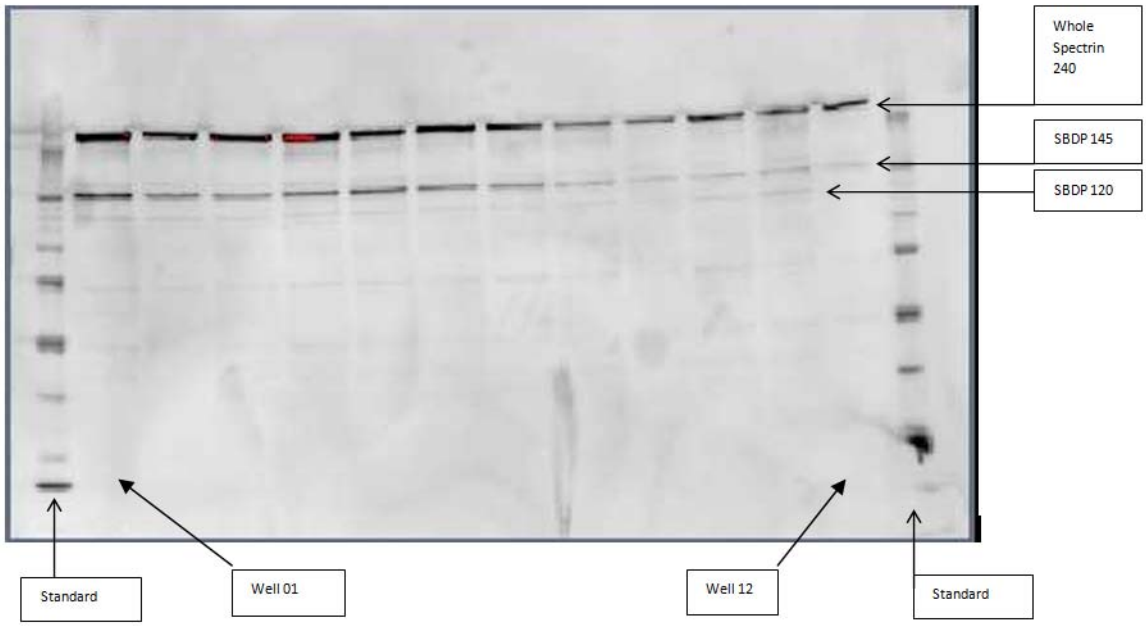

Figure 8: Photograph of Western Blot Gel Preparation for Brain Homogenate. Wells are numbered 1-12 from left to right. The well contained brain tissue lysate from the following animals (B denotes blast and S denotes sham blast) as follows: 1 = S2, 2 = B2, 3 = B3, 4 = S3, 5 = S4, 6 = S5, 7 = BB4, 8 = B5, 9 = B6, 10 = S6, 11 = B1, 12 = S1.

However, blast exposure did not alter the level of SBDP145 (P=0.5079 blast vs sham blast) or SBDP120 (P=0.398) found in brain tissue 8 hours after exposure (Figure 9).

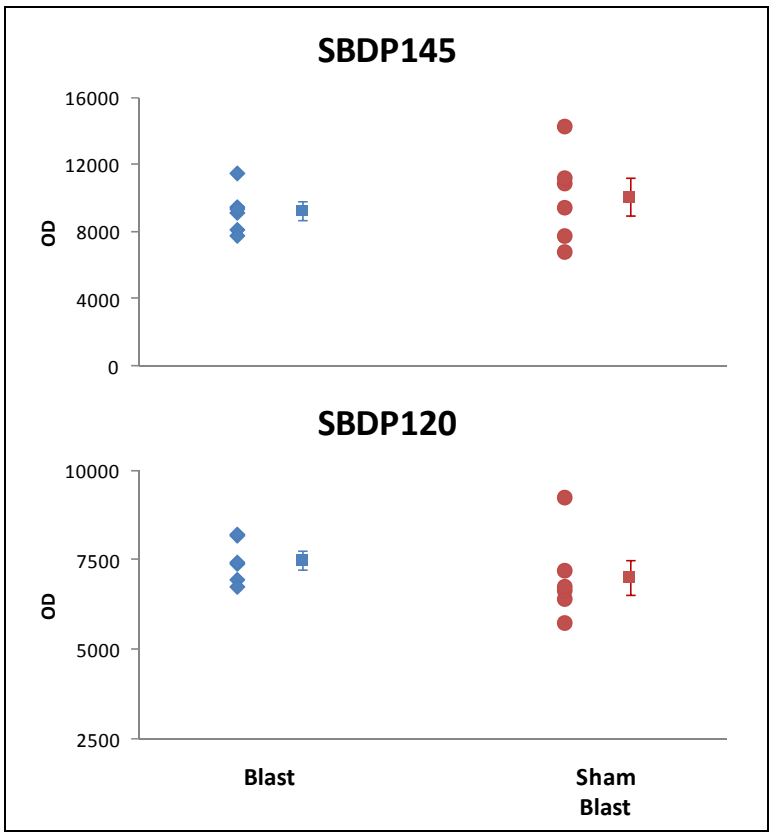

Figure 9: SBDP145 (upper panel) and SBDP120 (lower panel) levels in blast and sham blast exposed groups. Data for individual animals are shown as either diamonds (Blast) or circles (Sham) and the relevant group mean values ± SEM are shown as squares to the immediate right of the individual data. SBDP levels are expressed in standardised units of optical density to allow direct comparison between western blot gels.

The levels of both SBDP140 and SBDP120 in brain tissue of individual blast exposed animals fell within the range seen in control (sham blast) animals (Figure 9). The data presented in this thesis are based on group sizes of 6 animals in each of the blast and sham blast cohorts. A power calculation (Power 80%, alpha 5%) using the variance found in this study indicated that group sizes of 6 and 4 respectively would be required to identify a 50% increase in SBDP145 and 120 levels in brain tissue. Since increases of approximately 1.5-7.0 fold have been reported 3h after moderate fluid percussion injury (at which time SBDP levels were still rising (McGinn et al 2009, Reeves et al 2010), this study was adequately powered to identify a similar increase if it occurred after blast exposure. Finally, levels of SBDP240 were also measured in brain tissue from both groups of animals, and again there were no significant differences between blast and sham blast groups (P=0.2132).

3.3 αII-Spectrin breakdown products in plasma

SBDP145 and SBDP120 were detected in plasma samples before and 8 hours after blast and sham blast exposure. A typical western blot is shown in Figure .

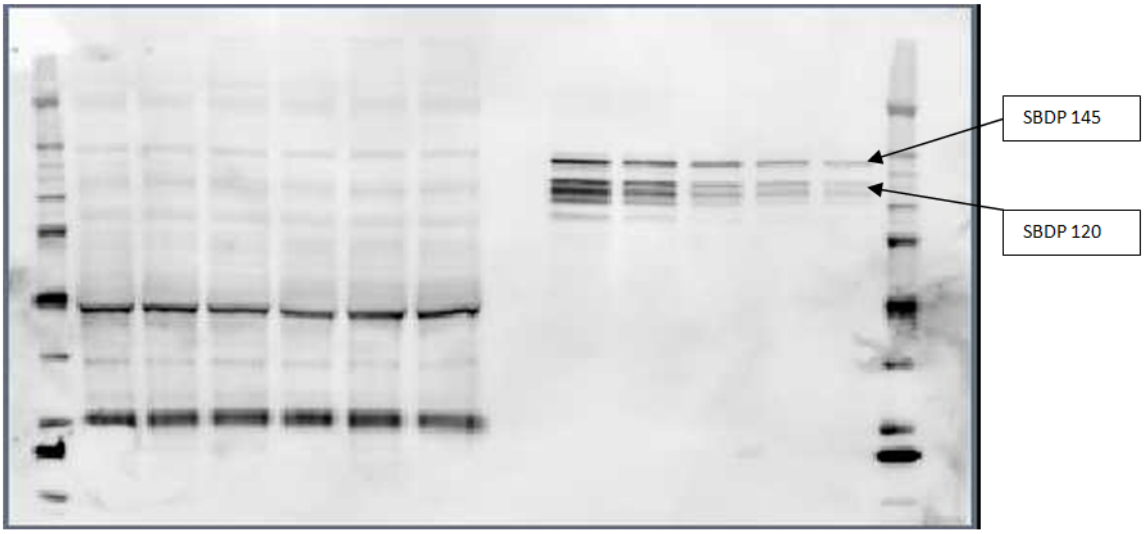

Figure 10: Photograph of Western Blot Gel Preparation for Plasma. Wells are numbered 1-12 from left to right. The well contained plasma from the following animals (B denotes blast and S denotes sham blast) as follows: 1 = S2 Pre-Blast, 2 = S2 Post-Blast, 3 = B2 Pre-Blast, 4 = B2 Post-Blast, 5 = S5 Pre-Blast, 6 = S5 Post-Blast, 7 = Blank, 8 = 800ng/ml, 9 = 400ng/ml, 10 = 200ng/ml, 11 = 100ng/ml, 12 = 50ng/ml.

Paired analysis showed that there was no significant change in either biomarker 8 hours after blast or sham blast exposure (Table 4, Table 5).

SBDP145					
Blast Group			**Sham Blast Group**		
Animal	Pre blast	Blast + 8 hours	Animal	Pre sham	Sham + 8 hours
B2	1533	1843	S2	1989	2020
B4	3397	2644	S5	1959	1738
B5	2944	3174	S7	1991	2275
B6	2630	2496	S8	2048	2209
B7	2232	2075			
B8	2708	2149			
Mean±SEM	2574±261	2397±196	Mean±SEM	1997±19	2061±120
Pre vs Post blast	P=0.3495		Pre vs post sham blast	P=0.5964	

Table 4 SBDP145 in plasma samples taken immediately before blast (or sham blast) exposure in individual animals (B2-B8 for blast and S2-S8 for non-blast groups). SBDP145 levels are expressed in standardised units of optical density to allow direct comparison between western blot gels. Group data are summarised as mean±SEM and a pre vs post blast (or sham blast) comparison made in each group using a paired t test.

SBDP120					
Blast Group			**Sham Blast Group**		
Animal	Pre blast	Blast + 8 hours	Animal	Pre sham	Sham + 8 hours
B2	1826	2295	S2	2246	2378
B4	3429	2879	S5	1982	2010
B5	3306	3335	S7	1836	2924
B6	3047	2881	S8	2255	2460
B7	2826	2560			
B8	2940	2433			
Mean±SEM	2895±233	2731±155	Mean±SEM	2080±103	2443±188
Pre vs Post blast	P=0.3346		Pre vs post sham blast	P=0.2341	

Table 5: SBDP120 in plasma samples taken immediately before blast (or sham blast) exposure in individual animals (B2-B8 for blast and S2-S8 for non-blast groups). For further explanation see legend to Table .

To minimise the impact of baseline variability in the levels of SBDP145 and 120 in plasma the post/pre blast fold change was calculated for each individual animal (Figure 11). Between group comparison of these fold changes again revealed that there was no significant difference between blast and sham blast groups for either SBDP145 (P=0.6088) or SBDP120 (P=0.2341).

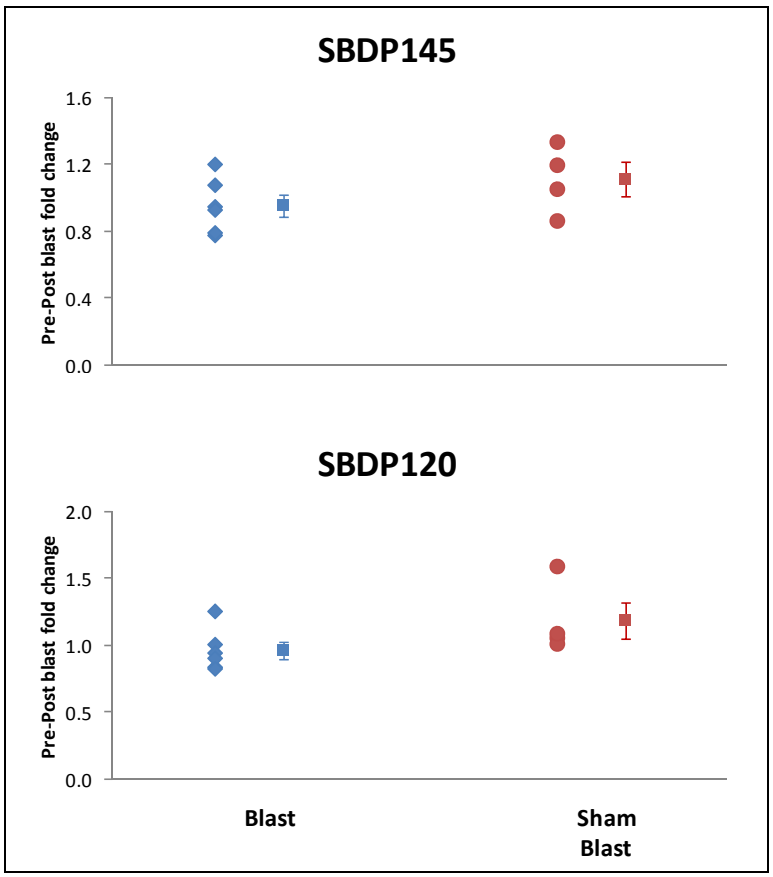

Figure 11: Fold increase (calculated as post blast / pre blast levels) in SBDP145 (upper panel) and SBDP120 (lower panel) in blast and sham blast exposed groups. Data for individual animals are shown as either diamonds (Blast) or circles (sham) and the relevant group mean values ± SEM are shown as squares to the immediate right of the individual data.

Chapter 4 – Discussion

Blast-induced neurotrauma has been an area of increasing concern as this type of injury may be missed on initial assessment and can develop over time (Lew et al 2006). The neurotrauma results in a varied range of symptoms including headaches, sleep disturbances, depression and anxiety (Ling et al 2009, Ruff et al 2009, Bogdanova & Verfaellie 2012). Hoge et al (2008) reports that of 2525 soldiers returning from Iraq, 4.9% suffered a loss of consciousness (LOC), while 10.3% reported altered mental status. 79% of those suffering LOC were exposed to a blast or explosion. Of the soldiers suffering injuries during deployment, 43.9% met the criteria for Post Traumatic Stress Disorder.

Blast-induced neurotrauma is viewed as a particular problem in casualties with minor, if any, other injuries. An additional group of casualties of significant concern are those with severe non-head injuries, in whom a relatively mild brain injury may not be apparent initially. In these latter patients a relatively mild brain injury could develop into a more serious condition when amplified by the effects of concomitant injury (hypotension, hypoperfusion, hypoxia and local or systemic inflammation).

Recent anecdotal evidence and case reports demonstrate the occurrence of clinically significant brain injury that is not detected by CT scanning (Belanger et al 2005) and that brain injury in the multiply-injured casualty can go undetected. An objective marker of TBI would be a useful diagnostic tool to identify those casualties who have suffered a brain injury where there are no external signs of such an injury for appropriate triage and treatment if available.

The aim of this project was to determine whether a single significant head focused exposure to a primary blast wave would be sufficient to cause neural damage that would be reflected in changes to SBDP concentration in brain tissue, CSF and plasma 8hrs post-injury. Assessing potential elevations of SBDP levels at an early time point after blast exposure would therefore determine whether SBDP were a suitable biomarker for the early detection of blast-induced TBI.

This study has demonstrated that a single blast exposure to the head in anaesthetised rats does not result in the presence of SBDP in either CSF or plasma. Interestingly however, SBDP were found in brain homogenate in both the blast and the control groups.

There are several possibilities for the results seen in this study:

- SBDP are not a relevant biomarker for blast-induced TBI
- The time of the sample collection post-injury was inappropriate
- The level of blast exposure was not sufficient to cause damage
- Primary blast injury to the head is not the only cause of blast related TBI

Alpha-II Spectrin is a major component of the cytoskeleton of axons and is cleaved by cysteine proteases Calpain and Caspase-3, resulting in necrotic and apoptotic cell death respectively. Several studies have demonstrated the presence of tissue, CSF and/or blood SBDP following brain injury in rats including injury resulting from cortical impact (Ringger et al 2004b), middle cerebral artery occlusion (Pike et al 2004), and penetrating ballistic-like injury (Zoltewicz et al 2013). Recent studies in human civilian TBI casualties have also demonstrated that SBDP are detected in CSF following severe TBI (Brophy et al 2009, Mondello et al 2010).

The presence of SBDP in the brain homogenate of the rats demonstrates that the process of spectrin breakdown has been initiated; however, the cause remains unclear. The presence in both sham and blast exposed animals may suggest that the test is in fact too sensitive and that small disturbances in the brain, for example due to prolonged anaesthesia, may result in spectrin breakdown. It is possible that, despite the rapid removal and freezing of the brains post-mortem, there was some degree of neuronal spectrin degeneration with the resulting formation of SBDP.

The utility of SBDP as a biomarker of severe TBI in humans has been demonstrated (Brophy et al 2009, Mondello et al 2010), however, its utility following blast-induced neurotrauma is unproven. Recent studies of mild and moderate TBI report the utility of biomarkers as a diagnostic tool, but interestingly SBDP are not reported in this cohort (Papa et al 2012).

It is possible that the degree of neurotrauma associated with the single high blast loading is insufficient for significant activation of calpain and/or caspase-3. It is also possible that the

mechanism for blast-induced neurotrauma is not associated with breakdown of spectrin and that another mechanism is the primary or more significant cause of damage. Interestingly though, two recent studies of low level blast exposure have demonstrated the presence of SBDP in brain tissue (Park et al 2011) and, following repeated low level blast exposure in humans, detectable levels of SBDP 150 in plasma (Tate et al 2013) although the time courses of these experiments is different to the current study. It is clear, however, that blast injury is not the cause of spectrin breakdown detected in this current model as there were no differences between blast and sham groups. The discrepancy between this study and that of Tate (2013) and Park (2011) is difficult to explain and may be due to the differences between the blast wave exposures. An explosive device was used in the human study, a shock tube used by Park et al (2011) and a compressed air device in this study. The compressed air device gives a very focussed insult to the head whereas the other studies have whole body exposures, and whilst there is no evidence of other blast related injuries, such as lung injury, it is possible that the sub-clinical effects (e.g. inflammation) are influencing brain injury. It is also possible that blast exposure, regardless of the degree of blast load, results in a more protracted SBDP response.

In the ideal world, if a biomarker is to be a useful clinical tool, then it needs to be readily identifiable in the sample, and the sample should be easy to obtain. Blood and urine samples are relatively easy to obtain, particularly in a trauma patient, so a biomarker present and detectable in blood or urine would be of great benefit. In this study however, no SBDP were detected in the plasma 8hrs post-injury.

CSF is not routinely taken from trauma patients, so would not be the first choice for biomarker analysis in clinical practice. In the research setting, however, where there is assessment for brain injury and evaluation of biomarkers, it is not unreasonable to use CSF samples for investigation. When CSF sampling is performed in human patients, the vast majority of CSF sampling is done via lumbar puncture. CSF withdrawal via the suboccipital route is exceedingly rare and usually only performed by an experienced neurologist or neurosurgeon. If any potential test is to become useful it will be necessary to consider time delay in biomarker production from damaged neurons in the brain to their appearance in CSF at the lumbar site.

In this study the analysis of SBDP in the CSF was hampered by the blood contamination (spectrin is also found in the cytoskeleton of erythrocytes) that occurred at the time of the sample collection despite the processes used to eliminate such contamination (e.g. centrifugation of the sample prior to freezing and storage). This is an interesting, and important, finding particularly if a test is to be used in a clinical setting. Even in the hands of very experienced clinicians some blood contamination is inevitable on occasion. This study has therefore demonstrated that the identification of SBDP in CSF using the methods described is a non-viable option as a clinical biomarker of TBI. Other studies however, have demonstrated SBDP in CSF (Brophy et al 2009, Mondello et al 2010), so it is possible that choice of assay is an important factor for biomarker analysis.

There is always the possibility that the timing of sample collection for analysis of SBDP in this study had missed the change in SBDP levels; either the peak of SBDP was in the very early acute phase post-injury (minutes to a few hours) or the SBDP may be expressed chronically. Thus peak levels may have returned to baseline by 8hrs post-injury or possible changes in SBDP would not be evident until at least 24hr post-injury.

Due to their size rats only have a small circulating blood volume and repeated blood-sampling throughout the 8hrs post-injury could have added an extra physiological burden (haemorrhagic shock) and complicated any interpretation of the findings. In this study, due to intermittent blood gas analysis, it was not possible to take serial samples for biomarker analysis. In addition it was not appropriate to take serial CSF samples over the 8hrs post-injury period. This procedure would have involved the placement of a, in-dwelling cannula at the level of the cisterna and this may have induced neurotrauma, thus confounding any results.

Assessment of brain tissue SBDP was only possible on termination of the study as brain biopsy was not appropriate in this case. Such direct tissue trauma could easily have resulted in the generation of SBDP such as that seen in rodent TBI models (Pike et al 2004, Ringger et al 2004b). The literature does give credence to the 8hr timescale used in the current study. In two clinical studies of severe TBI, there were significant elevations in CSF SBDP 145 and 150 by 6 hrs post-injury (Brophy et al 2009, Mondello et al 2010). In rodent models of TBI, significant elevations in SBDP have been shown in brain tissue between 3 and 24 hrs (Ringger et al 2004b, McGinn et al 2009, Zoltewicz et al 2013). The study by Park

et al (2011) demonstrated SBDP at 12 hrs post-injury, earlier time points were not assessed so it is not possible to conclude that 8hrs post-injury is a suitable time point for SBDP analysis, but it is unlikely that the lack of SBDP was due to inappropriate timing. It is acknowledged, however, that if the neurotrauma resulting from blast injury is mild in nature (even though the exposure was intended to be severe) this may explain the lack of SBDP.

One possible solution to the limitation posed by the need to take terminal samples of blood, CSF and post-mortem tissue analysis is increasing the numbers of animals used in the study, and sacrificing groups of animals at different time points. In an attempt to circumvent this problem a single time point was chosen that had been identified by a number of groups as being appropriate for detection of non-blast induced elevations of SBDP, (at the time of commencement of the present study there were no reports of the effects of blast-induced trauma on SBDP levels). Unfortunately the results of the study in this thesis, and reports published after completion of the experimental work in this study, now suggest that if SBDP do rise after blast-induced brain injury, the time course may be longer than 8hrs. This returns to the problem of needing several groups of animals culled at different time points. Whilst this may be the ideal, there is a cost to the animals, and this needs to be balanced against the benefits gained from such an approach using the principles defined by Russell and Burch (1959). This is especially relevant in a situation where the latency in marker expression renders it too late to be of use in the acute clinical setting.

In the current study the level of blast loading to the head was deliberately chosen to be high. A high blast level was used to ensure the fewest numbers of animals were used as, if a severe blast loading did not cause measurable neurotrauma, then neither would a single exposure to lower levels of blast. The blast exposure used in the study was 700kPa and it has been shown that 480kPa delivered by the same blast apparatus produces a moderate survivable blast lung injury (Brown et al 1993). Exposure greater than 600kPa results in acute severe pulmonary oedema (Watts and Kirkman – personal communication). It is unlikely therefore that the injury level was a factor for the results in the current study as 700kPa focussed over the chest would have resulted in 100% mortality.

Overall there were no significant differences in the physiological data between the two groups. The continual blood pressure monitoring, however, showed that immediately post blast injury there was a marked cardiovascular depression in two of the five animals in the blast group as shown in Figure 5. This depression is similar to the reflex combination of apnoea, bradycardia and arterial hypotension seen in studies where the thorax has been exposed to a primary blast wave (Guy et al 1998, Ohnishi et al 2001, Sawdon et al 2002). The response has not, however, been reported in TBI studies using Cortical Controlled Impact in either adult (Ringger et al 2004a) or immature (Aikman et al 2006) rat models of TBI. It has been shown, however, that mild and moderate TBI using fluid percussion results in a brief period of apnoea and a transient increase in arterial blood pressure (McMahon et al 2008, McMahon et al 2011). Hence, cardiovascular reflexes post-TBI may be dependant on the model used.

The absence of differences in primary respiratory parameters such a PaO_2, $PaCO_2$, and arterial Base Excess between the two groups suggest there were no long term cardiorespiratory effects from the blast, and that any anaesthetic related cardiorespiratory depression was similar between the groups.

There is still much debate as to the cause of blast-related neurotrauma and whether it is the shock wave that causes the damage, or whether it is rapid acceleration/deceleration, or impact of the head during the blast. Studies performed in Sweden suggest there is no link between a primary blast wave and neurotrauma. Soldiers firing heavy artillery, or being exposed to nearby explosions showed no evidence of TBI based on normal CSF:albumin ratios (a marker of blood-brain barrier integrity, and biomarkers T-tau and NFL (Blennow et al 2010). It is entirely plausible that blast-induced neurotrauma of military personnel is not just associated with the shock wave produced from explosions, and may in fact be due to several combined mechanisms resulting from an explosive injury.

Explosive blast is an extremely complex phenomenon that is difficult to predict except is a research environment with defined parameters. Primary blast waves on the battlefield and as a result of terrorist incidents are highly variable and unpredictable, and it has been recognised that blast waves reflecting off surfaces may actually amplify the wave by as much as eight to ten-fold (Mellor 1992, Leibovici et al 1996). Blast exposure within buildings or vehicles has also been recognised as producing a higher, more lethal, primary

blast wave due to the multiple reflections (Leibovici et al 1996). Even if body armour is worn, and personnel are behind cover or within an armoured vehicle, it is recognised that the pressure wave acts rather like a sound wave, reflecting off and flowing around hard surfaces (Born 2005), such that even hard cover may not be protective Wiener & Barrett 1986).

The effects of other forces such as energised fragments resulting in secondary blast injuries, and the blast wind causing tertiary injuries such as blunt head injury may also contribute to the neurotrauma. Indeed there is evidence to suggest that even the primary blast component of blast-induced neurotrauma is multi-factorial as head protection failed to prevent neuro-inflammation and reduce the neurological deficits following whole body blast exposure (Cernak 2010).

Further research is required therefore to determine whether concomitant injury will exacerbate and blast brain injury currently undetectable by SBDP. Future studies will determine whether haemorrhagic shock (hypotension and hypoperfusion) and hypoxia will cause blast neurotrauma which can be detected by evaluation of tissue and/or plasma SBDP. Both of these injury elements have a significant negative impact on outcome following severe head injury, although this is not as profound as the effect of hypotension (Chesnut et al 1993, DeWitt & Prough 2009). One factor contributing to morbidity and mortality is hypoxia, a regular symptom as a result of blast induced lung injury (BLI) (Long et al 2009). Incidence of BLI can be high, depending on the circumstances of the explosion. One study describes an incidence of BLI of 83% of survivors in a terrorist bomb attack. Despite the provision of supplemental oxygen, 46% of those individuals with BLI were suffering from extreme hypoxaemia (Pizov et al 1999).

In addition to concomitant injury future work may also want to explore the so-called Second Impact Syndrome (SIS), where another insult to the head can exacerbate the current injury, leading to a worse clinical outcome (McQuillen et al 1988, Bey & Ostick 2009)). One of the keys features of SIS is that a second or subsequent insult to the head can be seemingly innocuous. The first insult ‘sensitises’ the brain to injury, and the second insult magnifies the pathological result (Byard & Vink 2009). It is possible therefore that primary blast injury to the head could sensitise the brain to future insults which may, or

may not, be blast related. Thus there is scope to look at multiple-blast head trauma and blast injury combined with another insult; impact for example.

There is also scope to optimise the analysis of SBDP in the future, in the current study the optical density measurement allows only a semi-quantitative assessment of SBDP whereas the ideal would be to exactly quantify SBDP concentration.

If SBDP are shown to be diagnostic for blast-induced neurotrauma then it would be important to try and determine the predominant breakdown pathway as either necrotic (SBDP 145 predominant) or apoptotic (SBDP 120 predominant). This would then potentially allow for the targeted treatment, and future studies to evaluate such treatments.

A previously accepted non-specific treatment for CNS injury is the administration of the corticosteroid methylprednisolone in the first 8hrs after injury as it decreases inflammation by the inhibition of the production of free radical oxidation products (Demopoulos et al 1982). However, more recent research indicates that this treatment not only produces poor results in both clinical and research settings; it is also associated with risk at the doses given (Lykissas et al 2007). Subsequently there have been other suggestions for neuroprotection including the use of hyperbaric oxygen (Harch et al 2009), Aminoguanidine (Moochala et al 2004), and erythropoietin (Lykissas et al 2007) to name but a few. One potential disadvantage of generalised neuroprotective therapy is that its non-specific targeting may be less effective that targeted therapy as some individual pathological pathways are continuing while therapy is effectively causing damage limitation.

This suggests that targeted therapy may have potential for reducing neural damage. It has been shown experimentally that if the pathological pathway is known then, in some cases, it may be possible to slow or halt its progress. For example, it is known that neural injury of whatever inciting cause results in neuroinflammation (Schmidt et al 2005), and that the release of pro-inflammatory cytokines such as Tumour Necrosis Factor (TNF), Interleukin (Il) 1h and Il6 contribute to neural pathology. It has been possible in one study by using a novel small molecule inhibitor of pro-inflammatory cytokine upregulation (Minozac) to reduce inflammation and thereby improve long-term neurological outcome (Lloyd et al 2008).

In a similar fashion, it has been suggested that a calpain-specific inhibitor could be used to suppress the necrotic cell death pathway (Czogalla & Sikorski 2005). However, at an experimental level, calpain inhibition failed to suppress the excitotoxic pathways following neuronal injury, suggesting that the relationship between calpain activation and cell necrosis is more complex than a simple cause and effect (Adamec et al 1998). Indeed, the absence of calpain activation led to the failure in molecular restorative function, suggesting that calpain is not only involved in cell death pathways, but it also has an important role in cell homeostasis following injury.

In summary, a single blast exposure to the head in anesthetised rats does not cause neurotrauma of sufficient severity to cause the production of SBDP. Evidence not available at the commencement of the study suggests that this may be due to the 8hr post-injury time point chosen for the study. Regardless of this however, the results are still valid in that SBDP are not suitable as an early biomarker of blast-induced brain injury.

Conclusions

Blast injury is a common occurrence in modern warfare and an area of increasing concern is blast induced TBI. This remains a complex syndrome that is attracting world-wide research attention but although there have been some advances in its detection including the use of advanced imaging, cognitive testing and biomarkers, there is no definitive test for the presence of this TBI.

The pathogenesis of blast induced TBI is still poorly understood and it is unclear whether one component of an explosive event, a blast wave to the head, is capable of producing TBI on its own.

The breakdown products resulting from the proteolysis of the structural neuronal protein Spectrin during cell death appear to be promising as a specific marker for TBI and this study aimed to show if Spectrin Breakdown Products (SBDP) were produced following blast wave exposure.

SBDP 145 and 120 were identified in brain homogenate and in plasma in blast and control groups, but there were no significant differences between groups or, in plasma, between pre and post-blast levels. This suggests that blast wave exposure *per se* does not specifically lead to elevations in SBDP. This may have been due to failure to induce injury, or time delay to production. It appears that blast wave to the head alone is not capable of producing an injury that results in elevations in SBDP concentration in the acute phase following injury. SBDP do not therefore appear to be a reliable marker for any blast-induced TBI in the early stages of injury caused by blast wave exposure to the head.

SBDP have been reported in TBI where there has been primary blast wave to another area of the body causing concomitant injury, and they may still have a role in the assessment and management of those cases.

The prevalence of blast induced TBI in the military population means that a reliable test for TBI is still urgently required. The test could be used to screen for, and identify, apparently physically uninjured blast-exposed personnel to allow them time to recover. The test could also be used to initiate neuroprotective therapy in severely injured personnel in order to protect neurological function while more life-threatening injuries are being attended to.

The ideal test will be fully quantitative to allow determination of severity of injury, and the biomarker's change over time linked to prognosis.

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Appendices

APPENDIX 1 – Animal Identification in Blast and Sham Group

Animals in Blast Group	Animals in Sham Group
BLBR 08	BLBR 07
BLBR 09	BLBR 10
BLBR 13	BLBR 11
BLBR 14	BLBR 12
BLBR 15	BLBR 16

APPENDIX 2 – Assay agent composition:

Gel Electrophoresis

Lysis Buffer: Tris-HCl 50mM

NaCl 150mM

NP-40 1%, EDTA 2mM

Sodium Vanadate 2mM

Sodium fluoride 50mM

Aprotinin 10μg/ml (Sigma-Aldrich, Gillingham, UK)

Leupeptin 20μg/ml (Sigma-Aldrich, Gillingham, UK)

MOPS Running Buffer Stock Solution (1ltr):

50ml of MOPS running buffer (Bio-Rad Laboratories Inc, Hemel Hempstead, UK)

950ml of filtered water to give a 5% MOPS running buffer stock solution.

Western Blotting

Anode Buffer (100ml):

10ml 10x Tris/CAPS (Bio-Rad Laboratories Inc, Hemel Hempstead, UK)

15ml 100% methanol

75ml of double distilled H_2O

Cathode Buffer (100ml):

10ml 10x TRIS/CAPS

1ml 10% SDS

89ml ddH_2O