Blast injury in pigs

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The nature of combat means that soldiers will suffer a mixture of primary, secondary, tertiary and quaternary brain injuries making it difficult to study the effects of the blast over pressure (BOP) wave in isolation. We used a porcine model to assess the impact of a primary blast in the context of polytrauma. We performed histopathology to investigate structural changes, axonal degeneration and the early microglial immune response. We also used standard MR imaging and DTI techniques to assess WM damage. This study aimed to identify accurate and robust correlates between neuroimaging and histopathology findings, strengthening the use of neuroimaging as a reliable diagnostic tool in human blast injuries.

This work was in collaboration with DSTL Porton Down, Imperial College London and UCL. DSTL developed the porcine blast injury model and conducted the animal injury and resuscitation phases. I attended the experiments and retrieved the brains once the animals were sacrificed. I developed the imaging protocols with Marina Arridge at the Brain Imaging Centre at Imperial College London and performed the DTI analysis. With Professor Steve Gentleman, I co-supervised Ting Kwok perform the immunohistological preparation and I recorded and analysed the data with her.

Introduction

IEDs have become a major contributor to mortality and morbidity in the conflicts in Afghanistan and Iraq. Following discharge, veterans often present with symptoms consistent with mild TBI (Terrio 2009, Okie 2005). While the neuropathology underlying this cognitive impairment is currently unknown, it has been linked to a condition called chronic traumatic encephalopathy (Goldstein 2012), previously known as dementia pugilistica, in which chronically activated microglia cause a tauopathy in axons. This topic is

important as blast injuries continue to be the main threat to troops around the world whilst survival rates of blast victims are improving (Penn-Barwell 2015).

Pathophysiology of TBI

To fully understand the histopathology results presented here, it is necessary to describe what happens at a cellular level when an injury to an axon occurs. In the healthy brain, glutamate is produced by neurons and taken up by astrocytes. These astrocytes then convert the glutamate into glutamine and return it to the neurons where it is an alternative energy source. Injured neurons overproduce glutamate and, if they die, release glutamate into the extracellular space. When there is too much glutamate for the astrocytes to remove, it binds to neuronal receptors (such as NMDA) and induces an influx of Ca^{2+} and Na^{+} and an efflux of K^{+}. This ionic imbalance causes the cell membrane to depolarise. Intracellular Ca^{2+} levels rise leading to mitochondrial dysfunction, reduced ATP formation (see Figure 1), energy failure and ultimately cell death. Mitochondrial dysfunction leads to a release of reactive oxygen and nitric oxide species which cause oxidative stress and damage to membrane lipids, proteins and DNA. Free Ca^{2+} activates enzymes (calpains) that disrupt the axon's cytoskeletal filaments. This disruption causes impaired axonal transport and a build up of amyloid precursor protein (APP) (Rosenfeld 2012, Gentleman 1993).

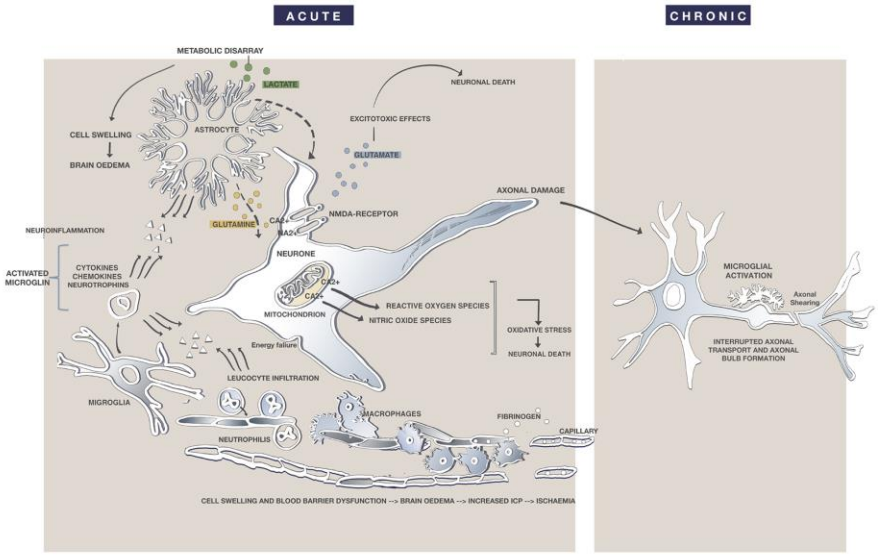

Figure 1. Pathophysiology of brain injury

Acute - The cycle of cellular events that occur when a neuron is injured and which leads to APP accumulating in the axons, microglial activation and fibrinogen leakage from blood vessels. Chronic - The activated microglia modulate tau metabolism leading to beta-amyloid plaque (different to APP accumulation) deposition and neurofibrillary tangles (McKee 2009).

Microglia are the immune cells of the central nervous system. Signals emitted from injured neurons activate microglia which then change shape (Figure 2). If there are dead cells present, the microglia become phagocytes. Activated microglia accumulate at the injury site and secrete inflammatory cytokines, chemokines that stimulate the migration of activated leucocytes into the brain. Infiltrated neutrophils maintain the immune response to injury, impairing the blood brain barrier's integrity which in turn leads to fibrinogen leakage into tissues, increased extracellular fluids, cell swelling and brain oedema. In the long term, for an unknown reason, in some individuals activated microglia remain in the brain and can cause chronic traumatic encephalopathy by modulating tau protein metabolism (Goldstein 2012). In this study, we looked

for APP as a marker of axonal injury, fibrinogen as an indicator of blood-brain barrier permeability and Iba1 (a microglia-specific calcium binding protein) to assess microglial morphology (Rosenfeld 2012, DeWitt 1995).

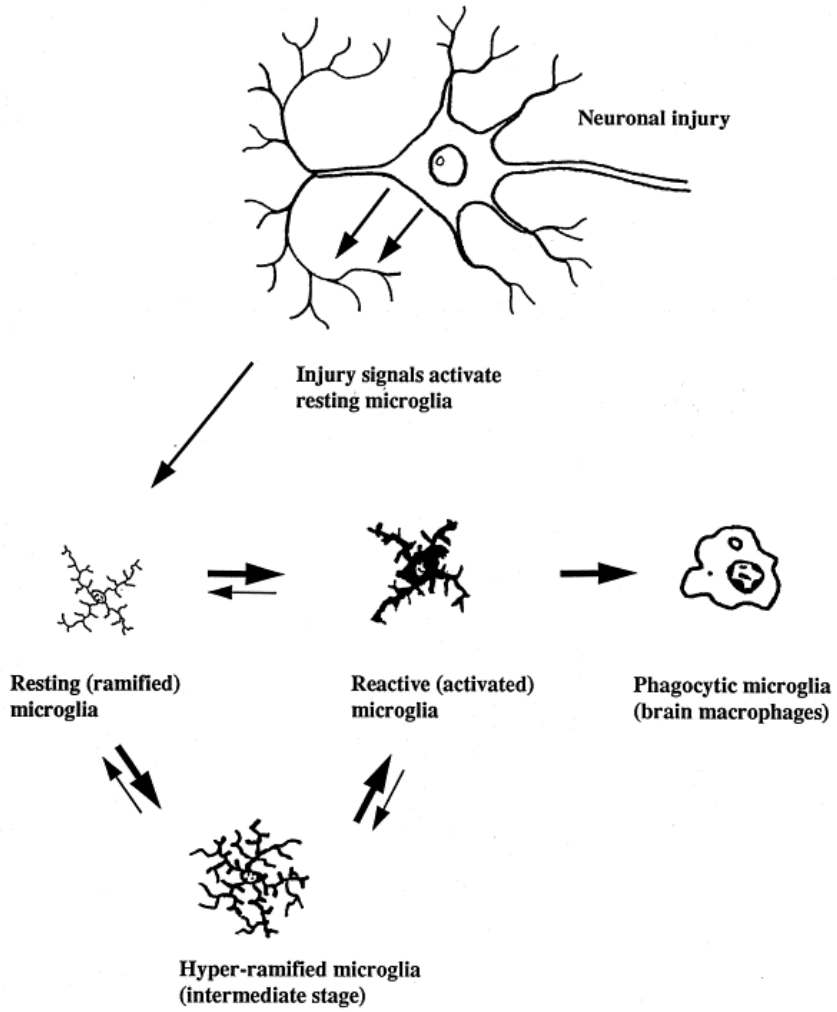

Figure 2. Functional plasticity of microglial (Streit 1999)

Injured or diseased neurons cause resting microglia to become activated by emitting injury signals. The degree of microglial activation varies with the severity of the neuronal injury. The mildest injuries may only cause hyper-ramification of microglia, but most types of neuronal damage will cause resting microglia to become reactive microglia. If neurons die, microglia transform into brain macrophages and remove the dead cells. If an injured neuron recovers, hyper-ramified and reactive microglia may revert to the resting form. Microglia-derived brain

macrophages probably do not revert to the resting state, but may undergo cell death (Streit 1999).

Haemorrhagic shock and resuscitation

Isolated blast injury is very uncommon and it usually occurs in the context of polytrauma. Approximately 4% of soldiers suffered from both TBI and haemorrhagic shock (HS) (Okie 2005) in combat operations in Iraq and Afghanistan. The presence of HS is known to worsen the morbidity and mortality significantly from TBI (Wald 1993). The worsened morbidity and mortality seen in TBI with HS may be due to secondary ischaemic damage as well as the effect of the loss of cerebral autoregulation. The current treatment for soldiers and civilians suffering from both TBI and HS is the infusion of crystalloid fluids, such as saline to restore BP and tissue perfusion. However, there is some evidence that this may worsen cerebral oedema causing intracranial hypertension and a reduction of brain compliance (Teranishi 2012, Hariri 1993).

Our injury model was designed to replicate the effects of battlefield polytrauma and the journey from injury and first-aid (Role 1), through evacuation (Role 2) to a medical facility (Role 3) (Garner 2009). The term "Role" or "Echelon" is used by NATO to describe the stratification of tiers of medical support. Role 1 medical support is integrated into a unit and includes the capabilities for providing first aid and immediate lifesaving measures such as stopping the haemorrhage. Role 2 is typically provided at a larger unit level, usually Brigade size, though it may be provided farther forward, depending upon the operational requirements. In general, it provides evacuation from Role 1 facilities. Role 3 is at Division level and above. It incorporates additional resources, including diagnostic equipment such as CT scanners, as well as specialist surgical and medical capabilities (NATO 1997). The resuscitation strategies and timelines used in this study replicate these echelons of medical support.

The porcine model

Animal models examining pathological changes have improved understanding of the fundamental pathophysiology underlying blast trauma. However, findings from these studies cannot be readily translated to humans. Most animal studies of bTBI have used rodents (Xiong 2013). However, there are a number of limitations to using these types of animals. Rodent brains are smaller and have a porencephalic structure; this limits the applicability of their findings to humans. The human brain has a gyrencephalic structure. The convolutions of the sulci and gyri will interact differently with any force acting on the brain and create a different pattern of injury.

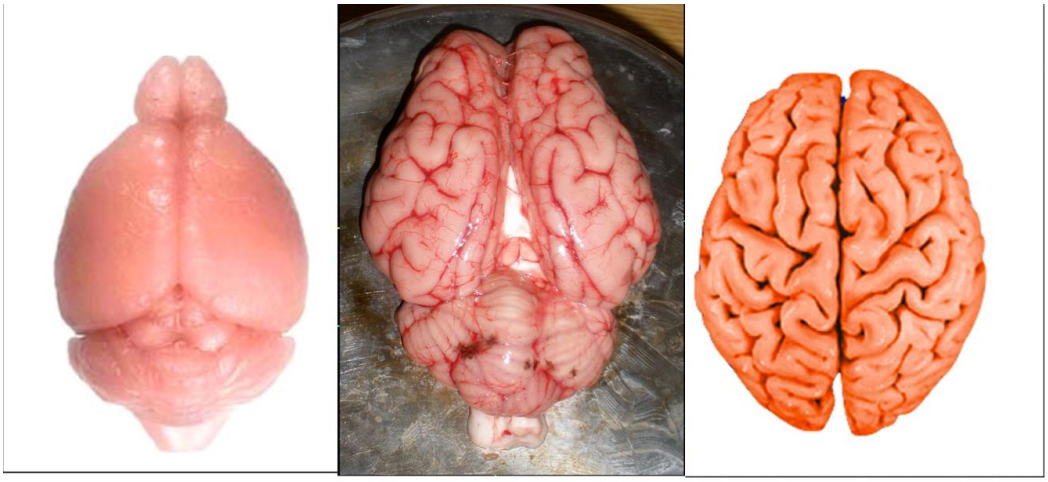

Figure 3. Comparison of rodent, pig and human brain

A porencephalic rodent brain and the gyrencephalic pig and human brain. The folds on the brain's surface will influence the transmission of energy and the location of the injury (adapted from Gholipour 2014, Heteroherent 2011).

In chronic traumatic encephalopathy following blast exposure, there is a predilection for injury at the base of the sulci, this illustrates the way that sulcal and gyral anatomy influence the location of damage (McKee 2014). Garner *et al*. (2009) developed a large-animal porcine model to address some of these limitations. Pigs have a gyrencephalic brain structure that is similar to

the human and also have comparable glial-to-neuron ratios, myelin levels and water content. Also, experiments have shown that pigs' brain tissue is analogous to human brain tissue when assessed biomechanically (Thibault 1998, Manley 2006).

We used a porcine model developed by Garner *et al.* (2009) to investigate the structural and early immune effects of military blasts. We gave ten pigs a peripheral injury, exposed them to either sham or blast conditions, limiting the secondary and tertiary blast effects, before controlled haemorrhages. Both groups of pigs were then given normal saline corresponding to Role 1 care, prior to being assigned to one of two resuscitation strategies. The early resuscitation group received packed red blood cells (PRBC) and fresh frozen plasma (FFP) one h after injury, corresponding to Role 2 care, these were continued in the late phase of the resuscitation (corresponding to Role 3). The late resuscitation group continued to receive crystalloid fluid to maintain BP whilst at Role 2 before receiving PRBC and FFP once at Role 3.

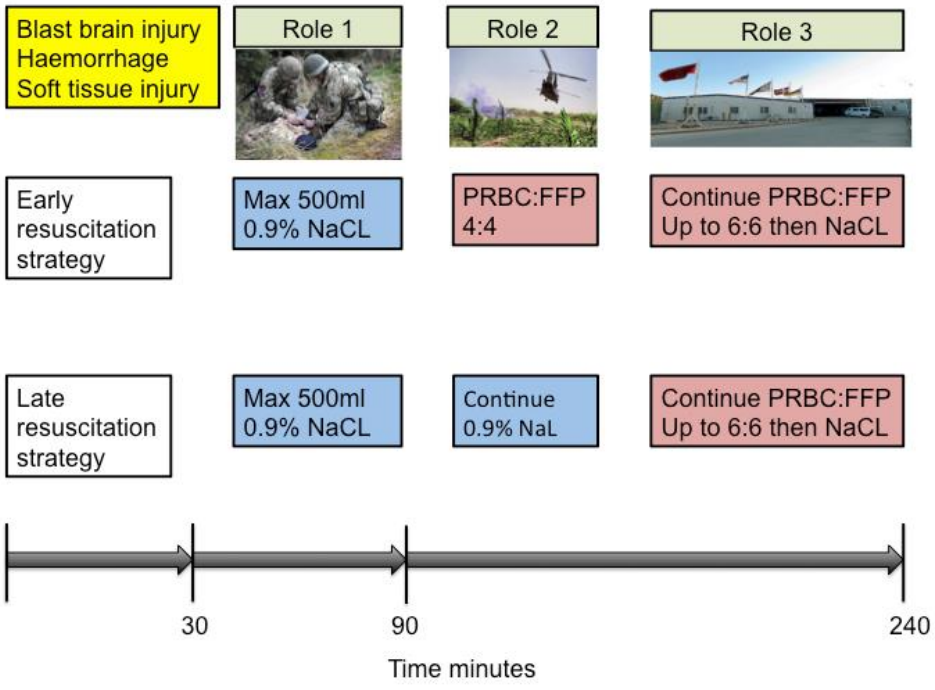

Figure 4. Timelines for fluid resuscitation in the early and late groups

An overview of the injury model showing the different fluids used by the early and late resuscitation strategies and their corresponding timelines.

This model replicates the timelines to Role 1, Role 2 and Role 3 medical care as set out by NATO.

Methods

All blast experiments were conducted by the Defence Science and Technology Laboratory (Dstl) at Porton Down. Garner *et al.* 2009 provides a detailed account of the development of this injury model, which combines blast, controlled haemorrhage and a soft tissue injury in a reproducible animal model, in order to carry out detailed physiological testing.

The study was conducted on 10 terminally anaesthetised large white pigs in accordance with the Animal Scientific Procedures Act (1986). The pigs were anaesthetised with Isoflurane (5%) in O_2N_2O (FiO_2 0.3) followed by Alfaxan (Saffan^{TM}), before experimentation. Arterial blood and central venous pressures were recorded throughout the experiment via intravascular cannulation. The injury and resuscitation model was divided into three phases: the shock phase, the pre-hospital phase and the in-hospital phase, to realistically simulate the experience of an injured soldier.

Shock phase (Pre Role 1)

After a 60 min recovery period following induction of anaesthesia, blood gases and cardiovascular measurements were made and the animal was randomly allocated to receive blast or sham (non-blast) treatment. The animals were wrapped in a Kevlar blanket to protect from secondary and tertiary blast effects and positioned outdoors on a trolley 2.15 m from a cylindrical charge of EDC1S explosive (2.2 kg), which was detonated remotely.

Figure 5. Blast Rig

The animal is seen here, on the right, wrapped in a Kelvar blanket on a sliding rail, which protected it from secondary and tertiary injuries. The high explosive charge was placed on top of the tube on the left.

Animals subjected to the sham blast were treated identically but not exposed to the blast. All animals then received a haemorrhage of approximately 30% blood volume loss and blunt injury to the muscle of the right thigh. The animal was then left to enter a 30 min shock phase during which a capped amount of 500 ml saline was given to prevent cardiovascular collapse and maintain the hypotensive target.

Pre-hospital phase (Role 1)

The treatment groups diverged at this point, those in the early resuscitation strategy group received up to 4:4 units of PRBC:FFP, which had been both forward and back cross-matched to the recipients. Animals in the late-resuscitation strategy group received saline to the same hypotensive BP target. At this stage, oxygen was used (at least FiO_2 0.3) to maintain an arterial concentration of 98%.

In-hospital phase (Role 2+)

After a 60 min simulated pre-hospital resuscitation phase, animals in the late-resuscitation group then received fluid to a maximum of 6:6 PRBC:FFP to reach and maintain a normotensive BP target, while a similar BP target was also employed in the early-resuscitation group. This resuscitation was continued for a further 150 min by which time all animals were sacrificed humanely with an overdose of pentobarbital (150 mg/kg i.v) and the heads removed for further analysis.

Tissue preparation

The heads of the animals were immediately removed and the soft tissues and

mandible were separated from the skull. The skull was perforated with a 1 cm cranial perforator in the frontal and occipital bones and diffusion fixed in 2% paraformaldehyde solution for two weeks. Perfusion and diffusion fixation are both accepted methods for fixing whole brains. Perfusion fixation requires paraformaldehyde to be pumped continuously through the arterial supply to the head (Dyrby 2011) whilst diffusion fixation is performing by submerging the brain in paraformaldehyde for a predetermined period of time (Miller 2011).

Diffusion fixation was chosen as perfusion with paraformaldehyde would have invalidated the concurrent investigations into porcine physiology following trauma. In addition, the effectiveness of diffusion fixation has been demonstrated in larger, human brains. After two weeks, the brains were surgically extracted from the skulls and then examined for apparent external damage. They were then suspended in TechAgar and stored at 4°C and scanned in a 4.7 Tesla MRI scanner. We performed MR imaging on 8 of the 10 brains (five blast and three sham animals).

Immunohistochemistry

We used a standard haematoxylin and eosin (H&E) staining procedure. Antibodies against Iba1, APP and fibrinogen, had not previously been used with porcine tissue, so the protocol was derived using experiments with antigen retrieval techniques and exposure times (see Supplementary Methods in Appendix 3). A Consultant Neuropathologist blinded to the group and resuscitation strategy of the animal examined the slides for structural damage, microbleeds, axonal pathology and microglial activation.

H&E stain

We examined all of the slices for structural changes, including oedematous pathology, alterations in cell morphology, and ependymal stripping. We looked for the presence of perivascular oedema, denoted by fibrous cavities surrounding the vessels in several regions including the orbitofrontal WM, hippocampus, corpus callosum, pons, medulla and cerebellum.

Fibrinogen

We used the presence of fibrinogen immunoreactivity to assess BBB permeability. In healthy subjects, fibrinogen is observed only within the vasculature. Increased BBB permeability leads to leakage of fibrinogen into the parenchyma, seen as a brown blush surrounding the vessel. We chose three standard sections throughout the brains and recorded all the cases of vascular leakage observed at 2 x magnification. We marked the presence and location onto a standardised outline of a porcine brain using graphics editing software (http://brainmuseum.org).

Amyloid Precursor Protein (APP)

We used APP to assess for the presence of axonal injury. When axons are injured axonal transport is interrupted and APP accumulates making the axon swell. We looked in the WM in the same three sections for each animal. We defined a focus as a distinct clustering of axonal bulbs and recorded their presence and location of the identified Foci onto a standardised outline of a porcine brain using graphics editing software (http://brainmuseum.org).

Iba 1

We stained the tissue with anti-Iba1 to observe changes in density and morphology of microglia. Semi-quantitative analysis of microglial profiles was performed to determine the locality and extent of the immunoreactive response. A severity scale of low (*), moderate (**) and severe (***) was set out, judged on intensity of clustering and degree of morphology change (Table 1), as shown in Figure 6.

Table 1. Severity scale of damage to microglia

Low (*)	Moderate (**)	Severe (***)
Microglia are mostly in a ramified state, with little retraction of processes and low density of cells	Microglia have slightly thickened and retracted processes but cells are evenly distributed, suggestive of early activation and little migratory response	Microglia have thickened and retracted processes, looking more like macrophages. Activated cells are often clustered indicative of widespread activation with proliferative and migratory responses

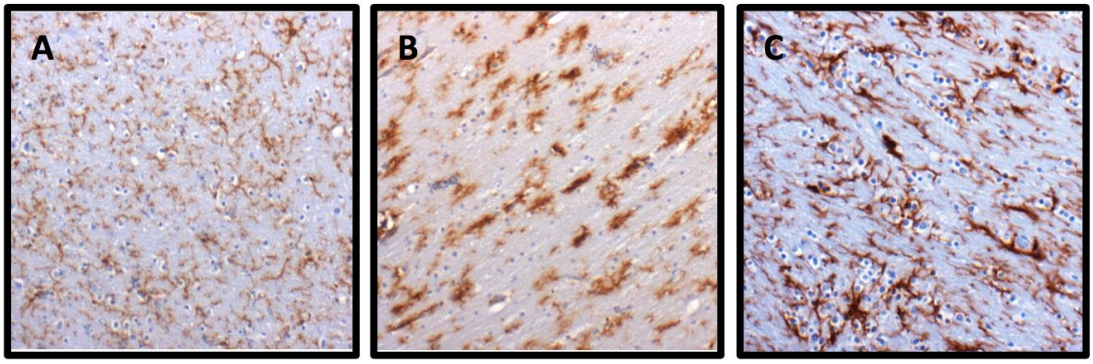

Figure 6. Visual impressions of the semi-quantitative rating of microglial activation: A) low (*); B) moderate (**); C) severe (***) 20 x magnification

1.1.1 Neuroimaging

We developed an *ex vivo* neuroimaging protocol to create high-resolution MPRAGE (T1) and gradient-echo (T2*) and DTI images to assess the extent of focal brain injury and haemorrhage. A Consultant Neuroradiologist, blinded to the pigs' blast injury status and resuscitation strategy, reviewed each of the standard structural scans (T1 and T2*sequences).

DTI analysis

Using a region of interest (ROI) approach we investigated FA within specified WM regions. We created ROI masks based on WM anatomy, in T1 space, for each animal. These regions were whole brain WM, the orbitofrontal WM, and the anterior internal capsule. These regions provide a representative measure of the degree of WM tract damage and are frequently disrupted by DAI (Mac Donald 2011). Informed by the histopathological results, we also created masks for the regions where we saw APP pathology. We extracted the mean FA value within the masks for each subject. SPSS was used to compare the mean FA in each of the regions between the blast and non-blast animals.

Results

Histopathology

Ependymal stripping

Stripping of the ependyma was identified in 4 of the six blast-exposed pigs, denoted by oedematous pathology underneath the ependyma with long fibrous attachments.

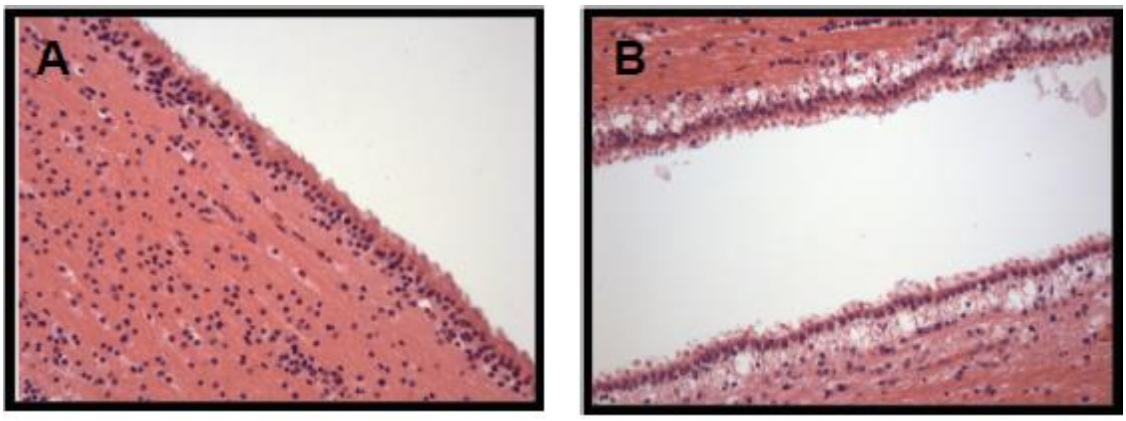

Figure 7. Ependymal stripping

(A) Normal ependymal compared with (B) Ependymal stripping

Hippocampal oedema

Two bTBI animals had hippocampal oedema that was not seen in the sham animals. One animal (B2) showed bilateral oedematous appearances in the dentate gyrus (DG) of the ventral hippocampi, and another (B10) had unilateral changes in the DG of the ventral hippocampus. In both animals with hippocampal oedema, there was associated microglial activation in the adjacent brain (Figure 8).

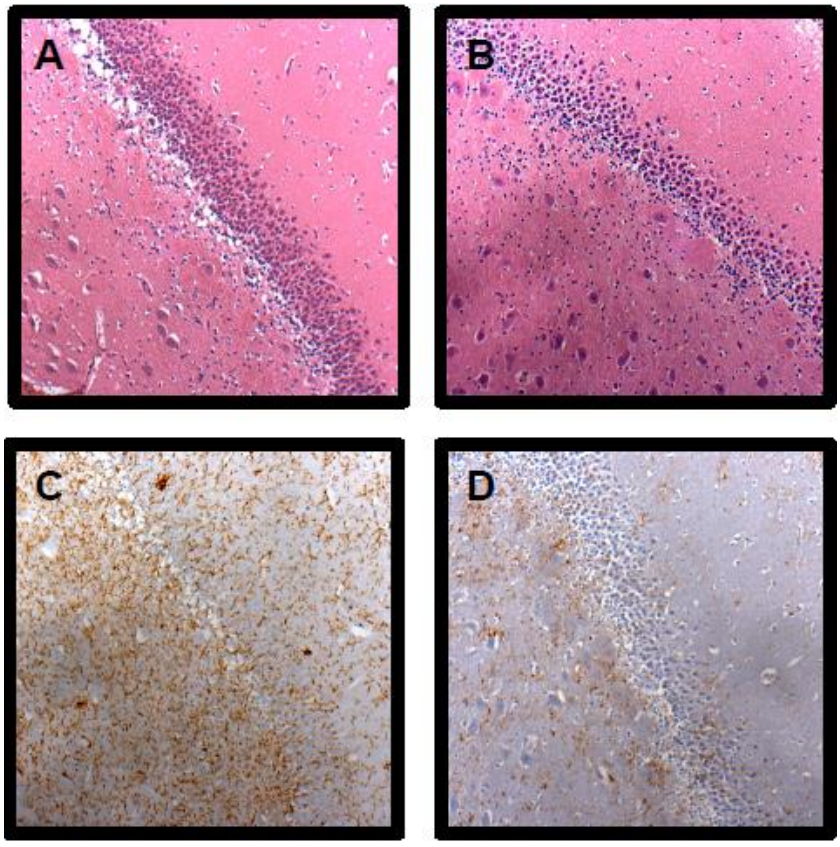

Figure 8. Hippocampal oedema with concurrent microglial activation

(A) and (C) are the slices from the same section through the hippocampus in pig B2. (A) H&E stained section showing fibrous structural pathology denoting oedema and (B) was stained with anti-Iba1 (brown colour) to show activation of microglia. (B) (D) Sections from animal B5 in which the oedema and microglial activation are not present.

Perivascular oedema

We observed perivascular oedema in both groups throughout the whole brain. Although in five of the six blasted pigs there were no microbleeds found, there were several microbleeds (extravascular erythrocytes indicating haemorrhage) found in the medulla of one of the blasted animals. This extravasation was associated with fibrinogen leakage. Both bTBI and sham groups displayed widespread fibrinogen leakage. There was no discernible pattern to the leakage, with this abnormality seen throughout the brains of all the animals (see Figure 9).

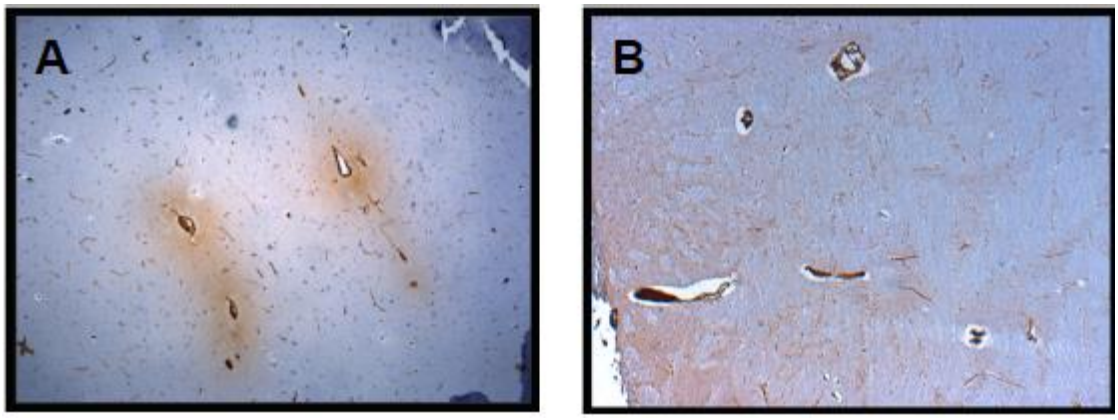

Figure 9. Fibrinogen leakage

(A) The brown blush around the blood vessel indicates fibrinogen leakage compared to (B) a typical vessel.

Amyloid Precursor Protein (APP)

All the pig brains, both blast and non-blast, displayed some APP immunoreactivity, with 8 out of 10 pigs showing widespread positive axonal varicosities. Axonal varicosities were mainly seen in the mid-coronal slice below the lateral ventricles, and in the internal capsule extending into the thalamus as shown in Figure 10.

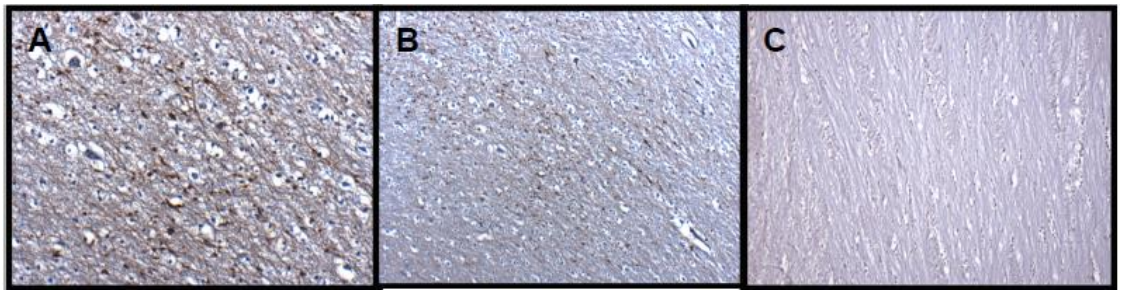

Figure 10. APP immunostaining at (A) 20x mag and (B) 10x mag compared to (C) normal WM without axonal injury

Iba 1

In animals exposed to a blast, there was evidence of focal microglial activation in areas of ependymal stripping as well as widespread activation of microglia in the sub-ependymal region (Figure 11). There was no evidence of sub-ependymal microglial activation in the sham animals. However microglial activation was seen in both bTBI and sham groups in other apparently undamaged parts of the brain, suggesting that a component of the injury model separate to blast caused microglial activation.

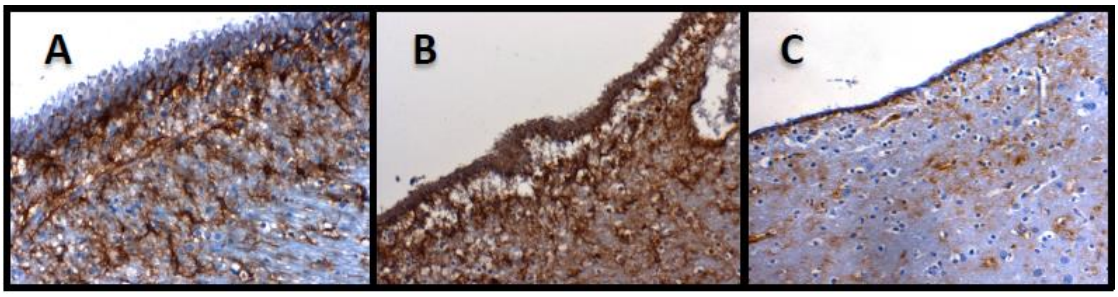

Figure 11. Microglial activation

(A) subependymal microglial activation and accumulation were seen in the blast pig (B1) without concomitant ependymal stripping; (B) subependymal microglial activation in the blast pig (B10) with ependymal stripping; (C) normal ependyma with ramified microglia.

Neuroimaging

The Gradient-echo and MPRAGE sequences showed no discernible difference between the two groups when reviewed (see Figure 12).

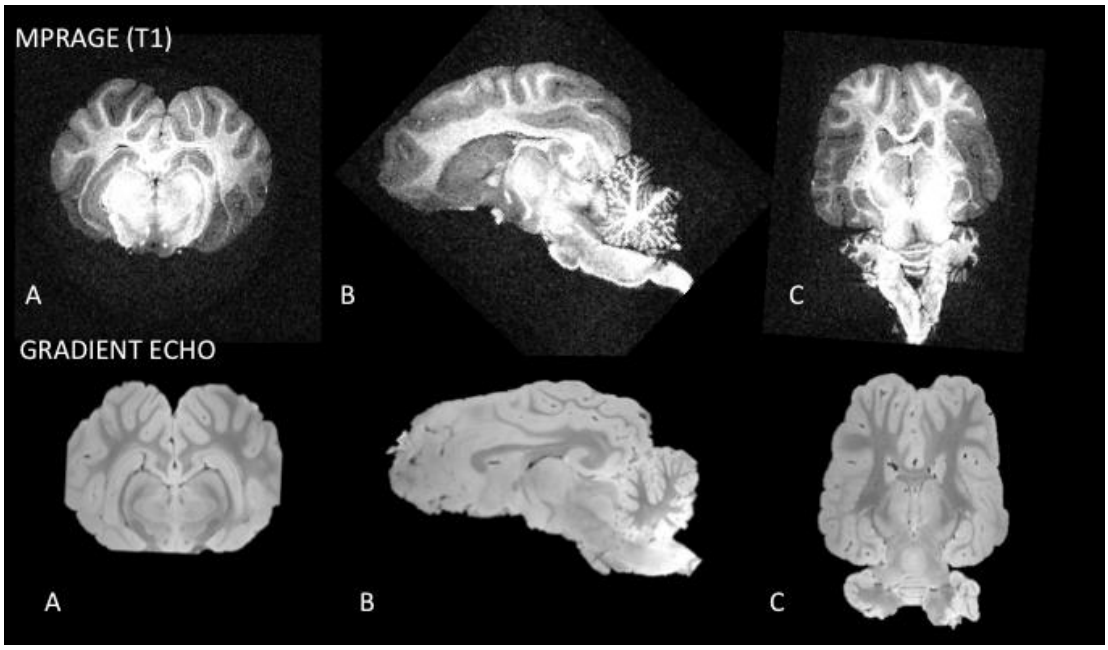

Figure 12. MR imaging in a pig brain

MPRAGE (T1) and Gradient Echo images of a pig brain in the (A) coronal, (B) saggital and (C) axial planes.

When the whole brain FA was compared for blast vs non-blast, we found a significantly lower FA in pigs with blast exposure (p=0.04), suggesting a difference in the WM integrity of the two groups (Table 5-2). However, no difference in FA was found between the two injury statuses in the ROI comparisons of the corpus callosum, the anterior internal capsule and the orbitofrontal WM (p=0.4, p=0.4 and p=0.2 respectively). Guided by the APP immunohistological results, we created ROI masks bilaterally in the internal capsule/thalamic areas. The more targeted analysis in this ROI yielded a significant FA difference between blast and non-blast brains, with a lower FA indicative of axonal injury being seen in the blast group when compared to the non-blast group (p=0.016) (see Table 2).

Table 2. Comparison of WM in blast vs. non-blast whole brain

ROI						
Injury status	**Pig**	**Whole brain**	**Corpus Callosum**	**Orbitofrontal WM**	**Anterior internal Capsule**	**Pathology led ROI**
Blast	B2	0.509	0.431	0.450	0.271	0.402
	B3	0.493	0.385	0.352	0.386	0.294
	B8	0.498	0.395	0.311	0.452	0.326
	B9	0.531	0.455	0.408	0.479	0.344
	B10	0.508	0.515	0.325	0.536	0.379
Non-blast	B5	0.531	0.434	0.329	0.434	0.399
	B6	0.514	0.398	0.365	0.409	0.424
	B7	0.539	0.493	0.342	0.445	0.480
Average blast FA		0.508	0.436	0.369	0.425	0.349
Average non-blast FA		0.528	0.442	0.345	0.430	0.434
t-test p-value		0.049	0.444	0.266	0.469	0.016

Discussion

The purpose of the blast injury in pigs study (BIPs) was to examine the effects of a primary BOP wave on the brain in a porcine model of polytrauma. The study has shown evidence that primary blast causes ependymal stripping with associated inflammation in the region of the lateral ventricles, helping to confirm that an isolated primary blast wave can cause brain injury. We found activation of the microglial cells throughout the brains of all animals raising important questions about the effects of polytrauma on the CNS and its treatment. Importantly the imaging results have confirmed that even at 4.7 Tesla, standard structural MR is not as sensitive to WM damage as DTI. More work is needed to develop DTI as a tool for use in trauma.

Neuropathology

Ependymal stripping

We found evidence of ependymal stripping in the region of the lateral ventricles. There was oedematous change underlying the areas of stripping as well as early activation of microglial cells indicating that the injury happened while the animals were alive. This finding supports previous work that has shown microglia activation within six hours of injury (Hoogland 2015).

De Lanerolle *et al.* (2011) demonstrated periventricular axonal injury and astrocyte infiltration two weeks after blast exposure in a porcine model of mild blast TBI (de Lanerolle 2011). Similar to our study, de Lanerolle and colleagues did not observe any obvious injury such as haemorrhage in these animals. Other authors have shown an association between ependyma injury and localised microglial cell activation (Sarnat 1995). These findings suggest that the blast-induced ependymal damage we observed triggers early immune activation.

There are several proposed mechanisms by which blast could cause brain injury, including spallation, implosion, and inertial effects (Nakagawa 2011, Leung 2008). Spallation is the disruption that occurs between materials of differing densities. As the BOP wave travels between materials, the

compression component is reflected at the material interface, leading to fragmentation of the denser material. Implosion occurs when gas bubbles in the tissue are compressed by the shockwave. The tissues collapse as the gas re-expands following the wave passage, the surrounding tissue is damaged. While the BOP wave propagates, lighter density masses will accelerate more than denser ones, resulting in large stress forces at the interface. This is known as the inertial effect. As such, the most vulnerable organs affected in the blast are those with air/liquid interfaces, such as the auditory canals, lungs and abdomen (Elder 2010b, Champion 2009). Previous investigators have hypothesised that pressure waves could be transmitted through the CSF spaces of the brain and spinal canal (Courtney 2009, Bauman 2009). The ependymal stripping that we have observed at the interface between the CSF-filled ventricles and the ependymal of the lateral ventricles supports the pressure wave transmission theory.

Clinical implications of ependymal stripping

The ependymal lining of the lateral ventricles has a role in controlling the composition and production of CSF as well as providing a reservoir of neural stem cells that can proliferate and migrate to areas of nervous tissue injury (Johansson 1999). The apical surface of the ependymal cells of the central nervous system have been shown to absorb and regulate the composition of CSF and the tight junction between ependymal cells act as a semi-permeable barrier to nervous tissue. Modified ependymal cells form the choroid plexus that produces CSF. Damaged ependymal may no longer be able to regulate the transport of fluid, ions and small molecules causing hydrocephalus. Tearing of the ependymal has been shown to leave discontinuities that become filled with the processes of subventricular astrocytes and can lead to extensive gliotic nodules (Sarnat 1995). Gliosis may change the compliance of the ventricular wall also leading to hydrocephalus. At the time of injury, a discontinuity in the tight junctions between the ependymal cells may predispose to infection, and ependymitis and ventriculitis are known to have high mortality rates (Lu 1998, Berk 1980*)*. The loss of the neural stem cell reserve may have implications for neuroregeneration. Future research should

be undertaken to determine if bTBI causes an ependymal injury in humans and if so whether there are higher rates of central nervous system infection and hydrocephalus. If future work confirmed ependymal injury, this would have significant implications for the design of personal protective equipment and the treatment of these injuries.

Hippocampal oedema

We also observed hippocampal oedema in two of the animals exposed to blast. This is in keeping with evidence from other studies which showed that the hippocampi are particularly susceptible to the effects of blast exposure (de Lanerolle 2011, Goldstein 2012, Miller 2015). Hippocampal injury is a well-documented consequence of nbTBI as well (Hicks 1993, Kotapka 1991). This vulnerability may be for several reasons: firstly, the hippocampus contains a large proportion of the CA1 fields of the cornu ammonis which are sensitive to trauma (Duvernoy 1988); secondly, the fronto-basal parts of the brain, which have extensive hippocampal projection fibres (Cavada 2000), are frequently damaged in moderate to severe TBI (Gennarelli 1998). This orbitofrontal damage may therefore result in transneuronal hippocampal cell death. In bTBI, damage to the hippocampi might be a direct result of the BOP wave, or could be secondary to hypoxia or impaired perfusion due to hypovolaemia. Previous studies looking at patients with hippocampal damage from epilepsy have found that they have poor memory (Addis 2007). Future research should be conducted to determine if these effects occur in trauma.

Perivascular oedema and generalised microglial cell activation

We saw perivascular oedema with fibrinogen leakage and widespread microglial activation in bTBI animals, but also in the sham group who had a soft tissue injury and IV fluid resuscitation but no exposure to blast. This suggests that these changes arose from another aspect of the injury model unrelated to the blast. Tissue oedema has been shown to occur in peripheral tissues following administration of IV fluids (Scallan 2010) and so it is possible that the changes we have observed are a result of the resuscitation strategy. Future research should be conducted to determine if the relationship between

perivascular oedema and IV fluid resuscitation as this could potentially worsen TBI outcome by increasing cerebral oedema, intracranial hypertension and reducing brain compliance (Hariri 1993, Teranishi 2012).

Widespread microglial cell activation in both blast and sham groups is another interesting observation that resulted from an aspect of the model unrelated to blast exposure. Hoogland *et al.* 2015 conducted a systematic review of 51 animal studies and showed that peripheral inflammatory stimuli can cause microglial cell activation. It is possible that inflammatory stimuli (cytokines) released by the soft tissue injury that the animals sustained activated the microglial cells in both groups.

Amyloid precursor protein

We found that both blast and sham pig brains showed early APP immunoreactivity, indicating that this result was not due to blast. However, there were some significant differences in the extent and location of reactivity seen between the two groups. The blasted pigs showed more extensive pathology in the orbitofrontal WM, the regions of the internal capsule and thalamus. This suggests that although blast is not the cause of the APP pathology it may exacerbate WM damage. De Lanerolle *et al.* (2011) noted similar pathology in pigs treated in a similar blast paradigm two weeks following a blast. Our finding of APP within four hours of blast is consistent with previous studies in nbTBI that has shown APP accumulation within three hours following injury (Sherriff 1994). The absence of a control group of pigs that had not received fluid resuscitation is an important limitation of the BIIPs study. The animals were sacrificed four hours after injury, if a longer survival time before sacrifice were possible, more APP accumulation may be detected, producing a clearer picture of the axonal injury. Future work should be conducted, comparing pig brains subjected to an isolated blast exposure and a group of normal pig brains to determine the role of the resuscitation strategy in APP pathology.

All histopathological analysis is subjective and, therefore, vulnerable to inter-observer variability and bias. In the BIIPs study, we limited our observations to

describing the presence or absence of individual pathologies and using semi-quantitative rating scales to make the results as reproducible as possible.

Imaging

Even at 4.7 Tesla, standard structural imaging did not reveal any areas of damage in any of the brains. Using DTI, however, we observed that the blasted pigs had a lower whole brain FA than the sham animals. Areas of the brain found to have more APP accumulation drove this difference in FA. FA and APP are markers of axonal injury (Warner 2010, Zhu 2014) (Gentleman 1993) and our work supports these findings. The absence of injury on structural MRI supports the previously stated view that standard structural imaging is not as sensitive as DTI when investigating WM damage and more work should be carried out to make DTI a readily available tool in the assessment of TBI. The imaging was performed on *ex vivo* brains and this may make the results difficult to translate into live human subjects. Also, the numbers of animals studied were small meaning that the findings need to be confirmed using a larger number of pigs and with a control group that had not received fluid resuscitation. High field strength MRI, using a 7 Tesla MRI demonstrates a hyperintense rim around the ventricles on FLAIR sequences (van Veluw 2015) and this may have a role in assessing ependymal integrity in the future.

Only one of the blasted pigs brains showed evidence of extravasation of erythrocytes, indicating haemorrhage in several areas of the medulla. These haemorrhages are analogous to microbleeds. Future work should be carried out to determine if there were factors, such as abnormal coagulation, that influenced this result.

Porcine model

We used a porcine model to examine the effects of blast on the brain because of similarities in gyral anatomy, glial-to-neuron ratios and the analogous behaviour of the tissues (Thibault 1998, Manley 2006). However there are significant differences in skull composition (Bauman 2009), size, shape and

integrity (Nakagawa 2011), which mean the findings may be different in humans. Pigs have thicker skulls and a different hindbrain orientation as well as larger sub-arachnoid spaces (Manley 2006), which may absorb and reflect energy differently. The neck of a pig is much thicker than that of a human, meaning that the whiplash-like forces that act on the head will be greater in humans. Pigs have hypercoagulable blood in comparison to humans and haemodilution further modulates coagulation. Therefore, the resuscitation targets used in this model may not produce the same effect in humans (Calzia 2012). Finally for practical reasons, we chose to diffusion fix the brains rather than perform perfusion fixation. Diffusion of paraformaldehyde throughout the brain would not have been instantaneous and so the cellular changes that we have observed may have occurred later than four hours after the blast injury.

Summary

In summary, we studied the effects of primary blast exposure in a porcine model of polytrauma. We found that an isolated BOP wave produces ependymal stripping with associated microglial cell activation within four hours of injury, as well as hippocampal damage in a subgroup of blasted animals. Standard MR imaging did not identify any structural abnormalities which mean that these injuries may be unrecognised. DTI identified the internal capsule and thalamus as areas with lower FA indicating more axonal injury.

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