Development of Novel Pharmacological Treatments for Intracranial Pressure Using Appropriate Experimental Models of Traumatic Brain Injury

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Doctor of Philosophy

Declaration

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Dedication

To those who give love and support to children and put the right seeds into their souls to grow.

Publications and Presentations

The following articles have been published or in preparation for publication or presentation during the period of my PhD candidature and sections of these articles have been included in the present thesis.

Journal papers:

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Abbreviations

ACE	Angiotensin-Converting Enzyme
AI	Axonal Injury
AMPA	Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid
ANOVA	Analysis of Variance
APP	Amyloid Precursor protein
ATP	Adenosine Triphosphate
ATPase	Adenosine Triphosphate-Hydrolases
BBB	Blood-Brain Barrier
Ca^{2+}	Calcium
CBF	Cerebral Blood Flow
CCI	Controlled Cortical Injury
CGRP	Calcitonin Gene Related Peptide
Cl^{-}	Chloride
CNS	Central Nervous System
CPP	Cerebral Perfusion Pressure
CSF	Cerebrospinal Fluid
CT	Computed Tomography
DAB	Diaminobenzidine Tetrahydrochloride
DAI	Diffuse Axonal Injury
DC	Decompressive Craniectomy
DNA	Deoxyribonucleic Acid
DVA	Diffuse Vascular Injury

EDH	Extradural Haemorrhage
ETCO2	End Tidal Carbon Dioxide
FPI	Fluid Percussion Injury
GCS	Glasgow Coma Scale
GPCR	G Protein-Coupled Receptor
GPs	Gaussian Processes
H+L	Heavy and Light
HTS	Hypertonic Saline
ICD	International Classification of Diseases
ICH	Intracerebral Haemorrhage
ICP	Intracranial pressure
IgG	Immunoglobulin G
IL	Interleukin
K^+	Potassium
LFP	Lateral Fluid Percussion
LFPI	Lateral Fluid Percussion Injury
MABP	Mean Arterial Blood Pressure
Mg^{2+}	Magnesium
mRNA	Messenger Ribonucleic Acid
Na^+	Sodium
NAT	N-Acetyl L-Tryptophan
NEP	Neutral Endopeptidase
NHS	Normal Horse Serum

NKA	Tachykinin (Neurokinin) A
NKB	Tachykinin (Neurokinin) B
NK1	Tachykinin-1 (Neurokinin) Receptor
NK2	Tachykinin-2 (Neurokinin) Receptor
NK3	Tachykinin-3 (Neurokinin) Receptor
NMDA	N-Methyl-D-Aspartate
NO	Nitric Oxide
NPγ	Neuropeptide γ
NPK	Neuropeptide K
$PaCO_2$	Arterial Blood Carbon Dioxide Tension
PAP	Post-Arteriolar Pressure
PaO2	Arterial Blood Oxygen tension
PBS	Phosphate Buffered Solution
PEEP	Positive Post-Expiratory Pressure
$P_{bt}O_2$	Brain Tissue Oxygenation
PE	Polyethylene
Pg	Progesterone
PGCS	Pediatric Glasgow Coma Scale
PNS	Peripheral Nervous System
PPT-A	Pre-Protachykinin-A
SAH	Subarachnoid Haemorrhage
SD	Standard Deviation
SDH	Subdural Haemorrhage

SEM	Standard Error of Measurement
SP	Substance P
SPC	Streptavidin Peroxidase Conjugate
SP-DE	Substance P Degrading Enzyme
Spm	Stroke per Minute
TAI	Traumatic Axonal Injury
TBI	Traumatic Brain Injury
TGF	Tumor Growth Factor
TNF	Tumor Necrosis Factor
TRPV1	Transient Receptor Potential Vanilloid 1
WDI	Weight Drop Injury
WHO	World Health Organisation

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Abstract

Traumatic brain injury (TBI) is the leading cause of death in the population below 40 years of age. Patients who survive TBI suffer from ongoing physical disabilities as well as mental and emotional deficits that significantly impact their quality of life. While a number of factors have been implicated in the brain injury cascade that is initiated by TBI, increased intracranial pressure (ICP) has been identified as one factor that is strongly associated with outcome. This is largely because increased ICP results in a fall in cerebral perfusion pressure (CPP) and in brain oxygenation ($P_{bt}O_2$), thus starving the brain of essential substrates and oxygen necessary for repair and recovery. Nonetheless, treatments targeting increased ICP are largely ineffective and have not changed for over 40 years. In part, this is because the mechanisms responsible for oedema formation after trauma are unknown and also because existing small animal models of TBI might not duplicate all the pathophysiological features of human TBI. The aim of this thesis was therefore to study changes in ICP and $P_{bt}O_2$ in two different experimental animal models of TBI, both large and small, and subsequently investigate the effects of different pharmacotherapies on these variables following TBI.

The thesis shows that TBI does not consistently produce increases in ICP in rodent models unless a haemorrhagic mass lesion is present. Accordingly, rodents may not be the ideal species for the development of ICP targeted pharmacotherapies. By then studying the effects of TBI on ICP, cerebral perfusion pressure (CPP) and $P_{bt}O_2$ in an ovine, large animal model, we noted that the sheep model of injury produces similar changes in these variables to clinical (human) TBI, and was therefore well suited to the development of ICP targeted pharmacotherapies. The targeted therapy we chose to investigate was the substance P, NK1 antagonists which have been previously shown in our laboratory to reduce blood brain barrier breakdown and oedema

formation following rodent TBI. We characterized the effects of two different NK1 receptor antagonists on ICP, $P_{bt}O_2$ and CPP in an ovine model of TBI at both moderate and severe injury levels, and compared the effects to those to that of the clinically used osmotic agents, mannitol and hypertonic saline. We noted that in contrast to the osmotic agents, the NK1 antagonists consistently reduced ICP and improved $P_{bt}O_2$ irrespective of the severity of injury. As a further comparison, we examined the effects of the putative neuroprotective compounds magnesium and progesterone on ICP and $P_{bt}O_2$ following ovine, moderate TBI, and noted that both agents were ineffective. This finding highlighted the importance of using large animal models of TBI to investigate novel interventional pharmacologies.

Having acquired a considerable amount of physiological data in a large animal model of TBI that largely replicated the temporal changes in ICP and CPP in human TBI, we then applied Gaussian processes for data analyses to investigate the dynamic interrelationship between $P_{bt}O_2$, ICP, and mean arterial blood pressure (MABP) and CPP after trauma. This facilitated the development of a contour plot describing these dynamic interrelationships and enabling the prediction of mean $P_{bt}O_2$ values for any given ICP and MABP, the identification of critical thresholds in ICP, and the physiological basis and refinement of the CPP formula. We noted that $P_{bt}O_2$ had critical thresholds that might be related to the compression of post-capillary venules, capillaries and precapillary met-arterioles, respectively. Real CPP thus depends upon the pressure within the vascular tree, and whether their flow has been restricted by increased ICP.

In conclusion, NK1 antagonists offer a novel intervention for increased ICP and reduced $P_{bt}O_2$ after TBI that is superior to existing, alternative therapies irrespective of injury severity.

CHAPTER 1

GENERAL INTRODUCTION

In industrialised countries, as well as in many non-industrialised countries, traumatic brain injury (TBI) is the leading cause of death in the population aged below 40 years (Fleminger & Ponsford 2005, Langlois *et al.* 2006), as well as the leading cause of death and disability in the population aged below 45 years of age (Hillier *et al.* 1997, Finnie & Blumbergs 2002, Werner & Engelhard 2007, Myburgh *et al.* 2008). The incidence of TBI is also increasing in those low and middle income countries where a considerable part of the population is moving from rural areas to urban areas and where there is also a rapid increase in the number of used motor vehicles (Maas *et al.* 2012). Considering the relatively young age of most victims, more productive years of life are lost from trauma than from cardiac and cerebrovascular disease, or from cancer (Rockett & Smith 1987).

The economic and social cost of head injury to the society is enormous with billions of dollars being spent each year on the management and rehabilitation of trauma patients. In the USA alone, 1.6 to 2 million people every year are affected by TBI and between 200 000 to 270 000 of them need to be hospitalized (Ghajar 2000, Whitmore *et al.* 2012). About 52 000 of these patients die, whereas about 80 to 90 thousand of them are affected with long-term disability (Ghajar 2000, Whitmore et al. 2012). Lifetime medical care costs of TBI patients in the US in 1985 alone were estimated to be more than $5 billion per year (Sosin *et al.* 1995, Sosin *et al.* 1996, Thurman & Guerrero 1999), while the total financial burden in the US is estimated at more than $60 billion annually (Corso *et al.* 2006, Rockhill *et al.* 2012, Whitmore et al. 2012). Although there has been an improvement in prevention and clinical management of TBI, it nevertheless remains one of the major community health problems worldwide and up to now there is no effective treatment for it.

Patients who survive TBI not only suffer from physical disabilities but also from ongoing mental and emotional symptoms such as cognitive deficits (Binder 1997, Rapoport et al. 2005), depression and mood disorders (Hanks *et al.* 1999, Chamelian & Feinstein 2006). Patients also demonstrate behavioral changes such as increased level of stress and a poor ability to cope with stressful situations (Bohnen *et al.* 1995, Bernstein 2002, Chamelian & Feinstein 2006). Difficulties with memory and attention, mental slowing and poor concentration are just some of the many other cognitive deficits (MacFlynn *et al.* 1984, Gentilini *et al.* 1985, Parasuraman *et al.* 1991). In many patients, there is often a significant increase in expressed emotions such as crying, bursts of anger, and increased levels of general anxiety (Ornstein *et al.* 2009, Tlustos *et al.* 2011). Other common symptoms with these patients are headaches, nausea and dizziness (Maskell *et al.* 2006). All these above mentioned neuropsychiatric sequelae outstrip the neurophysical (such as ataxia and incontinence) as the major cause of disability (Fleminger & Ponsford 2005). Regardless of the age of the patient, it is these changes in cognition and behavior that represent the greatest burden to families after a TBI (Ponsford *et al.* 2003).

Currently it is accepted that neurological deficits caused by TBI develop over time through two mechanisms (McIntosh *et al.* 1996, Gennarelli & Graham 1998, Stahel *et al.* 2005). The first is known as primary injury and is made up of mechanical events that occur at the time of the trauma. Secondary injury refers to the delayed biochemical and pathophysiological events initiated by the primary injury and developing over minutes to days (in some cases even months) after the primary injury. Since secondary injury, unlike the primary injury, develops over time, it provides an opportunity for pharmacological intervention to prevent or reduce the level of injury and subsequently improve a patient's survival and functional outcome. However, the factors that

make up the secondary injury must first be identified and interventions then developed to inhibit the injury process.

To date, a number of different secondary injury factors have been identified. Oedema, in particular, is one factor shown to be responsible for up to as much as half of all the associated mortality and morbidity (Feickert *et al.* 1999, Marmarou 2003). Brain oedema implies an abnormal increase of fluid within the brain parenchyma associated with an increase in tissue volume (Klatzo *et al.* 1980, Klatzo 1987). Oedema contributes to an increase in intracranial pressure (ICP), which may decrease brain tissue perfusion and result in localized hypoxia and ischaemia (Andrews & Citerio 2004). If increased ICP is left uncontrolled, the body's compensatory mechanisms can fail, which eventually will lead to delayed brain death (McIntosh et al. 1996, Feickert et al. 1999, Battison *et al.* 2005). While oedema and ICP increases are significant in all cases of severe brain trauma, oedema development is of particular concern in young victims of brain injury where even mild to moderate injury can result in profound oedema (Barlow *et al.* 2010).

Depending on the underlying mechanisms there are two principal types of brain oedema, vasogenic and cytotoxic (Klatzo 1967). Vasogenic oedema is the accumulation of protein-rich exudate derived from plasma in the extracellular space caused by increased permeability of the blood-brain barrier (BBB) (Betz *et al.* 1989). Cytotoxic brain edema is characterized by sustained intracellular water accumulation involving both astrocytes and neurons. In contrast to vasogenic brain edema, this type of edema occurs independently of the BBB integrity (Unterberg *et al.* 2004).

Among the different mechanisms that have been associated with the development of post-traumatic oedema, neurogenic inflammation has recently been implicated as an early one and less studied (Vink *et al.* 2003, Nimmo *et al.* 2004, Vink & Van Den Heuvel 2004, Vink *et al.* 2004). Neurogenic inflammation is the result of stimulation of C-fibers that causes the release of neuropeptides (Woie *et al.* 1993). Substance P (SP) and calcitonin gene related peptide (CGRP) are the major neuropeptides involved in the development of neurogenic inflammation (Newbold & Brain 1995), with CGRP recognized as an extremely potent vasodilator affecting both major vessels and the microvasculature (Newbold & Brain 1995) and SP recognized as increasing microvascular permeability (Newbold & Brain 1995). It has been proposed that sensory C-fibres are mechanically distended during the primary phase of TBI (Vink et al. 2003), causing the release of neuropeptides that facilitate plasma protein extravasation from blood vessels and subsequent oedema formation (Nimmo et al. 2004). The effects of SP in the brain are mediated primarily via the tachykinin NK1 receptors (Harrison & Geppetti 2001, Maubach *et al.* 2001) and experiments have shown that the NK1 antagonist n-acetyl L-tryptophan (NAT) could effectively resolve existing oedema and prevent further oedema development in experimental animal models (Donkin *et al.* 2009). Whether this translates to any decrease in ICP after TBI is unknown.

Laboratory studies have shown that different animal models exhibit different levels of similarity to the clinical situation in humans. In particular, rodent models have been extremely useful in studying secondary injury factors following TBI despite their vastly different CNS anatomy from humans. However, all rodents share the disadvantage of a small, lissencephalic brain (absence of the convolutions of the cerebral cortex) and small head size relative to body size. Given that

there has been little success in the translation of findings from rodent models to the clinical arena, we have chosen to use both a rodent and sheep model of TBI in the current studies. The sheep model of TBI (Lewis *et al.* 1996, Van Den Heuvel *et al.* 2004) allows direct measurement of ICP and brain oxygenation ($P_{bt}O_2$) after TBI in a gyrencephalic brain (cerebral cortex marked by convolutions) that has substantial white matter domains, thus more closely replicating the clinical situation.

The aim of this thesis is therefore to study changes in ICP and $P_{bt}O_2$ in two different experimental animal models of TBI, investigate the effects of NK1 antagonists on increased ICP and decreased $P_{bt}O_2$ and compare them to current and experimental interventions, study the dynamic interrelationship between ICP, MABP, CPP, and $P_{bt}O_2$, and finally investigate clinically observed critical thresholds of ICP and $P_{bt}O_2$. Before outlining the details of the present study, consideration will be given to (a) epidemiology, definitions and classification of TBI, (b) its pathophysiology, (c) traumatic brain oedema and increased ICP with current management protocols, (d) brain tissue oxygenation, (e) substance P in neurogenic inflammation and the potential of NK1 antagonists to treat increased ICP, and finally (f) an overview of different animal models of TBI in studying raised ICP.

1.1 Epidemiology

1.1.1 Incidence, Causes and Demography

Epidemiological data for TBI is available mainly from developed countries, though it is difficult to overestimate its importance in organizing care for survivors and improving preventive measures (Roozenbeek *et al.* 2013). Often it is difficult to determine the overall incidence of TBI, partially because most statistics are derived from specific locations and not all studies are done by the same methodologies and accuracy. Also mild head injuries that account for the vast majority of head injury (Weight 1998, Maegele *et al.* 2007) tend to be underreported partly due to poor identification or categorization of organ specific injuries (Masson *et al.* 2001) or sometimes just due to missed cases (Binder 1997, Asbury *et al.* 1998, Weight 1998). Nevertheless there is a considerable data available that gives more or less the whole picture of the problem worldwide as well as in different regions or countries.

Worldwide about 57 million people have been hospitalized because of TBI, and more than 5 million of them suffer long term consequences (Langlois et al. 2006). In the USA and Europe the annual incidence of TBI is about 500 per 100 000 (Maas et al. 2012). This results in 200 per 100 000 patients being hospitalized every year in most European countries (Maas et al. 2012). The mean incidence rate of both hospitalized and fatal cases of TBI in Europe is about 235 per 100 000 population, whereas in USA it is 103 per 100 000, in India it is about 160 per 100 000, and in East Asia it is about 334 per 100 000 (Gururaj 2002, Langlois & Sattin 2005, Langlois et al. 2006, Tagliaferri *et al.* 2006). In Australia overall incidence rate of TBI is about 226 per 100 000 population (Hillier et al. 1997, Myburgh et al. 2008). The incidence of head injury associated death is over 25 per 100 000 (Finfer & Cohen 2001, Myburgh et al. 2008).

The average mortality rate in Europe is over 15 per 100 000, ranging from as little as about 5 per 100 000 in Aquitaine, France in 1996 (Masson et al. 2001) to up to over 24 per 100 000 in a province in Italy (Servadei *et al.* 2002). In USA mortality rate is a little higher compare to Europe and it is over 18 per 100 000, in India it is about 20 per 100 000, while in East Asia it is up to 38 (Gururaj 2002, Langlois et al. 2006, Tagliaferri et al. 2006). Statistics about severity of TBI are more or less the same worldwide and mild head injuries account for up to 80 % of all cases, moderate injuries account for 10-12 % and severe cases account for 9-11 % (Masson et al. 2001, Maegele et al. 2007, Roozenbeek et al. 2013).

1.2 Definitions and Classification of Brain Injury

1.2.1 Definitions

The definition of TBI still has not been completely finalized to date and often varies in relation to specialties and circumstances (Bigler 2001). Different terms are frequently used interchangeably, like brain or head injury, or mild head injury or concussion (Bennett & Raymond 1997). Nevertheless, TBI generally refers to any trauma to the brain inflicted by an external mechanical force that is associated with a diminished or altered state of consciousness and can potentially lead to permanent or temporary impairments of mental or physical functions (van Baalen *et al.* 2003, Summers *et al.* 2009). The TBI itself can be classified as open or closed. Open injuries are those injuries where penetration of the skull occurs (as in gunshot injuries) and closed injuries are those without penetration of the skull and are caused primarily by blunt impact (Castellanos-Pinedo *et al.* 2012). Open injuries caused by penetrating forces often are localized and follow the path of the missile (Stratton & Gregory 1994). Closed injuries, however, occur more frequently than open injuries and often without an impact or blow to the head.

1.2.2 Classification and Severity Indices

While there have been a number of different classifications developed for clinical head injury, the most commonly used is the post-resuscitation Glasgow Coma Scale (GCS) developed by Teasdale and Jennett in 1974 (Teasdale & Jennett 1974). The GCS attempts to classify the severity of a head injury over an initial and continuing assessment period by evaluating a patient's motor, verbal and eye opening responses (Table 1.1). According to the GCS, head injury is classified as mild if the evaluation scores are in the range of 13 to 15, moderate if the scores are between 9 to 12, and severe if the scores are equal to or less than 8 (Teasdale & Jennett 1974).

Table 1.1 The Glasgow Come Scale where a patient is assessed against the stated criteria with points giving a score between 3 (indicating deep unconsciousness) and 15.

	1	**2**	**3**	**4**	**5**	**6**
Eyes	Does not open eyes	Opens eyes in response to painful stimuli	Opens eyes in response to voice	Opens eyes spontaneously	N/A	N/A
Verbal	Makes no sounds	Incomprehensible sounds	Utters inappropriate words	Confused, disoriented	Oriented, converses normally	N/A
Motor	Makes no movements	Extension to painful stimuli	Abnormal flexion to painful stimuli	Flexion / Withdrawal to painful stimuli	Localizes painful stimuli	Obeys Commands

Recently, some concerns have been raised about the sensitivity of the GCS in evaluating a mild head injury, as well as in severe head injury (Kraus & Nourjah 1988, Heim *et al.* 2004, Zuercher *et al.* 2009, Kevric *et al.* 2011, Namiki *et al.* 2011). The GCS was also initially developed for adults and since children under the age of 36 months still have poor verbal performance, a pediatric Glasgow Coma Scale (PGCS) has been developed which also assesses motor, verbal and eye opening responses.

International Classification of Diseases (ICD) is another classification of head injury and is published by the World Health Organisation (WHO). ICD is a patho-anatomical classification that is used for epidemiological health data gathering and hospital record keeping (Rutledge *et al.* 1998, Chen & Colantonio 2011, Nakahara & Yokota 2011). It mainly includes the type and the location of brain injury. In the Revised ICD-10 system, intracranial injuries are included under the three digit rubric S 06 which has 10 subcategories expressed with numbers from 0 to 9 (Table 1.2).

Table 1.2 The International Classification of Diseases -10 system.

ICD-10 S 0-6 (Intracranial Injury) with Subcategories	
S 06-0	Concussion
S 06-1	Traumatic Cerebral Oedema
S 06-2	Diffuse Brain Injury
S 06-3	Focal Brain Injury
S 06-4	Epidural Haemorrhage
S 06-5	Traumatic Subdural Haemorrhage
S 06-6	Traumatic Subarachnoid Haemorrhage
S 06-7	Intracranial Injury With Prolong Coma
S 06-8	Other Intracranial Injuries
S 06-9	Intracranial Injury Unspecified

1.2.3 Mild Traumatic Brain Injury

Mild traumatic brain injury accounts for 80-90 % of all head injuries in industrialised countries and most commonly affects teenagers and elderly people primarily as the result of falls and motor vehicle accidents (Sterr *et al.* 2006). Some of the most common neuropsychological symptoms after mild TBI are headaches, attention deficits, poor concentration, sleep disturbance, impaired verbal retrieval, memory difficulties, depressed mood and irritability, blurred vision and emotional distress (Lezak 1994, Sherer *et al.* 2002, Milders *et al.* 2003). Not all of these symptoms become apparent immediately after the injury, sometimes taking days or weeks until these symptoms start to appear. These post-injury symptoms are known as post-concussion syndrome or post-traumatic syndrome, which is defined as a transient condition reflecting a completely recoverable disorder of neuronal function (Binder *et al.* 1997, Binder 1997, Ingebrigtsen *et al.* 1998, Hou *et al.* 2012). Nonetheless, a few studies have shown that some victims show persistent deficits for much longer time periods (up to 4-5 years) (Boll & Barth 1983), including headaches, fatigue, dizziness, memory difficulties, and often some personality changes. People experiencing these symptoms frequently have difficulties in coping with their everyday life, which results in emotional distress (Eriksson *et al.* 2006). Often people become more distressed when they realize that they need to make more effort with considerable concentration to perform routine activities that they used to do automatically before the injury (Eriksson *et al.* 2009). These activities include such mental functions as planning, monitoring, listening, calculating, and interacting with others, amongst others. These individuals become tired more easily which gets even more burdensome when they are aware of their mental inefficiency. All these symptoms and experiences together result in even more increased

emotional distress and eventually can lead to anxiety or general depression (Eriksson et al. 2009, Schonberger *et al.* 2011).

1.2.4 Moderate Traumatic Brain Injury

Moderate traumatic brain injury accounts for 5-10 % of all head injury cases (Hillier et al. 1997, Miller 1993). With victims of moderate TBI, symptoms can vary depending on the age of the victim and the location of the injury (Pentland *et al.* 1986, Roy *et al.* 1986, Miller 1990, Myburgh et al. 2008). Many studies have shown that some of these symptoms can last for years, especially with children and adolescents (Max *et al.* 2000, Bloom *et al.* 2001). The most frequent deficits in moderate head injury include such cognitive deficits as memory impairment, depression, and decreased ability to concentrate (Levin *et al.* 1988a, Levin *et al.* 1988b, Sherer et al. 2002, Schonberger et al. 2011). Other problems that are experienced by patients with moderate brain injury are impairments in conceptualization and abstract thinking, difficulties in tracking thought processes and conversations, considerably impaired ability in planning, controlling and executing life activities as well as achieving goals (Banich *et al.* 2001, Milders et al. 2003). Studies have shown that victims of moderate brain injury can also demonstrate some loss of emotional and behavioral capability, such as inability to monitor their behavior, increased impulsivity or angry outbursts, and decreased capability to show emotions (Chamelian & Feinstein 2006). All these symptoms result in increased levels of psychological, emotional and behavioral distress as well as severe depression and lack of motivation (Bloom et al. 2001, Milders et al. 2003, Chamelian & Feinstein 2006). All these cognitive, emotional and behavioral difficulties occurring after moderate head injury often can end up as personality changes reported

by family members or friends of a victim (Ponsford et al. 2003, Chamelian & Feinstein 2006, Ponsford & Schonberger 2010).

1.2.5 Severe Traumatic Brain Injury

Although severe brain injuries account for about 5 % of all head injuries in industrialised countries (Miller 1993, Hillier et al. 1997), few victims survive and have good recovery (Lipper-Gruner *et al.* 2002, Wijdicks & Cranford 2005, Leon-Carrion *et al.* 2012). Victims exhibit a wide variety of deficits ranging from subtle difficulties with concentration and attention (Costeff *et al.* 1985, Couillet *et al.* 2010) to being left in a debilitating vegetative state (Wijdicks & Cranford 2005). Patients that recover from severe traumatic brain injury are often left with permanent cognitive, emotional, behavioral and physical deficits (Chamberlain 2006). Since the majority of severe brain injury patients are in the age group of 15-24 (Fleminger & Ponsford 2005), it often disrupts important developmental processes such as completing studies, attaining independence from parental support, and forming an independent social life. All these disruptions result in loss of self esteem, behavioral and personality changes with social isolation and they frequently end up as a persistent burden for families. Patients often experience difficulties with language, hearing, vision, memory, which often remain long-term, though some of them can become fairly subtle in time. Patients also demonstrate disorders with motor speech, which includes difficulties in pronunciation and word production (Theodoros *et al.* 1998, Wang *et al.* 2004).

Difficulties with social skills partially arise from deficiencies of self-monitoring and social judgment. An injured person's difficulties keeping up with conversation due to slowed information processing is one of the reasons that make these patients avoid social contact, which

in turn can create social anxiety (Massagli *et al.* 1996, Deb *et al.* 1999, Giacino & Kalmar 2005). Studies have shown that some of the symptoms described by these patients at interview often are overlooked. Patients reported feeling self-conscious regarding physical symptoms of their trauma, as well as having a continuing sense of loss because of failure to accomplish their goals. Sometimes patients described negative feelings from others, possibly due to lack of understanding of the consequences of brain injury (Deb et al. 1999). There is an increasing awareness of the long-term psychological disorders after brain injury, including post-traumatic stress disorder, depression, anxiety, delusional disorder, and drug misuse (Fleminger & Ponsford 2005, Chamberlain 2006).

Recovery for most patients often takes years after traumatic brain injury and after this patients face an uncertain future. Some patients still show improvement even as late as ten years after injury (Engberg & Teasdale 2004). Though physical disabilities play a huge role as a cause of disabilities for patients with severe traumatic brain injury, however for those who recover neuropsychological and behavioral consequences are of greater problems for themselves as well as their families, friends and community (Ponsford *et al.* 1995, Bloom et al. 2001, Fleminger & Ponsford 2005, Ponsford & Schonberger 2010).

1.3 Pathophysiology of Traumatic Brain Injury

The pathophysiological changes that occur after traumatic brain injury are complex. They are dynamic and occur at macroscopic levels as well as the cellular and molecular levels, and may be adversely influenced by events that occur after the initial injury (Adams *et al.* 1983, Adams *et al.* 1985). The mechanisms of injury are classified as either primary (mechanisms resulting in early

tissue deformation) or secondary (mechanisms involved in later onset of injuries). Although there is some overlap between these two categories, the mechanisms of TBI can be extended out to more than two phases; primary injury, the delayed consequences of the primary injury, secondary injury and recovery with functional outcome (Graham 2002). The severity and outcome of an injury also depends on such individual differences amongst victims such as age, pre-existence of any disease, physical fitness, psychological and nutritional status (Abou-Hamden *et al.* 1997, Bigler 2001).

1.3.1 Primary Traumatic Brain Injury Mechanisms

The primary injury is caused by the mechanical deformation of a skull, brain tissue and vasculature at the moment of injury caused by coming into contact with an object and/or by inertial forces producing acceleration or deceleration movement of the brain (Adams *et al.* 1981, Adams et al. 1983, McIntosh et al. 1996, Adams *et al.* 2000). Depending on the magnitude and direction of the force, primary injury may cause gross disruption of brain tissue and blood vessels at the macroscopic level in a focal, multifocal or diffuse pattern of involvement (Abou-Hamden et al. 1997, Finnie & Blumbergs 2002), while on the microscopic level, it may damage the neuronal and glial cells, axons, and capillaries (McIntosh et al. 1996). The consequences of primary injury may include metabolic changes, inflammatory reactions, cellular swelling, intracranial haemorrhage, raised intracranial pressure, cerebral ischaemia and cell death (McIntosh *et al.* 1989).

Biomechanics of brain injury

Biomechanics studies the forces and physical responses in dynamic or static biological systems. Force and stress are the most basic terms used to describe applied loads on tissue, and strain and deformation are the terms used to describe the resulting responses of tissues to these forces. Force is defined as the action of one body on another as a result of an impact that will cause acceleration of the second body. When forces are created in tissues, deformations can occur which greatly depend on the physical properties and the nature of the force itself. Deformation is used to describe the changes in shape of a body or tissue undergoing a force. The term stress is used to describe the distribution of an applied force relative to the area on which it acts. Generally there are two kinds of stress; normal stress, which acts perpendicular to the surface, and shear stress which acts tangential to the surface. When a force or load is applied on a biological system, it gives a specific response that partially depends on tissue tolerance. Tissue tolerance is the point at which a specific force or load can cause structural and functional failure in tissue (LaPlaca *et al.* 2007). Although determination of tissue tolerance standards needs data about the forces and mechanical deformation that causes structural and functional failure of the tissue, all these mechanical parameters are only partially understood (Goldsmith 2001). Development of more effective protective equipment and preventive measures will be enhanced with a better understanding of injury biomechanics and resulting brain response.

The severity and outcome of brain injury greatly depends on the nature, direction, duration and magnitude of affecting forces as well as on the anatomical site of impact. Primary mechanisms of brain trauma involve energy transfer to brain tissue by contact or acceleration/deceleration (inertial) movements (Adams et al. 1981, LaPlaca et al. 2007). These forces that are responsible

for the mechanical damage to the brain and other tissues can be viewed as either dynamic or static (Adams et al. 1983, Greve & Zink 2009, Nakagawa *et al.* 2011), with dynamic loading being the most common. The amount of shear stress and strain applied to the brain which results in focal, diffuse or a mix of both types of injury depends on whether the forces involve direct impact, acceleration, deceleration or rotation (LaPlaca et al. 2007, Nakagawa et al. 2011). During the impact to the brain the velocity of movement is passed on to the brain tissue, with rotational forces present in most cases. The impact typically involves rapid deceleration forces since the brain tissue comes to a sudden halt and as a result of these forces the brain tissue could be stretched and rotated within the skull (Nakagawa et al. 2011). The effects of such impact involves the brain tissues direct striking of the skull and shear strain which results in the pulling apart of axons and distraction of cell bodies that occurs as a result of the momentary deformation of the brain's shape and density (Adams et al. 1983, Goldsmith 2001).

Within the skull, the brain is fixed by the parasinusoidal granulations, parasagital bridging veins, tentorium and cranial nerves. The forward movement of the brain tissue inside the skull applies more force at the base of the frontal lobes and the tips of the temporal lobes, and surface contusions are therefore more common at these locations than in other areas in the brain (Bullock *et al.* 2006a). Bruising can occur also at the point of rapid deceleration as a result of the soft brain tissue's strong movement against the hard bony regions of the inside of the skull (Adams et al. 1981, Adams et al. 1985). This bruising is generally more prominent in the frontal and temporal lobes where the cortex rests on the rough surface of the skull (Mattson & Levin 1990, Bullock et al. 2006a).

Structural damage

Structural damage following TBI is considered as either focal or diffuse (Povlishock *et al.* 1994, Goldsmith 2001, Andriessen *et al.* 2010, Potapov *et al.* 2011). Focal injuries include neuronal and neurovascular damages such as cerebral haemorrhages and haematomas (epidural, subdural, subarachnoid, intraventricular, intracerebral), contusions, lacerations, brain stem injury, scalp lacerations and skull fracture; diffuse injuries include diffuse axonal injury (Yao *et al.*) and diffuse vascular injury (DVI) (Adams et al. 1981, Adams et al. 1983, Abou-Hamden et al. 1997, Besenski 2002, Bullock et al. 2006a, Bullock *et al.* 2006b, Bullock *et al.* 2006c).

Traumatic intracranial haemorrhage and haematoma

Traumatic haemorrhage (bleeding) or haematoma (blood clots) in the brain generally results from tearing of blood vessels following head injury (Bullock et al. 2006c, Bullock et al. 2006b, Gerlach *et al.* 2009). Haemorrhages generally are classified as extradural haemorrhage (EDH), subdural haemorrhage (SDH), subarachnoid haemorrhage (SAH), intracerebral haemorrhage (ICH) or intraventricular haemorrhage. Extradural haemorrhage is usually located directly beneath the point of injury and in most cases associated with a skull fracture (Povlishock et al. 1994, Marmarou 2003). Extradural haematomas are usually arterial in origin and in most cases occur in the temporal, parietal and frontal areas (Povlishock et al. 1994, Marmarou 2003, Gerlach et al. 2009). In most cases of EDH, the disruption of anterior or posterior branches of the middle meningeal arteries is the source of bleeding. Most EDH occurs in the second and third decade, when the dura is less tightly attached to the overlying cranium than in the newborn or elderly (Bullock et al. 2006b, Gerlach et al. 2009). Subdural haemorrhages are usually venous in origin and often occur in the frontal regions of the brain when the brain experiences deceleration

forces which result in stretching and tearing of parasagital bridging veins (Lobato *et al.* 1988, Gennarelli & Graham 1998, Muszynski *et al.* 1999, Bullock et al. 2006c). They are classified as acute, sub-acute or chronic depending on time of presentation following injury. Acute SDH presents within 48 hours, subacute SDH presents between 48hours and 2 weeks, and chronic SDH presents after 2 weeks (Gulsen *et al.* 2009, El-Fiki 2012). Subarachnoid haemorrhage is the most common form of structural damage seen in head injury (Finnie & Blumbergs 2002). SAH usually results from shear injury to the vasculature of the subarachnoid space or direct extension into the subarachnoid space by a contusion or haematoma (Besenski 2002). It also may occur as a consequence of disruption of superficial vessels at sites of contact injury (Fung *et al.* 2012). Generally SAHs are small although they can expand and become a considerable space-occupying lesion (Sawauchi *et al.* 2001, Zhao *et al.* 2009). Intracerebral haemorrhage is a well-defined collection of blood within the brain parenchyma as a result of stretching and rupture of small-calibre cerebral arterioles located within either the basal ganglia or the ventricles secondary to a contre-coup injury (Sawauchi et al. 2001, Erol *et al.* 2004, Maskell et al. 2006).

1.3.2 Secondary Traumatic Brain Injury Mechanisms

Primary injury initiates a complex series of parallel and interacting biochemical and metabolic changes that can severely exacerbate injury. These delayed changes are called secondary injury and last from minutes to days and even months. Though not fully understood, the ongoing interconnected pathological processes of secondary injury generally are perpetuated by early failure of cellular energy, blood-brain barrier disruption, stenosis of microvasculature (due to loss of vasodilators such as nitric oxide, and release of vasoconstrictors such as endothelin-1), ischemia, hypoxia, swelling of astrocytes, astroglyosis, neurotransmitters release, excitotoxicity

and abnormal ionic homeostasis, axonal injury, free radical overload, increased oxidative stress, calcium influx and intra-axonal calcium accumulation, cytoskeletal breakdown, oedema, increased ICP, inflammation and cell death (McIntosh et al. 1996, Abou-Hamden et al. 1997, Yi *et al.* 2008, Park *et al.* 2008). For patients who survive the primary injury, secondary injury is the leading cause of morbidity and mortality.

Metabolic changes following traumatic brain injury

Cerebral metabolism is altered following traumatic brain injury and the degree of these changes depends on the severity of the primary injury (Wu *et al.* 2004). Subsequently, it is well accepted that the severity of cerebral metabolic changes largely determines outcome after TBI (Cunningham *et al.* 2005). Following TBI, brain tissue metabolism and energy state are first reduced despite the increase need for energy to maintain biochemical homeostasis, (Vink *et al.* 1994, Wu et al. 2004, Cunningham et al. 2005, Werner & Engelhard 2007). This early metabolic reduction relates to disequilibrium of ion homeostasis, particularly reduced Mg^{2+} concentration, and reduced ATP production due to mitochondrial dysfunction and reduced respiratory rates (Vink *et al.* 1988, Vink et al. 1994, Tavazzi *et al.* 2005, Werner & Engelhard 2007). All these metabolic changes can lead to cellular swelling, increased intracranial pressure, cerebral hypoxia and delayed cell death (Teasdale & Graham 1998, Werner & Engelhard 2007).

Ionic changes

Brain cell response to TBI is a complex process in which ionic imbalance plays one of the major roles in both persistent cellular and axonal dysfunction. TBI results in Ca^{2+} influx into neural cells and axons following TBI (Greve & Zink 2009, Johnson *et al.* 2012) as well as a massive

influx of sodium (Na^{+}) and chloride (Cl^{-}), and an associated efflux of potassium (K^{+}) (Robertson *et al.* 2001). These ionic changes trigger blood-brain barrier breakdown as well as increased Na^{+}/K^{+} -ATPase activity with an associated increase of metabolic demand, thus creating a vicious circle of cell metabolism (Werner & Engelhard 2007). Increased concentrations of intracellular Ca^{2+} activates various proteases and lipases resulting in organelle and membrane damage, as well as disruption of protein phosphorylation, microtubule construction, protease formation, and other enzyme functions (Hayes & Dixon 1994, Greve & Zink 2009). In addition, increased concentration of Ca^{2+} can activate calpains resulting in further cellular damage (Iwata *et al.* 2004, von Reyn *et al.* 2009, Johnson et al. 2012). Under normal conditions calpains, as calcium mediated proteases, have a variety of cellular regulatory functions. However it has been shown that activated calpains are responsible for degradation of the inactivation gate of sodium channels, leading to further increase of intra-axonal calcium influx and related pathological changes (von Reyn et al. 2009). As key enzymes, calpains play a significant role in targeting axonal proteins, which can result in cytoskeletal breakdown and disruption of axonal transport in TBI (Ai *et al.* 2007). Inhibition of calpain enzymes has been found to alleviate axonal injury following TBI (Reeves *et al.* 2007). The increased calcium in mitochondria can also lead to mitochondrial depolarization, swelling and finally loss of function (Verweij *et al.* 2000). This in turn can cause the initiation of cell death either directly via apoptosis or indirectly through loss of oxidative phosphorylation and failure to produce ATP (Kim *et al.* 2003). All these ionic changes play a critical role in progression of secondary injury in both grey and white matter, particularly in the later development of cytotoxic oedema, programmed cell death and axonal disconnection (Park et al. 2008).

Excitotoxicity and oxidative stress

Release of excitatory amino acids such as glutamate, aspartate, and glycine, oxygen free radical reactions and nitric oxide production are closely interrelated events in the cell response to injury (Greve & Zink 2009). Release of excitatory amino acids facilitates Ca^{2+} influx through the n-methyl-D-aspartate (NMDA) channel, which subsequently promotes oxygen free radical production. It can also lead to increased production of nitric oxide (NO) that can participate in oxygen radical reactions as well as lipid peroxidation in adjacent cells. Notably, each can enhance the release and activity of the others, resulting in cell damage or death if endogenous protective mechanisms, such as free radical scavengers, are exhausted. Excessive glutamate can also cause a depolarization of traumatised cells, although it is still unclear whether increased glutamate concentrations are a result or cause of the initial depolarization (Greve & Zink 2009). Nonetheless, research has shown that glutamate plays a significant role in brain cell dysfunction and death following TBI and alterations in both presynaptic and postsynaptic receptors are essential to this excitotoxicity (van Landeghem *et al.* 2006, Greve & Zink 2009).

There are two main groups of glutamate receptors; amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (Galvin *et al.*), and NMDA receptors. Following TBI there is an increase in glutamate receptors neuronal hyperexcitability and calcium influx (Goforth *et al.* 1999). Glutamate NMDA receptor activation can also lead to increased production of nitric oxide, which can result in the production of highly reactive peroxynitrite by combining with mitochondrial superoxide anions. Peroxynitrite, being much more reactive than nitric oxide, can activate DNA breakup, lipid peroxidation, and amino acids disruption (Arundine *et al.* 2004, Arundine & Tymianski 2004). Reactive oxygen in peroxynitrite molecules can lead to membrane

lipid peroxidation by removing hydrogen atoms from unsaturated fatty acids. This can lead to cell membrane disruption, which in turn can lead to cell lysis and death. Research has shown that lipid peroxidation occurs within the first few hours of TBI and blocking NMDA receptor mediated peroxynitrite production has the potential to improve outcome of TBI patients (Aarts *et al.* 2002).

Oxygen radicals can also be produced by other mechanisms, and their prevalence can have destructive effects on neural or glial cells. Increased calcium influx and depolarization leads to an increase in arachidonic acid and free fatty acids. Metabolism of arachidonic acid in the cyclooxygenase pathway produces lipid hydroperoxides as well as oxygen radicals. Hydrogen peroxide can be produced by auto oxidation or degradation of catecholamines during secondary injury, which can also play a part in oxygen radical reactions (Greve & Zink 2009). In those cases when there is post TBI hemorrhage, available iron can also be a part in catalyzing free radical formation. The excessive production of reactive oxygen species and exhaustion of cellular antioxidant mechanisms can result in peroxidation of cellular and vascular structures, protein oxidation, breakup of DNA, inhibition mitochondrial electron transport chain and depletion of cellular energy resources. All these mechanisms of oxidative stress can eventually lead to apoptotic cell death (Chong *et al.* 2005, Werner & Engelhard 2007).

1.4 Traumatic Brain Oedema

Normal physiological equilibrium of extracellular and intracellular fluid and ions in the brain is maintained by regulated transport of water and solutes across the blood brain barrier (BBB), membranes of neuroglial cells and epithelia of the choroid plexus (Kahle *et al.* 2009). Changes in

the equilibrium of ions and water in the cerebral extracellular and intracellular spaces can lead to brain oedema, which is a pathological accumulation of water in cerebral extracellular (vasogenic oedema) and intracellular (cytotoxic oedema) spaces (Pappius 1965, Klatzo 1967). Since the contents of the brain are confined by a non-expandable calvarium, oedema can lead to increases in ICP, decreases in cerebral blood flow, decreases in brain tissue oxygenation, brain tissue herniation and finally result in increased mortality and morbidity after TBI (Unterberg et al. 2004, Simard *et al.* 2010). Brain oedema has been described as the major cause of death and disability following severe traumatic brain injury (Feickert et al. 1999, Marmarou 2003).

1.4.1 Classification of Oedema

There are four discrete fluid compartments in the brain, each with their own distinctive solute composition and volume, namely the intracellular fluid, the extracellular or interstitial fluid around cells, the cerebrospinal fluid (CSF) in the ventricular system and subarachnoid space, and the blood of the cerebral vasculature. These compartments are separated from each other by specialized cellular barriers which control the movement of ions and water from one compartment to the next and maintain the normal composition and volume of each compartment as well as the physiological normal equilibrium between them. Even minor changes in the ionic and water composition of these compartments can lead to significant disruption of the function of neuronal and glial cells (Kahle et al. 2009, Simard et al. 2010). Depending on the mechanisms and the compartment, oedema principally has been divided into vasogenic and cytotoxic types (Klatzo 1967). Other types of oedema have been described, namely osmotic or interstitial oedema, which refers mainly to etiology and the principal transported solute rather than location;

these are not critical during the early acute phase of TBI (Fishman 1975, Unterberg et al. 2004, Simard et al. 2010).

1.4.2 Vasogenic Oedema

Vasogenic oedema is defined as fluid accumulation in brain parenchyma with the fluid derived from cerebral blood vessels as a result of BBB disruption and subsequent opening of tight junctions in microvascular endothelia (Marmarou 2004). Vasogenic oedema fluid is extracellular and accumulates primarily in white matter, since the resistance to fluid flow is less in white than in grey matter. One of the characteristic features of the vasogenic oedema is being rich in proteins derived from plasma such as albumin, IgG, and dextran (Betz et al. 1989, Unterberg et al. 2004, Simard *et al.* 2007). Some of the mechanisms thought to contribute to vasogenic oedema are disruption of calcium signaling, uncoupling of tight junctions, and enzymatic degradation of basement membrane (Simard et al. 2007). Vasogenic oedema was considered as the predominant form of oedema after TBI due to BBB opening, especially in and around contusions, however later research has shown that cytotoxic oedema also plays an important role, especially in the later stages of oedema development (Unterberg et al. 2004).

1.4.3 Cytotoxic Oedema

Cytotoxic brain oedema is defined as sustained intracellular water accumulation in neuronal and glial cells primarily as a result of disrupted osmotic gradients across the plasma membrane, occurring independently of BBB breakdown (Unterberg et al. 2004, Bloch & Manley 2007). In contrast to vasogenic oedema, it occurs mainly in the grey matter and is commonly associated with cerebral ischaemia (Kimelberg *et al.* 1995). Under normal physiological conditions

neuroglial cells produce enough ATP to maintain the ionic gradient across the plasma membrane via the Na^+/K^+-ATPase (Unterberg et al. 2004). Brain injury often leads to energy depletion and subsequent Na^+/K^+-ATPase pump failure due to mitochondrial structural and functional impairment. This in turn results in increased efflux of K^+ and increased influx of Na^+ with Ca^{2+}, and Cl^-, which cannot be compensated for by the active ion-pump; this leads to the development of cytotoxic brain oedema (Unterberg et al. 2004, Liang *et al.* 2007). Since astrocytes outnumber neuronal cells about twenty times and can swell up to five times of their normal size, glial swelling is thought to be the main contributor to cytotoxic oedema following TBI (Kahle et al. 2009).

Cytotoxic oedema also contributes to the further development of transcapillary vasogenic oedema and further swelling of brain parenchyma (Simard et al. 2007). Compared to the intracellular space, the extracellular space in the brain is relatively small and comprises between 12 to 19% of brain volume (Go 1997). Cytotoxic oedema results in depletion of extracellular ions and water leading to a new ionic and water gradient across the BBB between the intravascular and extracellular spaces which acts as additional driving force for transcapillary efflux of water and osmotic active solutes (Liang et al. 2007, Simard et al. 2007). In addition, mechanical deformation following TBI can result in release of excitatory amino acids, which can activate ligand-gated ion channels allowing ions to move down the electrochemical gradients and so contribute to further development of cytotoxic oedema (Marmarou 2004, Marmarou 2007).

1.4.4 Osmotic or Interstitial Oedema

Osmotic or interstitial oedema was first proposed by Fishman in 1975 on the basis of his observations in patients with hydrocephalus (Fishman 1975). In the development of osmotic oedema, Na^+ transport across the BBB creates an electrical gradient for Cl^- and an osmotic gradient for water, thus refilling osmotic solutes and water in the extracellular space that previously was exhausted by developing cytotoxic oedema (Simard et al. 2007). As a result, the amount of accumulated Na^+ exceeds the amount of expelled K^+ resulting in net influx of Na^+ into oedematous brain (Young *et al.* 1987). Osmotic oedema is different from vasogenic oedema because abnormal Na^+ transport is not accompanied by protein accumulation, which occurs during vasogenic oedema (Todd *et al.* 1986a, Todd *et al.* 1986b). The speed of osmotic oedema development is important since only slowly developed osmolar gradients can be compensated in the brain. Osmotic oedema can also be observed in patients with abnormally high secretion of anti-diuretic hormone, which results in decreased serum osmolarity leading to osmotic brain oedema (Unterberg et al. 2004). Cerebral ischemia can create an increased osmolarity of the brain tissue that after cerebral reperfusion with isotonic fluids can also lead to the development of osmotic brain oedema (Katayama & Kawamata 2003).

1.5 Intracranial Pressure and Cerebral Oxygenation

Intracranial pressure (ICP) is the total pressure inside the skull created by the brain tissue, cerebral blood volume and CSF combined. In the average adult, the total volume of the skull is about 1475 mL of which brain is about 1300 ml, cerebral blood is 110 mL and CSF is 65 mL (Doczi 1993), representing 78-82%, 10-12%, and 8-10% of the total volume inside the skull, respectively (Sankhyan *et al.* 2010). The Monroe-Kellie hypothesis states that the sum of the

intracranial volumes of brain, cerebral blood and CSF is constant and that under normal physiological conditions, any increase in the volume of any one component is compensated by a corresponding decrease in another; this maintains normal ICP. However, within the rigid skull, the compensatory capacity of the cerebrum is quite small, on the order of 100 mL in regards to volume changes. Therefore, any increase in the volume of any of the components beyond the compensatory capacity of the brain (100 mL) will lead to an increase in ICP, which may or may not distribute relatively evenly throughout the intracranial cavity (Miller & Sullivan 1979, Rangel-Castilla *et al.* 2008).

1.5.1 Intracranial Pressure

The brain and spinal cord with its circulating blood and CSF are contained in a skull and vertebral canal that have almost no compliance in adults. The only small amount of capacity is presented by the inter-vertebral spaces. A non-linear sinusoid relationship between intracranial volume and ICP therefore exists as described by Lofgren (Lofgren & Zwetnow 1973).

Normal intracranial pressure values and components

The normal values of ICP vary with age and body posture. For healthy adults, normal values of ICP in the vertical position are negative and vary between -10 to -15 mm Hg, while in the supine position normal ICP values are less than 10 to 15 mm Hg (Albeck *et al.* 1998, Czosnyka & Pickard 2004). In younger children, ICP values vary between 3 to 7 mm Hg, and in term infants ICP values of 1.5-6 mm Hg are considered normal (Welch 1980, Dunn 2002, Sankhyan et al. 2010). In newborns ICP can be sub-atmospheric (Welch 1980). ICP values greater than 20 to 25 mm Hg are considered as intracranial hypertension and in most circumstances must be treated,

although in the presence of temporal mass lesions, herniation can occur even with ICP values less than 20 mm Hg (Andrews *et al.* 1988). Continued ICP values of 40 mm Hg or above are considered as critical and life threatening hypertension (Rangel-Castilla et al. 2008).

Given that the brain tissue volume is normally constant, ICP under normal physiologic conditions has two main contributors, that is CSF and cerebral vasculature (Czosnyka 2000). CSF underpins the baseline ICP and in pathologic states it can contribute to an increase in ICP due to obstruction of CSF outflow, or resistance to CSF flow between different cerebral compartments because of brain oedema or mass lesions (Smith 2008). Any change in the vascular component of ICP is associated with slight fluctuations of cerebral blood volume and is increased by cerebral hyperaemia, hypercapnia or increases in cerebral metabolism (Smith 2008). Generally cerebral blood volume is maintained within the normal range by cerebral autoregulation. Increased ICP results in increased pressure on the cerebral venous system, which leads to decreases in CBF and cerebral perfusion pressure (CPP) (Mollanji *et al.* 2002). This leads to an activation of autoregulatory mechanisms and consequent vasodilation, resulting in an increase in CBF (Mollanji et al. 2002, Schaller & Graf 2005), and potentially an increased ICP.

Increased intracranial pressure and its clinical role

Traumatic brain injury often leads to an increase in the volume of one or more intracranial contents, namely increases in intracerebral blood volume due to vascular engorgement or mass lesions, increases in brain tissue volume due to water accumulation (Biersteker *et al.*), or decreases in CSF outflow due to increased resistance. Laboratory research and clinical studies have shown that among these mechanisms brain oedema plays the major role in increases of ICP

after TBI (Marmarou *et al.* 2000, Smith 2008). Although both vasogenic and cytotoxic oedema occur following TBI, during the first hours of brain injury, vasogenic oedema dominates due to disruption of BBB (Marmarou 2003, Unterberg et al. 2004).

ICP values above 15 mm Hg are considered abnormal, although the definition of increased ICP may depend on the type of pathology and the age of the patient (Smith 2008). Accordingly, the treatment of increased ICP can be initiated at different levels depending on the pathology, although after TBI an ICP above 20 mm Hg is generally required before treatment is considered (Bratton *et al.* 2007e). In children up to 8 years of age, treatment is recommended when ICP is above 18 mm Hg, and in infants treatment is recommended when ICP is above just 15 mm Hg (Mazzola & Adelson 2002). Often, ICP distribution inside the skull is not even due to intraparenchymal pressure gradients between different brain compartments, which are present because of the falx, tentorium, mass lesions, or restricted by low CSF volume because of brain oedema (Rosenwasser *et al.* 1989, Mindermann & Gratzl 1998, Smith 2008). Increases in ICP lead to critical reductions of cerebral blood flow and CPP potentially resulting in ischemic cerebral injury. Increased ICP may also cause structural damage to the brain tissue, or herniation through either the foramen magnum or tentorial hiatus, by causing an actual shift of brain parenchyma (Smith 2008). Brain herniation can lead to additional pressure on the brain stem and cause hypertension, bradycardia, respiratory depression or death, if not treated. Unsurprisingly, a number of studies have shown that high ICP is strongly related with unfavorable outcome, especially when the period of increased ICP is extended (Saul & Ducker 1982, Balestreri *et al.* 2006).

ICP monitoring

According to current guidelines, ICP should be monitored in all patients with a severe TBI (Glasgow coma scale score of 3-8 after resuscitation) and abnormal computed tomography (Salazar *et al.*) scan, especially where complications such as mass lesions, contusions, swelling or herniation are present (Bratton *et al.* 2007d). ICP monitoring is also recommended to those patients with severe TBI but with a normal CT scan who are above 40 years old, have either unilateral or bilateral posturing, and/or systolic blood pressure below 90 mm Hg (Bratton et al. 2007d). Studies have shown that in patients with severe TBI, significant reductions in mortality and morbidity can be achieved by using intensive management protocols, which include ICP monitoring (Bratton et al. 2007d, Smith 2008). The ICP monitoring for surgical patients is commenced at the end of the surgery and is continued for as long as it stays high and requires treatment. Generally the average time period for ICP monitoring is 3 to 5 days, while in about 30% of patients secondary increase in ICP may be observed 3 to 10 days after TBI as a result of delayed intracerebral haematoma, cerebral vasospasm, hypotension and hypoxia (Unterberg *et al.* 1993, Rangel-Castilla et al. 2008).

There are two main methods for ICP monitoring in clinical practice, namely intraventricular catheter-tip microtransducer systems and intraparenchymal catheters. Compared to intraparenchymal catheters, ventricular catheters measure total ICP and allow periodic external calibration, therapeutic drainage of CSF, and administration of drugs (Zhong *et al.* 2003, Steiner & Andrews 2006). However, placement of the ventricular catheter may be somewhat difficult in those patients with ventricular displacement or effacement due to brain oedema or mass lesions (Smith 2008). Ventricular catheter usage is also associated with infection in up to 11% of cases,

resulting in increased morbidity or mortality (Lozier *et al.* 2002, Lo *et al.* 2007), and they may become blocked, particularly when there is a mass lesion and increased CSF protein (Birch *et al.* 2006, Smith 2008).

In contrast, intraparenchymal catheters can be sited either in the brain parenchyma or in the subdural space through a skull bolt and are as accurate as intraventricular catheters. The Camino ICP monitor uses a fiberoptic cable with a strain gauge to measure the pressure while the Codman microsensor uses two semiconductor strain gauges mounted on a thin titanium diaphragm at the tip of the catheter (Martinez-Manas *et al.* 2000, Koskinen & Olivecrona 2005). Although intraparenchymal catheters are pre-calibrated before their insertion (zeroed relative to atmospheric pressure), they cannot be calibrated *in vivo* and their output is subject to the zero drift of the sensor (Czosnyka *et al.* 1996, Stendel *et al.* 2003). Nonetheless they are widely considered very reliable and have the added advantage that they are easy to use and have minimal infectious or other complications (Martinez-Manas et al. 2000, Smith 2008).

1.5.2 Cerebral Autoregulation and Cerebral Perfusion Pressure

The brain is very sensitive to any changes in cerebral blood flow or perfusion due to its high metabolic demands. However, the autoregulatory mechanisms are well developed and maintain constant and adequate cerebral blood flow relatively independent of the mean arterial blood pressure (MABP) and CPP. Such autoregulation is achieved by the small arteries and pre-capillary arterioles, which can change their diameter according to a complex of physiological mechanisms. Under normal physiologic conditions and MABP between 60 to 150 mm Hg autoregulatory mechanisms maintain cerebral perfusion pressure within the range of 50 to 150

mm Hg in adults, and between 60 to 150 mm Hg in children (Steiner & Andrews 2006, Czosnyka & Pickard 2004, Duschek & Schandry 2007). CPP is calculated as the difference between the mean arterial pressure and intracranial pressure:

$$CPP = MABP - ICP$$

CPP can be increased by an increase in MABP, decrease in ICP or both, and vice versa. With normal pressure autoregulation, any decrease in CPP results in vasodilation of cerebral vasculature, maintaining consistent and unaffected CBF. This in turn can result in an increase in ICP, which is called the vasodilatory cascade. Similarly, any increase in CPP results in vasoconstriction in cerebral vasculature and a decrease in ICP. When CPP values are below 50 mm Hg or above 150 mm Hg, autoregulation is no longer able to maintain normal blood supply to the brain and cerebral blood flow may be compromised. Following TBI and depending on its severity, brain autoregulation can be impaired or in some cases even lost. CBF could then become more dependent on CPP and would more passively follow the changes of CPP (Rangel-Castilla et al. 2008). Impaired autoregulation after TBI may also lead to a more linear relationship between ICP and CPP (Rangel-Castilla et al. 2008). To date, most management guidelines recommend maintaining cerebral perfusion pressure above 60 mm Hg as one of the primary goals (Rosner & Daughton 1990, Bratton *et al.* 2007c). On the basis of analysis of randomized trials, the CPP target threshold should be set about 10 mm Hg above the critical threshold simply to avoid falls below them, with adjustments made based on monitoring of cerebral oxygenation and assessment of cerebral autoregulation (Clifton *et al.* 2002, Bratton et al. 2007c).

1.5.3 Cerebral Oxygenation

Brain tissue is heavily reliant on a constant supply of oxygen and glucose to maintain its normal aerobic metabolism and cellular integrity (Siesjo & Siesjo 1996, Bardt *et al.* 1998, Spiotta *et al.* 2010). While making up only 2% of total body weight, it consumes nearly 20% of the oxygen consumed by the whole body (Rolett *et al.* 2000, Stevens 2004). Over 90% of this oxygen is being used for mitochondrial aerobic metabolism. Following severe TBI the metabolic load on brain tissue is increased, while the oxygen delivery is impaired due to decreased cerebral blood flow, resulting in reduced aerobic metabolism and cellular hypoxia (Jaggi *et al.* 1990, Bergsneider *et al.* 1997, Martin *et al.* 1997, van den Brink *et al.* 2000, Glenn *et al.* 2003, Enriquez & Bullock 2004, Wu et al. 2004). Post-mortem histopathology studies have confirmed that up to 90% of patients who had a lethal outcome after TBI had ischemic or hypoxic brain injury (Spiotta et al. 2010). Furthermore, clinical studies have shown that in survivors of TBI there is a significant correlation between a patients' poor outcome and incidence, duration and level of cerebral hypoxia (van Santbrink *et al.* 1996, Kiening *et al.* 1997, Bardt et al. 1998, van den Brink *et al.* 1998, van den Brink et al. 2000, van Santbrink *et al.* 2003). Furthermore, in more than one third of patients, cerebral blood supply is compromised in the early stage, which may contribute to secondary damage to neuroglial cells (Bouma *et al.* 1992, Bouma & Muizelaar 1995). Outcome is significantly improved with management targeted at improving cerebral hypoxia (Palzur *et al.* 2004, Tolias *et al.* 2004).

Brain tissue oxygen partial pressure ($P_{bt}O_2$) is the partial pressure of oxygen in the extra-cellular fluid and indicates the amount of available oxygen for cellular oxidative processes (Nortje & Gupta 2006). There is a strong correlation between regional CBF and $P_{bt}O_2$, suggesting that

cerebral oxygenation reflects regional CBF and substrate delivery rather than oxygen use (Doppenberg *et al.* 1998). Following TBI, increases in ICP reduce blood flow to the brain tissue and so compromises the cerebral oxygenation; measuring $P_{bt}O_2$ may therefore help prevent hypoxic events and so improve patient outcome (Littlejohns *et al.* 2003). Indeed, a number of studies have shown that direct monitoring of $P_{bt}O_2$ can be an effective supplement to ICP monitoring in the management of patients with TBI, with therapies targeted at maintaining adequate oxygen tension combined with ICP/CPP monitoring have significantly better outcome compared to only ICP/CPP based therapies (van Santbrink et al. 1996, van den Brink et al. 1998, van den Brink et al. 2000, van Santbrink et al. 2003, Stiefel *et al.* 2005, Nortje & Gupta 2006, Stiefel *et al.* 2006a, Bratton *et al.* 2007f).

Normal and pathological values of brain tissue oxygenation ($P_{bt}O_2$)

To date, there is still some uncertainty with regards to $P_{bt}O_2$ thresholds due to such factors as usage of different probes (Licox, Neurotrend and Paratrend), different locations of placed probes, and not fully clarified critical values of hypoxia resulting in irreversible damage to the brain. Nonetheless, based on clinical and experimental studies using Licox technology, $P_{bt}O_2$ values are considered normal at or above 25-30 mm Hg, generally ranging between 35 to 45 mm Hg (Dings *et al.* 1996, Kiening *et al.* 1996, Sarrafzadeh *et al.* 1998, Stevens 2004, Lang *et al.* 2007). $P_{bt}O_2$ values within the range of 15 to 25 mm Hg are considered as moderate hypoxia, and $P_{bt}O_2$ values below 10-15 mm Hg are critical and considered as severe and life-threatening hypoxia (van den Brink et al. 2000, Menon *et al.* 2004, Nortje & Gupta 2006, Stiefel et al. 2006a, Longhi *et al.* 2007, Barazangi & Hemphill 2008). Indeed, $P_{bt}O_2$ values below 15 mm Hg have been shown to be associated with a 50% risk of death within 4 hours following TBI (van den Brink et al. 2000,

Stevens 2004). $P_{bt}O_2$ values below 5-6 mm Hg generally result in death within the first few hours after TBI (Valadka *et al.* 1998, Stevens 2004). Specifically, $P_{bt}O_2$ values below 10 mm Hg is associated with a 50% likelihood of death in less than two hours after TBI while $P_{bt}O_2$ values below 5-6 mm Hg for only 30 minutes poses a 50% overall risk of death (Bardt et al. 1998, Valadka et al. 1998, van den Brink et al. 2000). In contrast, $P_{bt}O_2$ values maintained above 35 mm Hg are associated with a good recovery (Stevens 2004). A significant reduction in mortality rate and improvement in functional outcome has been noted in patients who have been treated with a $P_{bt}O_2$-guided protocol ($PbtO_2$>25 mm Hg) as opposed to those patients who have been treated with a traditional ICP/CPP-guided protocol (Stiefel et al. 2005, Nortje & Gupta 2006, Meixensberger *et al.* 2003, Meixensberger *et al.* 2004). Furthermore, Meixensberger and colleagues (Meixensberger *et al.* 2001, Meixensberger et al. 2004) have specifically shown that low $P_{bt}O_2$ in the acute post-injury stage is correlated with poor neurological outcome two to three years after TBI. Thus, the monitoring of $P_{bt}O_2$ has high prognostic value and its simultaneous use with ICP monitoring is associated with a significant reduction in mortality and morbidity following severe TBI (Stiefel et al. 2005, Lang et al. 2007).

1.5.4 Correlation between ICP, MABP, CPP, and P_{bt}O2 and Their Thresholds

Severe TBI often results in secondary brain hypoxia and tissue ischemia, which can be the result of such factors as systemic hypotension, decreased CPP, arterial hypoxia, brain tissue oedema, and increased ICP (Menzel *et al.* 1999, Marmarou *et al.* 2005). Detection and treatment of increased ICP, decreased CPP, systemic hypotension and cerebral hypoxia is therefore the cornerstone of current management protocols in severe TBI. Current guidelines suggest that ICP greater than 20 mm Hg should be treated, and CPP values maintained between 50 and 70 mm

Hg. However, monitoring ICP gives only a limited amount of insight and does not record such other important parameters as cerebral blood flow and oxygenation. Delivery of oxygen and metabolic substrates, in particular glucose, does depend on CBF, and monitoring of CPP is therefore of extreme importance. However, maintaining CPP within a general recommended range does not guarantee that an individual brain will in fact get enough blood supply to maintain its metabolism. With improved technology, monitoring of $P_{bt}O_2$ will therefore become more commonplace.

Studies examining the correlation between $P_{bt}O_2$ and ICP or CPP are being undertaken, although the outcomes have been somewhat contradictory, in part due to the fact that the studies to date have analyzed the relationship between $P_{bt}O_2$ and each of the other parameters separately. Understanding the simultaneous, dynamic interrelationships between MABP, ICP, CPP, and $P_{bt}O_2$ will be essential for maintaining the optimal cerebral oxygenation in patients with moderate to severe TBI. Accordingly, the current study will utilize the simultaneous measurement of $P_{bt}O_2$, ICP, and MABP, along with a sophisticated Gaussian-based iterative program to characterize the dynamic interrelationships between these parameters and CPP, so as to elucidate the critical interrelationships between ICP and CPP in the management of $P_{bt}O_2$. And finally we will investigate clinically observed two different critical thresholds of ICP and reconsider the formula of CPP.

1.5.5 Management of Increased ICP after TBI

The first measurements of ICP were conducted in 1951 using an electromagnetic transducer in patients with intracranial lesions (Guillaume & Janny 1951), and subsequently Nils Lundberg

and his colleagues laid the foundation for current ICP monitoring (Lundberg 1960, Lundberg *et al.* 1965). Numerous studies have since established the association between increased ICP and poor outcome in both adults and children following TBI (Chambers *et al.* 2006, Catala-Temprano *et al.* 2007, Padayachy *et al.* 2010). Several mechanisms can lead to increased ICP after TBI, such as intracranial bleeding, vasogenic and cytotoxic oedema of brain parenchyma, vascular congestion due to impaired autoregulation and uncoupling between cerebral metabolism and blood flow (Barzo *et al.* 1997b, Marmarou et al. 2000). In pediatric TBI there is a continuing debate whether hyperemia contributes to brain oedema, and thus increased ICP or not (Zwienenberg & Muizelaar 1999).

ICP management strategies/protocols

The main goals of ICP treatment is to maintain ICP at less than 20-25 mm Hg, keeping CPP above 60 mm Hg by controlling blood pressure, and avoiding those factors that can exacerbate increased ICP (Rangel-Castilla et al. 2008). Therapies targeted at ICP/CPP include surgical evacuation of intracranial mass lesions, optimization of venous outflow, reducing high temperature, controlled ventilation support and mild hyperventilation, administration of hyperosmotic solutions such as mannitol and hypertonic saline, cerebrospinal fluid drainage, barbiturates, and decompressive craniectomy (Winter *et al.* 2005, Adamides *et al.* 2006). Some European countries apply the Lund protocol, which targets the intracranial volume to reduce ICP and improve the cerebral perfusion around contusions (Naredi *et al.* 2003). By reducing MABP, and maintaining cerebral microcirculation by drugs such as clonidine, the Lund protocol accepts periods of lower CPP (<50 mm Hg). This approach does confirm the vital importance of appropriately managing increased ICP (Eker *et al.* 1998, Naredi et al. 2003, Grande 2006).

Management of mass lesions

TBI often can be associated with intracranial mass lesions, which can lead to development of increased ICP, cerebral hypoxia, brain compression or midline shift. While surgical evacuation of mass lesions is widely considered as urgent for such patients (Bullock et al. 2006b, Bullock et al. 2006a), there is a still an ongoing debate about the significance of surgical evacuation in intraparenchymal lesions as opposed to extraparenchymal lesions (Li *et al.* 2010), with a more conservative approach recommended for the intraparenchymal lesions (Compagnone *et al.* 2005). Nonetheless, some studies have shown that surgical removal of mass lesions is associated with better outcome in patients with intracerebral mass lesions (Zumkeller *et al.* 1992, Choksey *et al.* 1993, Bullock et al. 2006a). There are a number of randomized controlled trials currently underway to provide more definitive answers with respect to the role of surgical intervention of intracerebral hemorrhage (Li et al. 2010).

Optimization of cerebral venous outflow

Elevating the head by up to 30° is now a standard treatment in the management of TBI so as to minimize the resistance of venous outflow and assist with the movement of CSF from the intracranial space into the spinal compartment (Rangel-Castilla et al. 2008). While some investigators suggest that keeping the patient's head in a flat position could improve CPP, most studies have shown that head elevation to 30° can decrease ICP without reducing CPP (Rosner & Coley 1986, Feldman *et al.* 1992). Some observational studies have actually shown that elevation of the head to 30° not only reduces ICP, but may also increase CPP, although with no significant improvement in cerebral oxygenation (Ng *et al.* 2004).

Reducing high temperature

Experimental studies have shown that high temperature post-TBI can worsen the neurologic injury and outcome in experimental animals (Dietrich *et al.* 1996). Clinical observational studies confirm that patients with high temperature following TBI show a significant correlation between high temperature and poor neurologic outcome (Jones *et al.* 1994). Increases in body temperature by every C° can increase the basal metabolic rate by up to 13% and result in a significant vasodilation. This type of vasodilation in brain parenchyma can lead to an increased CBF and increased ICP. Fever generally is being controlled by antipyretics and cooling blankets, with infection being treated with appropriate antibiotics (Rangel-Castilla et al. 2008).

Ventilatory support

Following severe TBI most patients may have respiratory dysfunction and therefore require mechanical ventilation to maintain normal arterial gases, in particular oxygen and carbon dioxide. Specifically, up to 36% of comatose patients with TBI have respiratory dysfunction and hypoxia and require mechanical ventilation (Rangel-Castilla *et al.* 2010). More than a half of TBI patients with spontaneous breathing also have breathing abnormalities such as tachypnoea, irregular breathing, and periodic episodes of hypoventilation (North & Jennett 1974). Periodic breathing was not related to the anatomical location of the injury (Rangel-Castilla et al. 2008). Hypoxia, hypercapnia and periodic episodes of hypoventilation after severe TBI can lead to significant increases in ICP, and controlled ventilation can reduce it by maintaining arterial blood gases within normal limits, in particular carbon dioxide (Miller & Becker 1982). However, mechanical ventilation may increase ICP when positive end-expiratory pressure (PEEP) is used to improve oxygenation. PEEP increases cerebral venous pressure by obstructing the venous

return, increasing cerebral blood volume and so increasing ICP. In patients who had low lung compliance due to lung injury, there were minimal impacts of PEEP on ICP (Caricato *et al.* 2005).

Hyperventilation

The main mechanism involved in reduction of cerebral blood volume is the reactivity of cerebral vasculature to carbon dioxide, which is generally preserved after TBI (Peterson & Chesnut 2009). Hyperventilation, defined as controlled hypocapnia with $PaCO_2$ levels below 35 mm Hg (Adamides et al. 2006), is used to induce vasoconstriction of cerebral arterioles by decreasing $PaCO_2$ and so decreasing the cerebral blood volume and ICP (Adelson *et al.* 2003a, Stocchetti *et al.* 2005). While overall cerebral blood volume can be decreased by acute hyperventilation, it also may result in cerebral ischemia (Diringer *et al.* 2002, Singhi & Tiwari 2009). The effects of hyperventilation on ICP are limited in time given that the vasoconstrictive effect of hyperventilation lasts only up to 20 hours due to CSF pH equilibration to the new level of $PaCO_2$ (Singhi & Tiwari 2009); it therefore has a limited use in the management of ICP. Furthermore, after CSF pH equilibrates to a new level of $PaCO_2$, cerebral arterioles may dilate again which may lead to rebound phase of increased ICP (Stocchetti et al. 2005). Nevertheless, acute hyperventilation of a short duration can be life saving in emergency management of severe TBI in patients with signs of brain herniation or acute severe increases in ICP (Adamides et al. 2006).

Osmotherapy with Mannitol and Hypertonic Saline

Hyperosmolar therapy is currently one of the primary interventions in management of increased ICP following TBI. Its history dates back to 1919 when Weed and McKibben showed that

intravenous administration of 30% saline could decrease the size of brain tissue (see (Adamides et al. 2006). Later, hypertonic solutions of urea were used to reduce increased ICP, but this treatment sometimes resulted in rebound increases in ICP (Javid & Settlage 1956, Adamides et al. 2006). The use of urea was accordingly abandoned and mannitol subsequently recommended as a replacement. In the 1980s, hypertonic saline (HTS) was recommended as a second hyperosmolar agent to treat increased ICP (Gunnar *et al.* 1988, Worthley *et al.* 1988). To date there is not enough data to evaluate their comparative effectiveness in reducing ICP (Rangel-Castilla et al. 2008). Due to its rapid effects on increased ICP, hyperosmolar therapy is especially indicated for patients with acute rises in ICP. Mannitol and hypertonic saline (HTS) remain the two main hyperosmotic agents in clinical use for the treatment of intracranial hypertension.

Mannitol

Mannitol is an osmotic diuretic that is generally administered as an intravenous bolus of 20% hypertonic solution, drawing excess water out of brain tissue and so decreasing increased ICP (Helmy *et al.* 2007, Meyer *et al.* 2010). Effects of intravenous administration of mannitol on increased ICP can be seen in as soon as 2 to 5 minutes after administration, and the peak effect is reached between 20 to 60 minutes after administration (Rangel-Castilla et al. 2008). Generally, effects of mannitol on ICP can last from 1.5 up to 6 hours depending on clinical conditions and dosages of the treatment (Knapp 2005). The usual dosage for mannitol administration varies between 0.25 g/kg to 1 g/kg depending on such factors as the level of increased ICP and the urgency of its reduction, and usually is not recommended with arterial hypertension. Some studies have shown that higher doses of mannitol may result in better outcome in patients with severe TBI, especially those with cerebral herniation or subdural hematoma (Cruz *et al.* 2001,

Cruz *et al.* 2004). Mannitol administration can be repeated every 2 to 6 hours to sustain its effects on increased ICP. In those cases, the usual dosage is between 0.25 to 0.5 g/kg (Rangel-Castilla et al. 2008).

Although the exact mechanisms of mannitol effects are not completely understood, rheologic and osmotic effects are the two known mechanisms by which mannitol is thought to exerts its effects on increased ICP (Muizelaar *et al.* 1984). The rheologic effects of mannitol largely depend on the state of cerebral autoregulation (Muizelaar et al. 1984). In patients with relatively intact autoregulation mannitol administration leads to cerebral vasoconstriction, which maintains CBF constant, as well as decreasing ICP (Rangel-Castilla et al. 2008). Because of these rheologic effects mannitol may reduce ICP within a few minutes after its administration and have more effects in patients with low CPP (McGraw & Howard 1983, Muizelaar et al. 1984, Rosner & Coley 1987). The osmotic effects develop more slowly (over 15-30 min) due to the time-period needed for establishment of an osmotic gradient between the plasma and the intracellular fluid (McGraw & Howard 1983, Takagi *et al.* 1984, Schwarz *et al.* 1998). By increasing serum osmolarity, it draws edema fluid from brain tissue into the vascular compartment, and this effect can last for up to 6 hours (Marshall *et al.* 1978, Brown *et al.* 1979). The optimal serum osmolarity is between 300 to 320 mOsm and keeping it at less than 320 mOsm will avoid many of the side-effects of mannitol therapy (Bullock 1995, Rangel-Castilla et al. 2008).

Side-effects of mannitol administration include plasma expansion and the consequent decrease of hematocrit, which may increase the deformability of erythrocytes and therefore reduce blood viscosity, increase CBF and so cerebral oxygenation (Muizelaar *et al.* 1983, Rosner & Coley

1987, Mendelow *et al.* 1985). Mannitol may also open the blood-brain barrier, crossing the BBB and entering the brain parenchyma where it can draw water into the brain tissue and aggravate cerebral vasogenic edema (Rangel-Castilla et al. 2008) and exacerbate increases in ICP (Wakai *et al.* 2005). Other complications of mannitol administration include acute renal failure, hyperkalemia due to its diuretic effects, and hypotension (Wakai et al. 2005). Accordingly, mannitol treatment should be supplemented with fluid replacement to avoid the intravascular volume depletion caused by the diuretic effects of mannitol. Most side-effects of mannitol are related to its higher doses and extended period of treatment, therefore its administration should be stopped in time to avoid the side-effects (Bullock 1995, Helmy et al. 2007).

Hypertonic saline

Administration of HTS reduces increased ICP at earlier stage mainly by instant haemodynamic effects and later primarily by an osmotic effect (Adamides et al. 2006, Bratton *et al.* 2007b). The dosage of administered HTS varies between 3% and 23.4%, and creates an osmotic gradient across the BBB to draw water from the interstitial space of the brain tissue into the cerebral intravascular space, thus reducing intracranial volume and ICP (Rangel-Castilla et al. 2008). HTS may also dehydrate both endothelial cells in the cerebral vasculature and erythrocytes, which increases the diameter of the cerebral microvasculature and deformability of erythrocytes leading to expansion of plasma volume and improved blood flow (Shackford *et al.* 1992, Shackford *et al.* 1994, Kempski *et al.* 1996). It may thus not only decrease ICP, but also improve intravascular volume and increase systemic blood pressure. In patients with relatively intact cerebral autoregulation, compensatory vasoconstriction may subsequently lead to reduced CBV and decreased ICP. HTS treatment may also improve pulmonary gas exchange and inflammatory

response (Rabinovici *et al.* 1996, Hartl *et al.* 1997c, Hartl *et al.* 1997a). In traumatised brain, HTS reduces the adhesion of leukocytes (Hartl et al. 1997c).

Hypertonic saline is preferred in those patients who are hypovolemic and have systemic hypotension (Rangel-Castilla et al. 2008) since mannitol is contraindicated in these patients because of its diuretic effects. Also compared to mannitol, HTS is less likely to cause rebound cerebral oedema, since it's less likely to cross the BBB (Bhardwaj & Ulatowski 2004). Potential side effects of HTS treatment may include such hematologic and electrolyte abnormalities as decreased platelet aggregation with prolonged coagulation time, which may lead to secondary bleeding, hypokalemia, and acidosis due to hyperchloremia (Doyle *et al.* 2001). Before HTS administration, hyponatremia must be excluded to avoid the potential risk for central pontine myelinolysis (Bratton et al. 2007b).

Barbiturate coma

Barbiturate coma may be effective in reducing increased ICP after TBI mainly by decreasing brain tissue metabolism, reducing CBF, and inhibition of free radical mediated lipid peroxidation (Demopoulos *et al.* 1980, Kassell *et al.* 1980). However, the use of high-dose barbiturate therapy to control increased ICP refractory to other treatments is still arguable (Helmy et al. 2007). One of the main disadvantages of barbiturate treatment is that barbiturates can result in significant hypotension, which can be very detrimental to cerebral blood supply. The other problem is that the long half-life of barbiturates makes clinical evaluation complicated after discontinuation of treatment (Bader *et al.* 2005). Other possible complications of barbiturate therapy include hypokalemia, sudden cardiovascular collapse, respiratory depression, hepatic and renal

dysfunction (Cairns *et al.* 2002). Hypotension caused by barbiturates is treated primarily with volume replacement and if necessary with dopamine (Singhi & Tiwari 2009). Though barbiturate treatment can control increased ICP, there is not enough data to support a significant improvement in outcome in patients with severe TBI (Bader et al. 2005). Also there is a persistent concern that reducing mortality by barbiturate treatment may end up increasing poor outcomes such as persistent vegetative state or severe disability (Helmy et al. 2007). Finally, there is not enough data to recommend the use of barbiturates for prevention of the development of intracranial hypertension in pediatric TBI (Adelson *et al.* 2003b). Because of all the above-mentioned concerns, barbiturate treatment is currently limited to the most extreme clinical cases (Adelson et al. 2003b).

Cerebrospinal fluid drainage

CSF drainage can lower ICP directly by decreasing intracranial volume and eventually by creating an available space for oedema fluid drainage. When intracranial compliance is already reduced due to brain oedema, even small amounts of CSF drainage can significantly reduce ICP (Rangel-Castilla et al. 2008). Though CSF drainage can be helpful in reducing increased ICP, in the presence of diffuse brain oedema and related ventricular collapse, CSF drainage has much less usefulness. In this respect, neurosurgical evaluation of ventricular volume is important before CSF drainage. Generally 10 to 15 mL per hour is the primary rate for CSF drainage (Li et al. 2010). Several clinical studies have shown that CSF drainage from ventriculostomy can be an effective method for an immediate reduction of critically increased ICP (Kerr *et al.* 2001, Timofeev *et al.* 2008). CSF drainage can be done either continuously, using gravity and often set to a level at 5 to 10 cm above the center of the head, or intermittently whenever there is an ICP

jump above clinically accepted thresholds (Singhi & Tiwari 2009). In those cases when an increase in ICP is not related to an intracranial mass lesion, cerebral drainage can be a very effective first-line treatment to reduce increased ICP (Timofeev et al. 2008). Some studies in pediatric TBI have shown that continuous drainage was associated with a higher volume of drained CSF and lower ICP, compared to intermittent drainage (Shore *et al.* 2004). CSF drainage is discontinued when ICP is normalized and sustained within normal limits at least for 48 hours (Li et al. 2010).

Decompressive craniectomy

Surgical decompression was first suggested in 1901 by Kocher, and since then decompressive craniectomy (DC) has continued to be intermittently used with a number of different variations (Li et al. 2010). DC has been used to treat increased ICP in patients with TBI as well as in subarachnoid hemorrhage, cerebral infarction and spontaneous hemorrhage (Cheung *et al.* 2005, Rangel-Castilla et al. 2008). It consists of surgical removal of a fraction of the calvaria, which allows herniation of brain tissue through the opening and the relief of increased ICP. The outcome depends on the clinical and radiologic severity of brain injury as well as on the timing and type of surgery. DC can significantly reduce increased ICP in most of patients who have intractable increased ICP that is nonresponsive to conventional therapy (Polin *et al.* 1997, Aarabi *et al.* 2006). It can also be beneficial to CBF and cerebral oxygenation (Stiefel *et al.* 2004, Bor-Seng-Shu *et al.* 2006). However DC can result in complications including hydrocephalus, hemorrhagic swelling, subdural hygroma, and even paradoxical herniation when DC is followed by lumbar puncture (Engberg *et al.* 1989, Aarabi et al. 2006). Though there are limited results from randomized studies to confirm or disprove the effectiveness of DC, some reports have

shown that DC may be effective in those cases where ICP control could not be achieved by other current medical treatments (Rangel-Castilla et al. 2008). There is nonetheless controversy with respect to whether DC should be unilateral or bilateral, and how broad it should be (Singhi & Tiwari 2009). Accordingly, there is a wide variability in the indications for DC in different centers (Bullock et al. 2006a, Bullock & Povlishock 2007, Li et al. 2010).

Other experimental pharmacological treatments - progesterone and magnesium

Progesterone

A number of studies have now shown that progesterone may have neuroprotective effects in TBI (Meyer et al. 2010). Experimental studies have revealed that progesterone can not only regulate excitotoxicity, reform the BBB, and reduce brain tissue oedema, but also regulate inflammatory processes and decrease apoptosis (Vagnerova *et al.* 2008). Stein and his colleagues were first to suggest that influence of sex hormones such as progesterone and estrogen may be the reason for better recovery in female rats compared to males after TBI (Stein 2001). Later Roof and his colleagues have shown that rats treated with progesterone following TBI developed less cerebral oedema compared to control animals and also had less intracranial bleeding and decreased secondary neuronal loss (Roof *et al.* 1992). Even progesterone treatment at 24 h after TBI was beneficial (Roof *et al.* 1996). Later studies examined the effects of progesterone on cognitive function following TBI and demonstrated that rats treated with progesterone not only showed better outcome than animals in the control group, but also were very close to the levels of sham animals (Roof *et al.* 1994). Although cerebral oedema could be decreased with short-term treatment with progesterone, longer treatment regimens were needed to achieve improvements in functional outcome (Shear *et al.* 2002). Higher doses of progesterone resulted in less cerebral

oedema (Wright *et al.* 2001), with the range between 8 to 16 mg/kg being the most effective compared to either lower or higher doses (Goss *et al.* 2003). However, some studies have demonstrated that abrupt withdrawal of progesterone treatment may result in a rebound effect, suggesting that the progesterone dose should be considered as an important approach for the final withdrawal of the treatment (Cutler *et al.* 2006).

Later studies in different brain injury models have shown that progesterone not only attenuates brain oedema formation following TBI, but also decreases astrocytes infiltration to the injury site and decreases the extent of brain injury (Garcia-Estrada *et al.* 1993, Garcia-Estrada *et al.* 1999, Vink & Van Den Heuvel 2004, O'Connor *et al.* 2005). However, other studies have shown that progesterone treatment had no positive effects following TBI (Toung *et al.* 2004, Gilmer *et al.* 2008). Furthermore, some studies have questioned the therapeutic window for progesterone treatment considering that most of the animal studies were done with progesterone administration within 2 h after TBI, which is not a realistic timeframe in most cases of human TBI (Loane & Faden 2010). Nonetheless, progesterone has undergone small human trials with some trends toward improved outcomes being noted despite no indication of a statistically significant reduction in ICP (Wright *et al.* 2005, Wright *et al.* 2007, Xiao *et al.* 2008, Shahrokhi *et al.* 2010). A phase III trial of progesterone in TBI has recently commenced. Accordingly, we have included progesterone as one of the experimental drugs to be assessed for its effects on ICP in our sheep model of TBI.

Magnesium

Among intracellular cations, magnesium is the second most common after potassium and in general it is the fourth most common in the body (Fawcett *et al.* 1999). As a co-factor, magnesium plays one of the basic roles in hundreds of enzymatic reactions involving nucleic acid synthesis and energy metabolism. Magnesium plays a fundamental role in such physiological processes as calcium channel gating, binding of hormone receptors, regulation of adenylate cyclase and transmembrane ion exchange, control of vasomotor tone and muscle contraction, neurotransmitter release and neuronal activity. Magnesium has been likened to a physiological calcium antagonist in many of its actions (Iseri & French 1984, Altura 1994). In humans magnesium is mainly distributed in bone tissue (53%), the intracellular compartments of muscle (27%) and soft tissue (19%), and less than 1% of it is found in serum and red blood cells (Elin 1994, Fawcett et al. 1999). Serum magnesium is about 0.3% of total body magnesium and exists in three states; ionized (62%), protein bound (33%), and complexed to anions (5%) such as phosphate and citrate (Elin 1987). Serum magnesium estimations may not be accurate since equilibrium between tissue pools generally is reached slowly (Elin 1994).

A number of studies have now shown that magnesium may play an important role in the pathophysiology of TBI (Fawcett et al. 1999, Thurman & Guerrero 1999). Studies in experimental animals have shown that magnesium levels are decreased following TBI and that magnesium administration can improve the outcome whether it was given before or after TBI (Vink *et al.* 1987, McIntosh *et al.* 1988, Bareyre *et al.* 2000, Saatman *et al.* 2001). Furthermore, in animals with low or artificially decreased brain magnesium concentrations, outcome was the worst after TBI, whereas outcome in animals with no alteration in brain magnesium

concentration after TBI were intermediate, and finally were best in animals treated with magnesium (Vink et al. 1988, Temkin *et al.* 2007, Winn *et al.* 2007). Similar reduced levels of serum and brain total and ionized magnesium concentrations are often below normal values after clinical TBI (Memon *et al.* 1995). Magnesium administration can improve the outcome in experimental TBI when it is given as a single bolus up to 24 h after the injury (Heath & Vink 1999b, Temkin et al. 2007). While the mechanisms of action are unclear, it is known that magnesium can inhibit the release of presynaptic excitatory neurotransmitters, potentiate presynaptic adenosine, and block both voltage-gated calcium channels and NMDA channels (Fawcett et al. 1999, Temkin et al. 2007, Winn et al. 2007). Furthermore magnesium can improve cerebral blood flow by causing relaxation of vascular smooth muscles, and improve tissue survival and reduce neurological excitation following acute brain insults (Wolf *et al.* 1991, Hallak *et al.* 1992). Given that magnesium plays such a multifactorial role in TBI, with many effects having an impact on BBB integrity and potentially oedema formation, we have included magnesium as one of the experimental drugs to be assessed for its effects on ICP in our sheep model of TBI.

1.6 Substance P

Substance P (SP) was first described by Euler and Gaddum in the early part of the last century as an unidentified substance extracted from equine brain and intestine that showed a smooth muscle stimulatory effect on the jejunum and potent hypotensive action in the rat (US & Gaddum 1931). The unidentified substance was called substance P, where P supposedly referred to the powder that was obtained from the extraction (Gaddum & Schild 1934). Later studies described substance P as a sensory neurotransmitter associated with pain transmission based on its high

concentrations in the dorsal roots of the spinal cord (Harrison & Geppetti 2001). Application of electrical stimuli to the isolated spinal cord of newborn rats was shown to produce increased substance P in the perfusate (Otsuka & Konishi 1976), with subsequent studies demonstrating that SP is released not only from central but also from peripheral endings of primary afferent neurons (Hokfelt *et al.* 1975b, Otsuka & Yoshioka 1993). Since then, it has been shown that SP is not only located in the central and peripheral nervous systems, but also in endothelial cells, inflammatory cells, fibroblasts and smooth muscle cells (Nessler *et al.* 2006).

1.6.1 Structure, Synthesis and Metabolism of Substance P

Structure

Chang and colleagues identified the undecapeptide structure of SP after purification of the physiological activity from bovine hypothalamic tissue(Chang *et al.* 1971). SP shares the same carboxyl terminal sequence as other members of the tachykinin family, such as neurokinin A (NKA) and neurokinin B (NKB) (Otsuka & Yoshioka 1993, Harrison & Geppetti 2001). The amino acid sequence of these peptides is:

Substance P: **Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-MetNH$_2$**

Neurokinin A: **Hys-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-MetNH$_2$**

Neurokinin B: **Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-MetNH$_2$**

Synthesis

Mammals derive SP from the pre-protachykinin-A (PPT-A) gene, being duplicated from a common ancestral gene and also encoding for NKA, neuropeptide K (NPK) and neuropeptide γ

(NPγ) (Carter & Krause 1990). Three distinct messenger ribonucleic acids (mRNA) are produced as a result of alternative splicing of the PPT-A gene transcript, that is αPPT-A, βPPT-A, and γPPT-A, which all encode for the SP precursor sequence (Carter & Krause 1990). SP and its mRNAs are largely expressed in CNS and PNS, with αPPT-A being expressed more in CNS, and the mRNAs of βPPT-A and γPPT-A expressed more in peripheral tissues (Kotani *et al.* 1986). In the CNS, SP immunoreactivity has been demonstrated in the telencephalon, basal ganglia, hippocampus, amygdale, diencephalon, hypothalamus, mesencephalon, pons and spinal cord (Shults *et al.* 1984). In the periphery, SP immunoreactivity has been shown in the trigeminal ganglia, dorsal root ganglia and intrinsic neurons of the intestine (Lee *et al.* 1985, Gibbins *et al.* 1987, Sternini *et al.* 1995). Any damage to neuronal cells can result in induction of neuropeptide gene expression that leads to changes in biosynthesis of the neuropeptides (Hokfelt *et al.* 1994).

SP synthesis occurs in ribosomes, and then, after being packaged in storage vesicles, is transported to the terminal endings of central as well as peripheral branches of primary sensory neurons (Brimijoin *et al.* 1980, Harmar & Keen 1982, Merighi *et al.* 1988). SP immunoreactivity is more apparent in the peripheral branches compared to the dorsal roots (Harmar *et al.* 1980) and may be associated with various vesicles in terminals within the brain as well as in the spinal cord (Pelletier *et al.* 1977, Pickel *et al.* 1977, Barber *et al.* 1979). In the sensory ganglion cells, SP is produced and transferred towards the terminal regions of the peripheral branches by axonal transport mechanisms (Brimijoin et al. 1980).

Metabolism

SP is metabolized by different enzymes including neutral endopeptidase (NEP), angiotensin-convering enzyme (ACE) and SP degrading enzyme (SP-DE) (Matsas *et al.* 1984, Probert & Hanley 1987, Skidgel & Erdos 1987). NEP and ACE are considered to be the main two enzymes involved in SP cleavage (Nadel 1991), with NEP involved in SP metabolism in the brain, spinal cord, and in the peripheral tissues, while ACE is involved in SP metabolism mainly in the plasma, CSF, and the substantia nigra where it continues to cleave the fragments released from NEP (Hooper & Turner 1987, Sakurada *et al.* 1990, Wang *et al.* 1991, Di Maria *et al.* 1998). Both NEP and ACE hydrolyze the same bond that separates the peptide from its terminal carboxyl region that is needed for binding to the tachykinin receptors (Skidgel & Erdos 1987).

1.6.2 Tachykinin Receptors

Examination of the pharmacological properties of currently known tachykinins has revealed three distinct receptors for the known tachykinins, namely the NK_1, NK_2 and NK_3 receptors (Teichberg *et al.* 1981, Maggi *et al.* 1987, Regoli *et al.* 1987, Hokfelt *et al.* 2001). These tachykinin receptors belong to the family of G protein-coupled receptors (GPCRs), which are a large family of membrane-bound receptors mediating signal transduction. Due to their widespread distribution, tachykinin receptors are involved in many different biochemical and physiological processes including regulation of neurotransmission, inflammation, pain, cell growth, cell differentiation, and oncogenesis. While binding to all tachykinin receptors, SP preferentially binds to the NK_1 receptor compared to NKA and NKB (SP>NKA>NKB). Similarly, NKA binds preferentially to the NK_2 receptor (NKA>NKB>SP) and NKB to the NK_3 receptor (NKB>NKA>SP) (Hokfelt et al. 2001). The tachykinins can therefore activate all three

receptors to a different degree depending not only on their affinity for the different receptor subtypes, but also on the receptor availability and peptide concentration (Regoli *et al.* 1994). The different tachykinin receptors can be found on many different cell types and their response to the different tachykinins depends on the type of the cell they are expressed on (Rosso *et al.* 2012).

NK_1 receptors

The tachykinin NK_1 receptors are observed in both mammalian central nervous system as well as in several peripheral tissues (Wolf *et al.* 1985, Dam & Quirion 1986, Danks *et al.* 1986, Saffroy *et al.* 1988, Maeno *et al.* 1993). In the CNS, NK_1 receptors have been found with relatively high expression in the striatum, nucleus accumbens, caudate-putamen, and superior colliculus, while moderate expression of the NK_1 receptors have been found in the cortex, amygdale, hippocampus, inferior colliculus, olfactory bulb, lateral nucleus of the hypothalamus, interpeduncular nucleus, nucleus of the tractus solitarus, raphe nuclei, substantia nigra, and medulla oblongata (Shults et al. 1984, Dam & Quirion 1986, Otsuka & Yoshioka 1993). In the PNS, NK_1 receptor expression has been shown in dorsal root ganglia, intrinsic neurons of the intestine, and in unmyelinated axons in the rat glabrous skin (Sternini et al. 1995, Andoh *et al.* 1996, Carlton *et al.* 1996, Li & Zhao 1998). At the cellular level, NK_1 receptors are found in a number of different cell types including neuronal cells, astrocytes, microglia, oligodendrocytes, vascular endothelial cells and myocytes (Cook *et al.* 1994, Maggi 1997, Palma *et al.* 1997, Rasley *et al.* 2002).

There is a high level of similarity in the NK_1 receptor sequence between humans and other animal species, including rats, mice, guinea pigs and man (Gerard *et al.* 1993). Indeed, protein

sequencing has shown a 92% similarity between human and rat NK_1 receptors (Gerard *et al.* 1991). Despite this similarity, there are distinct variations between species when selective NK_1 antagonists are used. These differences between humans and rats are considered due to different amino acids at positions 116 and 290 (Val and Ile in humans, and Leu and Ser in rats, respectively) (Fong *et al.* 1992, Sachais *et al.* 1993). Furthermore studies have shown that in humans the NK_1 receptor is a single copy gene on chromosome 2, which has a relatively long 5′ untranslated region compared to the other tachykinin receptors, and preceded by a single TATAAA sequence (Yokota *et al.* 1989, Hershey & Krause 1990).

Activation of the NK_1 receptor is involved in such physiological processes as smooth muscle contraction and relaxation, plasma protein extravasation, vasodilation, salivary secretion, airway contraction, and transmission of nociceptive responses (Regoli et al. 1994, Germonpre *et al.* 1995, Holzer-Petsche 1995, Maggi 1995a, Santos & Calixto 1997, Holzer 1998). They have also been associated in oncogenic mechanisms such as mitogenesis, angiogenesis, cell migration, and metastasis, which may explain the influence of SP on the microenvironment of a tumor (Rosso et al. 2012). There is also evidence supporting the involvement of NK_1 receptors in sensory transmission as well as in the mediation of different immunological and inflammatory processes (Lembeck & Holzer 1979, Maggi 1997, Quartara & Maggi 1997, Juranek & Lembeck 1997). Finally the NK_1 receptors play a significant role in oedema formation, with SP binding to the receptor having been shown to increase vascular permeability and extravasation of plasma proteins (Campos & Calixto 2000).

SP binding to the NK_1 receptors first leads to phosphoinositide hydrolysis, which results in calcium mobilization and activation of mitogen-activated protein kinase (Rollandy *et al.* 1989, Pradier *et al.* 1993, Luo *et al.* 1996, DeFea *et al.* 2000). After binding, the NK_1 receptor is internalized, which can be fully reversed within 30 min (Mantyh *et al.* 1995). This internalization phenomenon has been used in subsequent studies as a marker for SP release (Hokfelt et al. 2001). NK_1 receptor expression is modified by peripheral inflammation (Abbadie *et al.* 1996, Abbadie *et al.* 1997). Specifically, many more neurons in lamina I show NK_1 receptor internalization following mechanical stimulation when inflammation is present, with the internalization of NK_1 receptors extending into the deeper layers and demonstrating an extended rostracaudal distribution (Hokfelt et al. 2001). Presumably, inflammation causes a restructuring of dorsal horns circuits.

1.6.3 Pathophysiological Effects of SP

Depression

The first reports of antidepressant effects of SP antagonists came from Kramer and colleagues (Kramer *et al.* 1998). In a multicenter, double-blind controlled clinical trial, the NK_1 receptor antagonist MK-869 was equally as effective as the reference drug, a serotonin reuptake inhibitor paroxetine, for treating depressive disorder, with no significant differences in adverse effects. Moreover, in contrast to paroxetine, MK-869 did not cause any sexual dysfunction. Animal studies have also demonstrated that SP is related to mood control (Rupniak *et al.* 2000). While the reported effects of MK-869 in depression are still not fully understood, NK_1 antagonists can produce an increased firing rate of 5-HT neurons compared to either the selective serotonin inhibitors or monoamine oxidase inhibitors (Blier & de Montigny 1999, Dong & Blier 2001).

Notably, about half of all serotonin (5-HT) neurons in the dorsal raphe nucleus in the human brain demonstrate SP co-localisation (Baker *et al.* 1991, Sergeyev *et al.* 1999). The NK_1 receptor antagonists may thus have antidepressant effects related to its actions on 5-HT neurons (Blier *et al.* 2004).

Learning and memory

Peripheral administration of SP has been shown to have neurotrophic and memory-enhancing effects, which are related to general dopamine activity changes, as well as specific increases in extracellular dopamine in the nucleus accumbens (Huston & Hasenohrl 1995). Subcutaneous administration of SP can dose-dependently induce active and passive avoidance conditioning in mice (Severini *et al.* 2002). Specifically, in a single trial passive avoidance task, 0.75 pmol/g SP enhanced retention, while lower or higher doses showed much less efficacy (Severini et al. 2002). Other studies have shown that administration of SP did not change the rate of learning in an active avoidance task; however it increased the extinction of learning (Schlesinger *et al.* 1983). In an appetite motivated learning task, animals administered SP maintained the task better than animals in the control group, demonstrating that SP can improve task performance by facilitating memory (Schlesinger *et al.* 1986). These findings were confirmed in later studies showing that enhancement of inhibitory avoidance learning can be produced by administration of the SP N-terminal fragment, SP(1-7), but not C-terminal fragment, SP(6-11) (Hasenohrl *et al.* 1990). Once again, higher or lower doses had much less of an effect, reinforcing that the facilitating effect was dose dependent. Finally, studies have shown that SP administration can block the amnestic effect of diazepam in rats showing impaired retention on an inhibitory avoidance task (Costa & Tomaz 1998).

Psychological stress and anxiety

In the CNS, SP has been localized to neurons located in the hypothalamus, hippocampus, mid-brain, basal ganglia, and limbic system, including the hippocampus and amygdale, which all contribute to emotional reactions, learning, memory, recognition and recollection of spatial relationships (Ronnekleiv *et al.* 1984, Menetrey & Basbaum 1987, McRitchie & Tork 1994, Seress & Leranth 1996). SP is also co-localized with other neurotransmitters in different regions of the CNS including with serotonin in the raphe nucleus, with dopamine in the midbrain and striatum, and with corticotropin releasing hormone in the hypothalamus (Otsuka & Yoshioka 1993, Sergeyev et al. 1999). Because of its broad distribution, it has been suggested that SP plays a role in number of psychiatric and psychological disorders including schizophrenia, affective disorders, different types of phobias, anxiety and mood disorders (Quartara & Maggi 1998, Rupniak & Kramer 1999, Stout *et al.* 2001). Several animal studies have since confirmed that SP may be involved in the pathology of anxiety and depression (discussed above). For example, NK_1 receptor antagonists could block behavioral changes induced by stress in guinea pigs and had anxiolytic effects in various animal models of chronic mild stress (Kramer et al. 1998, Papp *et al.* 2000). The NK_1 receptor antagonists have also shown anxiolytic effects in animal models of the social interaction test (File 2000). A human study carried out on civilians during a war attack has shown elevated levels of plasma SP in subjects with high levels of anxiety (Weiss *et al.* 1996), while first human clinical trials subsequently have demonstrated that NK_1 receptor antagonists could have potent anxiolytic effects (Kramer et al. 1998). This comes to show that SP is related to mechanisms of anxiety disorders and therefore NK_1 receptor antagonists may have the potential to treat such disorders.

Pain

The association between SP and pain first was proposed by Lembeck and Holzer who suggested that SP could be released from peripheral sensory nerve fibers (Lembeck & Holzer 1979). Later studies confirmed that SP is produced in a subset of small diameter capsaicin sensitive sensory neurons located in the dorsal roots of the spinal cord (Hokfelt *et al.* 1975a, Seybold 2009). Noxious stimulation or inflammation of these sensory neurons initiates release of SP into the dorsal horn leading to activation of NK_1 receptors as a first step in pain transmission (Maggi 1995b, White 1997). This response is significantly reduced either by pretreatment with NK_1 receptor antagonists or by SP-depleting agents like capsaicin (Inoue *et al.* 1998). Subcutaneous administration of NK_1 antagonist also attenuates progressive hypersensitivity of spinal flexor motoneurons induced by stimulation of inflamed tissue, which may suggest the involvement of SP in mediating hypersensitivity during inflammatory processes (Ma & Woolf 1997). However, other studies on NK_1 receptor knockout mice have shown that the level of SP and NK_1 receptor involvement in nociception may depend on the level of pain intensity, since there is no difference in response between knockout mice and control animals after a certain level of induced pain (De Felipe *et al.* 1998, Zimmer *et al.* 1998). Subsequent studies have not made the same conclusion, having demonstrated that normal animals subjected to an intense period of noxious stimulation showed an amplification of nociceptive reflexes, which did not occur in animals lacking NK_1 receptors (Cao *et al.* 1998, Severini et al. 2002). These authors suggested that SP is not only involved in pain transmission, but also in the modulation of nociceptive inputs and whether any stimulus will be experienced as a pain (Severini et al. 2002).

Classical inflammation

SP is related to stimulation and augmentation of numerous inflammatory mechanisms, including plasma extravasation, leukocyte activation, endothelial cell adhesion molecule expression, mast cell activation and cytokine production (Vishwanath & Mukherjee 1996, Quinlan *et al.* 1999). Various reports have shown that SP can play a role in the secretion of interleukins (IL) such as IL-1, IL-6, and IL-12, tumor necrosis factor (TNF)-α by myeloid cells, upregulate interferon (INF)-γ production by T cells, as well as downregulate anti-inflammatory cytokines including transforming growth factor (TGF)-β (McCormack *et al.* 1996, Marriott & Bost 2001). SP antagonists may also change host responses against different infections (Nessler et al. 2006). For example, mice with salmonellosis treated with SP antagonists had a limited Th1 response and an increased bacterial burden compared with untreated control animals (Kincy-Cain & Bost 1996). In a post-treatment reactive encephalopathy model of Trypanosome brucei, animals with deficient NK_1 receptors also showed significantly reduced clinical impairment and more severe neuroinflammatory responses (Kennedy *et al.* 1997).

1.6.4 Neurogenic Inflammation

Neurogenic inflammation is the process whereby mechanical or chemical stimulation of the unmyelinated C-fibers leads to an axon reflex that results in the release of neuropeptides, including SP, and the subsequent development of typical inflammatory symptoms such as pain, vasodilation and plasma extravasation (Abelli *et al.* 1991, Geppetti *et al.* 1995, Bertrand *et al.* 1993, Trevisani *et al.* 2004). Moreover, antidromic stimulation of these sensory nerves leads to release of SP from their peripheral terminals (Brodin *et al.* 1981, White & Helme 1985). These processes are particularly prominent on the microvascular system where they can cause

vasodilation of small arterioles, extravasation of plasma proteins in microvasculature, and leukocyte adhesion to endothelial cells of post-capillary venules (Geppetti et al. 1995, Holzer 1998). Some of the other tissue specific responses of neurogenic inflammation are inotropic and chronotropic effects on the heart, smooth muscle responses in the urinary bladder, ureter, and iris, and bronchoconstriction in the lungs (Abelli et al. 1991, Bertrand et al. 1993, Geppetti et al. 1995).

While a number of neuropeptides are involved in the development of neurogenic inflammation, SP is recognized as one of the primary mediators and has been directly linked to the resultant increased vascular permeability (Lembeck & Holzer 1979, Pernow 1985, Severini et al. 2002). Increased baseline plasma extravasation has been noted in mice pretreated with either NEP or ACE inhibitors (Lu *et al.* 1997, Emanueli *et al.* 1998). NEP and ACE play a major role in the metabolism of SP, and their inhibition would increase SP concentration (Harrison & Geppetti 2001). Calcitonin gene related peptide (CGRP), which co-exists with SP in primary afferent neurons, is another of the released neuropeptides and promotes oedema development via its potent vasodilatory actions (Rosenfeld *et al.* 1983, Wiesenfeld-Hallin *et al.* 1984, Brain & Williams 1985). However, CGRP is less potent than SP in production of flare and wheal, and produces a slower onset and longer lasting intense vasodilation (Foreman 1987). Another neurokinin involved in neurogenic inflammation is neurokinin A, which may increase vascular permeability and have vasodilatory effects (Hua *et al.* 1984). It is a more potent bronchoconstrictor than SP. Somatostatin, vasoactive intestinal polypeptide and neurokinin B are other neuropeptides that have been implicated in neurogenic inflammation (Foreman 1987), however their roles are less certain.

These neuropeptide-containing primary sensory neurons are sensitive to capsaicin, which is a pungent component present in capsicum (Szallasi & Blumberg 1999). The capsaicin receptor, the transient receptor potential vanilloid 1 (TRPV1) receptor, is a seven transmembrane domain protein that acts as a non-selective cation channel that can be stimulated by endogenous heat (>43°C), protons, mechanical stimuli, and various endogenous ligands(Bevan & Geppetti 1994). While capsaicin can selectively stimulate a subset of primary sensory neurons, which will release sensory neuropeptides, higher concentrations of capsaicin can either permanently destroy the sensory neuron (in neonatal animals) or transiently deplete the nerve terminals of neuropeptides (adult animals), which can prevent the development of neurogenic inflammation (Szolcsanyi & Mozsik 1984, Szallasi & Blumberg 1999). Vink and colleagues took advantage of this property of capsaicin by depleting sensory nerve neuropeptide levels in adult rats and subsequently demonstrating that neurogenic inflammation plays an important role in early cerebral oedema development in response to mechanical injury (Vink et al. 2003, Nimmo et al. 2004). Specifically, capsaicin pre-treatment significantly attenuated BBB permeability and oedema formation in rats following TBI. Later studies in naïve animals demonstrated that SP increases following TBI, and that administration of the NK_1 receptor antagonist, n-acetyl-L-tryptophan (NAT), inhibited neurogenic inflammation, as demonstrated by reduced posttraumatic BBB permeability and vasogenic oedema formation, and improved functional outcome (Donkin et al. 2009). While the NK_1 antagonists clearly reduce oedema after experimental TBI, oedema is not easily monitored in clinical TBI. In contrast, ICP is widely used to monitor TBI in patients and $P_{bt}O_2$ is gaining increasing importance. However, no studies have shown that a reduction in oedema using NK_1 antagonists will translate to a reduction in ICP or an improvement in $P_{bt}O_2$.

This thesis will definitively answer this question using both small (rodent) and large (sheep) animal models of TBI.

1.7 Experimental Models of Traumatic Brain Injury

Animal models of traumatic brain injury are used to replicate some sequence of events occurring in real life human injuries, but under controlled circumstances, thereby facilitating an understanding of its pathophysiology. Denny-Brown and Russell originally separated experimental brain injury into two main groups and named them acceleration-concussion and percussion-concussion (Denny-Brown & Russell 1941). The percussion-concussion models were the forerunner to later developed animal models such as fluid percussion, impact acceleration, weight-drop, rigid indentation, inertial acceleration, and dynamic cortical deformation (Gennarelli 1994, Povlishock et al. 1994). Different animal models were developed because no single animal model could fully reproduce all aspects of the complex pathophysiological changes occurring in humans following TBI. Indeed, each animal model can only replicate certain aspects of human trauma; therefore the design and choice of a specific model depends on the goal of the experimental study. There are, however, some aspects of human TBI that all animal models should contain, such as a quantifiable and reproducible controlled mechanical force to induce injury, reproducible injury that replicates some of the components of human injury, and correlation between the measurements of injury outcome and the intensity of the mechanical force causing the injury (Cernak 2005).

The differences between species with respect to brain anatomy and geometry, gyral structure, craniospinal angle and receptor distribution determines the specific differences in

pathophysiological, behavioral, immunohistological and metabolic responses in different species. For example, there is a difference in the pattern and distribution of neurotransmitter receptors between humans and rodents, which may be one of the reasons that some researchers question the rodent's credibility as a model for studying TBI (Gennarelli 1994). Rodents also have a lissencephalic brain and an underdeveloped tentorium compared to humans, which makes it arguable whether they are useful in the study of post-traumatic brain oedema development, intracranial pressure changes and cerebral oxygenation (Gabrielian *et al.* 2011). Nonetheless, the majority of researchers to date have used rodent models of TBI because they are a convenient choice with advantages such as relatively small size and low cost, allowing for a large number of morphological, biochemical, cellular, and functional studies that could not always be possible with large animals due to ethical, technical and financial restrictions (Povlishock et al. 1994).

1.7.1 Fluid Percussion Injury

Fluid percussion injury (FPI) was initially developed as a midline injury for use in the cat and rabbit (Stalhammar *et al.* 1987, Hayes *et al.* 1987, Hartl *et al.* 1997b) and was later modified for use in the rat, both as a midline and a lateral injury model (Dixon *et al.* 1987, McIntosh *et al.* 1987, McIntosh et al. 1989). It has since become one of the most frequently used models of experimental TBI (Thompson *et al.* 2005). The injury is produced by application of a rapid fluid pressure pulse to the intact dura through a craniotomy, made either centrally between bregma and lambda or lateral to the sagittal suture over the parietal cortex (McIntosh et al. 1989, Marmarou & Shima 1990). Although central and lateral FPI have many similarities, studies have demonstrated distinct differences between the two models (Povlishock et al. 1994, Iwamoto *et al.* 1997). The LFPI model mainly produces unilateral injury, while central FPI causes bilateral

injury (Gennarelli 1994, Iwamoto et al. 1997). Studies have also shown that for LFPI, the distance of the craniotomy from the sagittal suture may dictate the type and extent of tissue damage (Vink *et al.* 2001). Some of the most frequently occurring pathological changes for both models of FPI are alterations in CBF, increased permeability of the BBB, intracranial hemorrhage, tissue tears, and axonal damage (McIntosh et al. 1987, McIntosh et al. 1989, Pfenninger *et al.* 1989, Tanno *et al.* 1992, Schmidt & Grady 1993, Wang *et al.* 1997, Graham *et al.* 2000). Both models also have some disadvantages, which limits their usage as a model, including increased severity or morbidity due to disproportional involvement of the brain stem (particularly in the midline model), development of neurogenic pulmonary oedema, restricted biomechanical control, a steep injury curve and, like all animal models, the inability to mimic the whole complexity of human injury (Cernak 2005).

1.7.2 Impact-Acceleration Injury

The main purpose of developing the impact-acceleration model of brain injury was to reproduce the TAI commonly related to severe clinical TBI (Morales *et al.* 2005). The injury is induced by dropping a fixed weight through a Plexiglas guide tube onto the rat's skull, which is protected by a stainless steel disc to minimize the incidence of skull fractures (Marmarou *et al.* 1994, Sawauchi *et al.* 2003). The anaesthetized animal is resting on a foam cushion throughout the procedure to allow for deceleration after the impact. Studies have shown that the elastic properties of the foam are very important since they contribute to the type and severity of the injury (Piper *et al.* 1996). Impact produced in this model of injury causes acceleration of the head and results in diffuse shear forces within the brain (Marmarou et al. 1994). The severity of the impact is directly related to the mass of the weight and the height from which it is released

(Marmarou et al. 1994, Piper et al. 1996, De Mulder *et al.* 2000). Depending on the severity of injury, this model can produce coma, axonal swelling, subarachnoid hemorrhage, fragmented dendrites, focal contusions and neuronal damage (Foda & Marmarou 1994, Heath & Vink 1995, Povlishock *et al.* 1999, Folkerts *et al.* 1998, Stone *et al.* 1999, Stone *et al.* 2000). A number of behavioral changes have been observed in this model of TBI including deficits in neurological reflexes, beam balance, inclined plane, forelimb placement and the rotarod (Beaumont *et al.* 1999, Rancan *et al.* 2001, Heath & Vink 1995). The impact-acceleration model of brain injury is now one of the most frequently used injury models due to its ability to produce traumatic axonal injury, acute subdural hematoma, concussion and dysfunction of the BBB.

1.7.3 Controlled Cortical Impact Injury

The controlled cortical impact (CCI) model of brain injury has been used in rodents and sheep and is described as a rigid percussion model of TBI (Povlishock et al. 1994, Gennarelli 1994, Dixon *et al.* 1991, Smith *et al.* 1995, Anderson *et al.* 2003). The advantage of this model is that it allows having more control over the velocity of an impact and the depth of resulting deformation, which allows control of injury severity without brain stem deformation, resulting in minimal mortality (Morales et al. 2005). Compared to impact-acceleration injury, this model also avoids any potential rebound injury. The injury is delivered to the intact dura via a craniotomy using a compressed air-driven metallic piston that deforms the underlying brain tissue (Dixon et al. 1991, Goodman *et al.* 1994). Vertical adjustment of the crossbar holding the cylinder allows the user to control the depth of cortical deformation, which generally varies between 1 and 3 mm (Cernak 2005). The CCI model often causes a more focused brain injury, which mimics a spectrum of contusion injuries, and intraparenchymal petechial hemorrhages, subdural or

epidural hematomas, axonal injury, and coma of varying length (Lighthall 1988, Gennarelli 1994, Dixon et al. 1991). The CCI model can produce changes in brain oedema, increased ICP, decreased CPP, decreased CBF, and coma (Cherian *et al.* 1994, Gennarelli 1994, Bryan *et al.* 1995, Baskaya *et al.* 1997).

1.7.4 Large Animal Models of TBI

The use of large animal models of TBI allows for improved targeting of a number of distinct mechanisms of injury compared to small animals, including inertial injury modeling with a magnitude enough to produce DAI (Duhaime 2006). The gyrencephalic structure of the brain in large animals more closely replicates the human brain and allows for more specific examination of both grey matter and white matter changes following TBI. Anatomical and physiological similarities between the brains of large animals and humans also allows for more accurate comparison of the brain's response to injury at different stages after TBI. However, large animal models also have disadvantages including high cost, more difficulties in handling and housing, require far more complex care, and finally they are less well-characterised than rodents. Also, there are more ethical hurdles in the use of large animals. Anesthetic aspects are also more difficult in large animals and must be selected with consideration of the animal age as well as species. Finally, functional outcomes after TBI are much less characterised in large animal models compared to rodents.

The sheep model of TBI was developed in our laboratory by Lewis and colleagues (Lewis et al. 1996) and has been subsequently well characterized in terms of pathology (Van Den Heuvel *et al.* 1998, Finnie *et al.* 2001). A humane stunner is used to produce an injury targeted at the left

temporal region of an anesthetised sheep placed in the sphinx position, with the head allowed free rotational and lateral movement following TBI (Lewis et al. 1996). This type of injury induces widespread axonal injury, intracranial hemorrhage within different locations, increased ICP, reduced CPP, cerebral hypoxia and decreased blood pressure (Lewis et al. 1996, Van Den Heuvel et al. 1998, Finnie et al. 2001). There is a high degree of variability compared to small animal models due to variations of biomechanical forces during the injury and because of different dynamic responses of the head. However, the ovine model of TBI replicates a number of pathophysiological changes occurring in human TBI, including changes in ICP, MABP, CPP, cerebral oxygenation, all of which will be investigated in the present thesis.

1.8 Synopsis

In the present thesis, we will initially investigate ICP and CPP changes in both small (rodent) and large (ovine) models of TBI to ascertain which is the most suitable to develop interventional therapies targeting ICP. Having established the validity of the large, sheep model of TBI, I will thereafter characterize changes in brain oxygenation following TBI and its correlation with ICP, MABP and CPP. We will then investigate the dynamic interrelationship between $P_{br}O_2$, ICP and MABP/CPP, reexamine the formula for CPP, and try to elucidate critical thresholds for ICP and $P_{br}O_2$. Our intent is to establish acceptable thresholds for ICP and determine the values at which ICP should be treated.

Following this characterization, I will examine the effects of two different NK_1 antagonists on ICP and brain oxygenation and compare their efficacy to currently used treatments, namely hypertonic saline (HTS) and mannitol. We will also study the effects of magnesium and

progesterone on increased ICP and brain oxygenation following TBI, given that these experimental compounds have been touted as multi-potential interventional therapies that reduce oedema and have an increased likelihood of success in human TBI.

A brief summary will precede each experimental investigation, along with a summary of the methodological protocol, which will be fully outlined in chapter two. Although each chapter will report results specific too that chapter, it is expected that many of the results will have implications not only for the present investigation but also for other aspects raised in the thesis. This will result in some overlap across the chapters in interpretation and discussion. Finally, a concluding general discussion will integrate the major conclusions drawn from each chapter.

CHAPTER 2

GENERAL METHODS

2.1 Animal Care

2.1.1 Ethics

All experimental protocols were approved by the Animal Experimental Ethics Committees of the University of Adelaide and the Institute of Medical and Veterinary Sciences according to guidelines established for the use of animals in experimental research as outlined by the Australian Health and Medical Research Council.

2.1.2 Animals

Two different animal species were used in the study, namely male Sprague-Dawley rats and adult male merino sheep. The rats (n=48) weighing between 400-450g were group housed in a conventional rodent room at 24°C on a 12-hour light-dark cycle, and had access to standard rodent food and water *ad libitum*. Sheep (n=82) weighing between 45-60kg were held at an animal facility and were fed and watered accordingly.

2.2 Rat Traumatic Brain Injury

2.2.1 Anaesthesia

Isoflurane

Isoflurane (Isoflo^{TM}, Abbott Australia Pty Ltd) was obtained as a volatile liquid from Lyppards Veterinary Supplies (Adelaide, Australia) and stored below 25°C away from direct heat and sunlight. General anaesthesia was induced by placing animals in a transparent plastic induction chamber and delivering 3% isoflurane in oxygen (30%) and nitrogen (70%) via a calibrated vaporiser at a flow rate 1.2 L/minute. After a surgical level of anaesthesia was reached, the animal was moved to the surgical table and placed in the supine position on a thermostatically

controlled electrical heating pad that maintained the animal's body temperature between 37-38°C throughout the experiment. Maintenance of anaesthesia was achieved via a rodent nose cone covering both the mouth and the nose delivering 2% isoflurane in oxygen and nitrogen mixture ($3O_2:7N_2$) at a rate 1.2L/minute.

Urethane

Urethane (urethane, Sigma-Aldrich Inc, Australia) was obtained as an aqueous solution from Lyppards Veterinary Supplies (Adelaide, Australia) and stored below 25°C away from direct heat and sunlight. Urethane was used for long-lasting maintenance anaesthesia and was administered through a venous catheter at a rate of 1.2gm/kg/5minute. Unlike isoflurane, urethane produces long-lasting (6-8h) steady level of surgical anaesthesia with minimal physiological effects on cardiovascular and autonomic respiratory systems.

Lignocaine

Lignocaine (lignocaine hydrochloride, 2%, MavLab^{TB}, Australia) was supplied as an aqueous solution by Lyppards Veterinary Supplies (Adelaide, Australia) and stored at room temperature. It was used in all rats to provide local anaesthesia prior to surgical incision. Lignocaine was administered subcutaneously via a 25 gauge, 12.5mm needle at a dose of 0.3ml to 0.5ml of 0.4% solution per injection site.

Pentobarbital

Pentobarbital (pentobarbitone sodium, 60mg/ml, Rhone Merieux Pty Ltd, Australia) was obtained as an aqueous solution from Lyppards Veterinary Supplies (Adelaide, Australia) and

stored at room temperature. Rats were administered pentobarbital via intraperitoneal injection using a 25 gauge 12.5mm needle at a dose of 60mg/kg. Animals were restrained by grasping the loose skin of the back and neck and the needle inserted into the left caudal area of the abdominal cavity, thus avoiding any vital organs (Van Dongen et al. 1990, Waynforth et al. 1992).

2.2.2 Induction of Rodent TBI

Surgical preparation

Animals were anaesthetised with isoflurane as described above and the animal's neck (the area over the throat), head and medial area of the right thigh were shorn to remove excess hair. A tracheostomy was performed and the animals were subsequently ventilated at a rate of 90 strokes per minute (s.p.m.) at approximately 225ml/minute and end tidal carbon dioxide (ETCO2) was monitor via a capnograph. The animal's right femoral artery and vein were cannulated using a polyethylene cannula (PE 50; inside diameter = 0.58 mm, outside diameter = 0.97 mm) with the arterial cannula used for monitoring of blood pressure and sampling of arterial blood for gas analysis, while the venous cannula was used for urethane administration after switching from isoflurane to urethane for maintenance anesthesia. Urethane produces a long-lasting (5-6hr) steady level of surgical anaesthesia and has minimum effects on lowering the systemic arterial blood pressure (Maggi & Meli 1986a, Maggi & Meli 1986b, Davis 2008). 1% lignocaine was injected subcutaneously prior to all surgical incisions.

A 2 cm sagittal incision was then made on the skin overlying the dorsal skull at the midline and the tissue retracted to permit a craniotomy to be performed laterally. A 5mm in diameter craniectomy was trephined into the skull centered 3mm right of the sagittal suture and midway

between the bregma and lambda, with the dura kept intact at the opening. A specially designed steel-bolt (analog to female Leur-loc) with 4mm inside diameter and 5mm outside diameter was screwed and secured into the craniectomy using cyanoacrylate adhesive.

Fluid percussion injury

Immediately prior to trauma, animals were placed onto a foam block in a prone position and the steel-bolt was filled with isotonic saline. The bolt was then attached to the LFP device (Dixon et al. 1987) and a moderate to severe trauma was induced. The LFP device consists of a Plexiglas cylindrical reservoir with 4.5cm diameter and 60cm length bounded at one end by a Plexiglas, rubber-covered piston mounted on O-rings, and at the other end fitted with a 2cm long metal housing on which a pressure transducer was mounted and connected to a 5mm tube that terminated with a male Leur-loc like fitting. At the time of injury the tube-fitting was connected to the bolt that had been fixed over the dura on the rat's skull. The whole system is filled with isotonic saline at room temperature and all bubbles carefully removed from the system. The injury was induced by releasing a metal pendulum from a pre-determined angle, striking the piston of the device. This produces a pulse of saline within the reservoir that is rapidly injected into the epidural space of a closed cranial cavity producing brief displacement and deformation of neural tissue. In the present study, a moderate to severe injury was initiated from 13.5 degrees generating a pressure pulse of 2.6-2.8 atmosphere (atm). Immediately following trauma, the bolt was detached from the LFP device and the animal was placed on a 37°C heating pad, and its tracheal tube re-attached to the ventilator.

Weight drop injury

Animals were injured using the Marmarou impact-acceleration model of diffuse TBI, which has been described extensively elsewhere (Marmarou et al. 1994). Rats were anaesthetized, intubated, ventilated, and their core temperature was maintained at 37.5 ± 0.5°C as described for the LFP animals. The skull was then exposed after midline sagittal incision (2 cm) and soft tissue retraction. A drop of cyanoacrylate adhesive was then applied to the skull along the midline, mid-way between the lambda and the bregma sutures, and a protective stainless steel disc (10 mm diameter x 3 mm thick) was fixed centrally upon the skull. The stainless steel disc acted as a helmet during trauma to reduce the incidence of skull fractures. Immediately prior to trauma, animals were secured in a prone position onto a foam block of uniform density (depth 11.5 cm) and placed under the injury apparatus, ensuring that the brass weight was centrally aligned above the protective steel disc. Moderate to severe traumatic brain injury was induced by releasing the brass weight (450 g) from a height of 2 m via a cylindrical PVC conduit and allowing it to impact onto the steel disc attached to the animal's skull. The animal was rapidly relocated after the initial impact to prevent any further contact from the rebounding weight. The stainless steel disc was subsequently removed and the animal re-attached to the ventilator.

Ventilation and induction of hypoxia

After injury, ventilation rate (90 s.p.m.) and minute volume (225ml/minute) of normoxic mixture (30% O_2, 70% N_2) were periodically adjusted following post-trauma arterial blood gas analysis to maintain a blood $PaCO_2$ of approximately 35mm Hg. The groups that were exposed to a secondary hypoxic episode were ventilated for either 15minutes or 30minutes with a hypoxic mixture of 10% oxygen and 90% nitrogen to achieve a blood PaO_2 of between 30-35mm Hg.

Following the hypoxic episode animals were ventilated with a normoxic mixture of oxygen and nitrogen (30% O_2 and 70%N_2) for the remainder of the monitoring period.

2.2.3 Intracranial Pressure Monitoring

Intracranial pressure was monitored using a Codman Microsensor ICP Transducer probe which is an intraparenchymal strain-gauge device that uses a Microsensor tip connected with a nylon catheter (1.2mm diameter; Codman and Shurtleff, Inc., DePuySpine™). The probe was connected to the Codman ICP Express monitoring system (DePuySpine™), which is a digital system that relays the ICP as a digital signal-value recording in mmHg and systolic and diastolic pressures relative to this value. After being attached to the monitoring system, the probe was calibrated in sterile water according to manufacturer's instructions before inserting into the brain of each experimental animal.

Following injury, the ICP probe was inserted into the brain tissue so that the tip of the probe was in depth of 2-3 mm into the brain parenchyma. In the LFP animals, this could be achieved through the existing bolt inserted in the craniectomy, while for weight drop injured animals, a craniectomy was rapidly performed over the left parietal cortex and the bolt mounted in place. To prevent CSF leakage following the probe insertion, the bolt was sealed using bone wax. ICP was continuously monitored using a MacLab data acquisition system (MacLab 2e) for 4 hours.

2.2.4 Arterial Blood Pressure Monitoring

Mean arterial blood pressure was monitored with a MacLab data acquisition system (MacLab 2e). A Statham-type pressure transducer was connected via a polyethylene tube to the animal's

arterial catheter (PE 50). The pressure trace was relayed from the transducer to the MacLab via a bridge amp and all data recorded using a personal computer.

2.2.5 Arterial Blood Gas Analysis

The Osmetech OPTI blood gas analyser (CCA, Helena Laboratories, Australia Pty Ltd) and Osmetech OPTI cassettes were used for arterial blood gas analysis. Arterial blood samples (0.2-0.25ml) were obtained via the femoral arterial cannula via a 3-way tap. Blood gas analysis was conducted three times during each experiment; the first analysis was done 5 minutes before the injury, the second analysis was done 10min after the injury, and the third one was done 2 hours after the injury. PaO_2, $PaCO_2$, pH, SO_2, tCO_2, tHb, Hct, Na^+, K^+ and BE were measured during each arterial blood gas analysis.

2.2.6 Oedema Measurement

Brain water content was assessed by the wet weight–dry weight method (O'Connor *et al.* 2006, Donkin et al. 2009) at 5 h after focal or diffuse trauma. Rats (n = 6 per group) were decapitated under pentobarbital anesthesia and their brains removed. The cerebellum and brainstem were then rapidly removed and the cerebral hemispheres placed in glass vials. After weighing, brains were dried at 100°C for 24 h. The dry weight was then obtained and brain water content calculated according to the formula

$$\text{Water} = (\text{wet weight} - \text{dry weight})/\text{wet weight} \times 100$$

2.2.7 Perfusion

At the conclusion of the 4 h monitoring, animals were killed by injection of pentobarbital (300mg/kg i.p.) and their brains perfused using 4% paraformaldehyde. Animals were placed on the supine position on a wire rack and a bilateral thoracotomy was performed to expose the heart. A blunt 19 gauge, 37mm needle was inserted into the apex of the left ventricle and guided onto position within the ascending aorta. Heparin (5000 i.u. in 1ml of normal saline; David Bull Laboratories, Mulgrave, Victoria, Australia) was injected slowly into the aorta over 30 seconds and the right atrium incised to permit vascular flushing. Then 4% paraformaldehyde (400-500ml as required) was flushed through the animal to complete the perfusion process. Brains were subsequently removed for histology.

2.3 Histology and Immunohistochemistry

2.3.1 Tissue Processing

Paraffin embedding and sectioning

All specimens were post-fixed in 10% neutral buffered formalin (Fronine Laboratory supplies) for a minimum of 48 hours before further processing. Processing consisted of 20 minutes in each graded ethanol bath (50%, 70%, 80%, and 95% for once and 100% for twice), followed by two xylene baths for 1.5 hours each, and four paraffin baths of increasing time (30, 60, 60, and 90 minutes). Samples were then embedded in paraffin wax. Serial 5µm sections were cut using a microtome (Microm, Walldorf Germany), floated onto APT (Cat. No. A-3648, 500ml; Sigma-Aldrich, Sydney, Australia) coated slides, and dried over a heater for 15min.

2.3.2 Albumin Staining

From selected wax blocks, 5µm sections were cut using a microtome (Microm, Walldorf Germany) and immunolabelled with albumin primary antibody (1:20 000 in NHS; goat polyclonal antibody, Cat No: 0113-0344, Cappel Laboratories) by overnight incubation at room temperature. After washing the slices in PBS, slices were then incubated with a Biotinylated horse anti-Goat IgG (H+L) conjugated secondary antibody (1:250 in NHS, Catalog No: BA-9500, Vector Laboratories) for 1h at room temperature, and the subsequent immunocomplex visualised using diaminobenzidine tetrahydrochloride (DAB) as a chromogen in a peroxidase reaction (Sigma-Aldrich, Sydney, Australia).

2.3.3 Amyloid Precursor Protein Immunohistochemistry

5µm sections were sliced from selected wax blocked sections using a microtome (Microm, Walldorf Germany) and immunolabelled with amyloid precursor protein (APP) primary antibody (1:1000 in NHS; monoclonal antibody 22C11, Chemicon) by overnight incubation at room temperature. After washing the slices in PBS, slices were then incubated with an anti-mouse IgG-HRP conjugated secondary antibody (1:250 in NHS, Sigma-Aldrich) for 1h at room temperature, and the subsequent immunocomplex visualised using diaminobenzidine tetrahydrochloride (DAB) as a chromogen in a peroxidase reaction (Sigma-Aldrich, Sydney, Australia).

2.4 Sheep Traumatic Brain Injury

2.4.1 Anaesthesia

Thiopentone

Thiopentone (thiopentone sodium, Abbott Lab, Australia Pty Ltd) was obtained as an aqueous solution (2.5%) from Lyppards Veterinary Supplies (Adelaide, Australia) and stored below 25°C away from direct heat and sunlight. It was used as induction anaesthesia and administered intravenously via the internal jugular vein at a dose of 5mg/kg, reconstituted in 20ml of 0.9% sterile saline, until loss of consciousness sufficient to allow intubation was achieved.

Isoflurane

Isoflurane (Isoflo^{TM}, Abbott Australia Pty Ltd) was obtained as a volatile liquid from Lyppards Veterinary Supplies (Adelaide, Australia) and stored below 25°C away from direct heat and sunlight. General anaesthesia with isoflurane was induced by delivering 2-3% isoflurane in oxygen (30%) and nitrogen (70%) via a calibrated vaporiser at a flow rate 4L/minute. Maintenance of anaesthesia was achieved via an intubation tube delivering 1-1.5% isoflurane in oxygen and nitrogen mixture ($3O_2$:$7N_2$) at a rate 4L/minute in combination with ketamine.

Ketamine

Ketamine (ketamine hydrochloride, Parnell Lab, Australia Pty Ltd) was supplied as an aqueous solution by Lyppards Veterinary Supplies (Adelaide, Australia) and stored below 25°C away from direct heat and sunlight. Ketamine was used for maintenance anaesthesia in combination with isoflurane at a dose 4mg/kg/hr and was administered through the venous cannula.

2.4.2 Induction of Ovine TBI

Surgical preparation

Induction anaesthesia was administered through the jugular vein using 1g thiopentone, diluted in 20ml 0.9% saline. Aliquots were given until a sufficient level of anaesthesia was achieved to allow intubation. After an adequate level of anaesthesia was achieved, animals were placed in the supine position and intubated using a size 8 endotracheal tube, which was connected to a ventilator (Ohmeda 7000 Ventilator; Ohmeda Madison, WI, USA). Anaesthesia was initially maintained using isoflurane at a concentration of 2-2.5% and with a flow rate of 4L/minute. The tidal volume was calculated using the following equation:

Tidal volume (ml) = Animal Mass (kg) x 10 (ml/kg)

The respiratory parameters were monitored and adjusted so that the end-tidal CO_2 was maintained between 35-45mm Hg. This initial maintenance anaesthesia with isoflurane was later supplemented with ketamine (4mg/kg/hr), which allowed a reduction in the concentration of isoflurane from 2-2.5% to 1-1.5%, thus reducing its hypotensive effects (Fish 1997).

After animals were intubated, a 7-8cm midline incision was made on the left upper thigh and the femoral artery and vein exposed and separated. A small cut was then made on the wall of artery and a size 22 cannula was inserted into the vessel and fixed using No 2 silk double-ties both proximally and distally. The venous cannula was similarly inserted and the wound closed with a continuous suture using No 2 silk tie. The arterial line was connected with a pressure transducer and the arterial blood pressure recorded continuously via the MacLab data acquisition unit.

During each experiment arterial lines *were* also used to collect blood samples for arterial blood gas analysis. The venous line was connected to a 3-way tap and used for administration of ketamine and also vehicle or drug treatment.

After femoral artery and vein cannulae were inserted, the sheep was turned into the prone sphinx position and restrained to the table with the head resting on a customized chin support. On the right hip, an area was clipped and a monopolar diathermy return pad was placed and secured with a tape. The animal's head was then clipped and a midline incision (8-10cm) was made on the scalp using the diathermy machine (Solid State Electrosurgery with Isobloc. Model: SSE-2 K, ValleyLab Boulder, Colorado, USA). The scalp was retracted and sagittal and coronal sutures were exposed on the skull. On both sides, 15mm lateral from the midline and in front of the coronal suture; two burr-hole spots were marked and drilled for only 1-2mm in depth. On the left side of the head, after having previously clipped, the midpoint between the supraorbital process and external auditory channel was selected as the impact target and was marked for the accurate positioning of the captive bolt device before injury. Due to relatively low incidence of skull fractures no skull cap was used in our study.

Impact Acceleration Head Injury

The ovine impact acceleration head injury model that was used in the current study was initially developed in our laboratory by Lewis and colleagues (Lewis et al. 1996) and has been extensively characterized by van den Heuvel, Finnie and colleagues (Van den Heuvel *et al.* 1999, Finnie *et al.* 1999, Van Den Heuvel *et al.* 2000). Diffuse head injury is induced using a Captive humane bolt stunner (Model KML, Karl Schermer & C0., Germany). This device was approved

for use in livestock animal euthanasia by the American Medical Association and produces immediate loss of consciousness (1993, Nolen 2011). It utilizes a blank cartridge positioned proximally to a captive bolt and when discharged propels the bolt in the muzzle of the stunner device. The bolt weighs 385g and at its distal end has a mushroom-shaped head (4cm in diameter), which contacts to animal skull during the impact. The muzzle velocity depends on the charge level of a cartridge; in our experiments we have used a number 17 Red charge which induces moderate to severe head injury as established in previous studies (Lewis et al. 1996, Finnie et al. 2001, Finnie & Blumbergs 2002). The biomechanical characteristics of this injury have been described in detail by Anderson and colleagues (Anderson et al. 2003).

Immediately prior to impact, the endotracheal rube was disconnected from the ventilator and the chin support was removed allowing free motion of the head due to impact. The humane stunner device was positioned at the target site over the left temporal bone and discharged, causing a rapid accelerating/decelerating lateral and rotational movement of the unrestrained head. After injury, the endotracheal tube was immediately reconnected to the ventilator following impact. Scalp bleeding at the site of impact was controlled by using gauze with manually applied pressure. The head was subsequently secured on the chin support for ICP and LICOX probes insertion.

2.4.3 Intracranial Pressure Monitoring

Intracranial pressure was monitored using a Codman Microsensor ICP Transducer probe which is an intraparenchymal strain-gauge device that uses a Microsensor tip connected with a nylon catheter (1.2mm diameter; Codman and Shurtleff, Inc., DePuySpine™). The probe was

connected to the Codman ICP Express monitoring system (DePuySpine^{TM}), which is a digital system that relays the ICP as a digital signal-value recording in mmHg and systolic and diastolic pressures relative to this value. After being attached to the monitoring system, the probe was zeroed and then calibrated in sterile water according to manufacturer's instructions before inserting into the brain parenchyma of the animal for each experiment. Between experiments the tip of the probe was stored in normal saline solution within the calibration cylinder.

Following the injury the burr hole (2.5-3mm in diameter) was completed on the left side of the skull (15mm lateral to midline and in front of the coronal suture) and that the dura kept intact at the opening. A previously prepared and specifically modified No14-gauge intravenous cannula was placed into the hole in a way that the hub of the cannula was at the level of external surface of the skull and the cut-end of the plastic cannula was at the level of a dura. The probe was fixed using acrylic superglue. A small puncture was made on the dura using a 23-gauge needle and the ICP probe was inserted into the brain tissue so that the tip of the probe was in depth of 15 mm into the brain parenchyma below the dura. To prevent CSF leakage following the probe insertion the gauge was sealed using bone wax (Ethicon, W810). ICP data was continuously monitored for 4.5 hours using the MacLab data acquisition system.

2.4.4 Cerebral Tissue Oxygenation Monitoring

Brain tissue oxygenation ($P_{bt}O_2$) and temperature were monitored using a LICOX oxygen catheter-microprobe (LICOX CC1.R, 0.5mm diameter, Integra Neuroscience, Plainsboro, New Jersey). The LICOX CMP 'Smart Card' (distributed with each probe) was inserted into the LICOX CMP Tissue Oxygen Monitor, so that the calibration data recorded during manufacturing

would be integrated with the probe. The probe was then checked for calibration accuracy in a solution (bubbled with O_2 and N_2, respectively) of sterile water and in air. After each experiment the probe was kept wet in the packaging in the refrigerator according to manufacturer's instructions.

The second burr hole was completed on the right side of the sheep skull (15mm lateral to sagittal midline and just in front of the coronal suture) and the dura left intact at the opening. A specifically modified No 14-gauge intravenous cannula was placed into the hole in a way that the hub of the cannula was at the level of the external surface of the skull and the cut-end of the plastic cannula was at the level of a dura. The probe was fixed using acrylic superglue. The dura was then punctured using a 23-gauge needle and the LICOX probe inserted into the brain tissue so that the tip of the probe was 35mm below the dura and inside the brain white matter. This depth was determined during preliminary experiments and ensured that the sensor of $P_{bt}O_2$ was in white matter and the temperature sensor was within brain tissue. To prevent CSF leakage following the probe insertion the gauge was sealed using bone wax (Ethicon, W810). After insertion, the LICOX probe required 15-20 minutes for stabilisation in accordance with the manufacturer's instructions. The LICOX monitor was linked to the MacLab data acquisition unit and continuous recording of cerebral oxygenation and temperature were obtained for 4hr.

2.4.5 Arterial Blood Pressure Monitoring

Mean arterial blood pressure was monitored with a MacLab data acquisition system (MacLab 2e). The animal arterial catheter was connected to a Statham-type pressure transducer and the arterial blood pressure was continuously recorded via the MacLab data acquisition unit onto a

laptop computer running LabChart. The pressure trace was relayed from the transducer to the MacLab via a bridge amp.

2.4.6 Arterial Blood Gas Analysis

The Osmetech OPTI blood gas analyser (CCA, Helena Laboratories, Australia Pty Ltd) and Osmetech OPTI cassettes were used for arterial blood gas analysis. Arterial blood samples (0.6-0.7ml) were obtained via the femoral arterial cannula using a 3-way tap connecting the cannula with the transducer. PaO_2, $PaCO_2$, pH, SO_2, tCO_2, tHb, Hct, Na^+, K^+ and BE were measured during each arterial blood gas analysis. Arterial blood gas analysis was conducted 5 times during each ovine experiment. The first analysis was done 10min before the injury, and another four analysis were done every hour after the injury.

2.4.7 Perfusion

Perfusion for ovine experiments was performed while animals remained under general anaesthesia. For each sheep perfusion, 10L of 4% paraformaldehyde was administered via a pressurized container (120-140mm Hg pressure). Animals were transferred to the post-mortem room and placed on a dissecting table where exposure of both common carotid artery and internal jugular vein was made on both sides. Perfusion was achieved by flushing 4% paraformaldehyde under pressure through both carotid arteries and with the jugular veins cut to allow blood outflow. After fixation was complete, the skull was opened and the brain removed and kept in 5L 4% formalin solution for at least for two weeks.

2.5 Drug Treatments

All the drug solutions and the vehicle (0.9% saline) were prepared by an independent researcher to ensure the blinded nature of the study, and were administered 30min post-injury through the femoral venous catheter.

2.5.1 NAT

The NK-1 antagonist n-acetyl L-Tryptophan (NAT) (Sigma-Aldrich, Sydney, Australia) was stored at 2-8°C and prepared by dissolving NAT in saline at a ratio of [0.0125g NAT: 5ml saline]. The solution pH was adjusted to neutral (pH=7.4) using NAOH. NAT (2.5 mg/kg) was administered 30 min post-injury at a total volume of 1ml/kg via a femoral venous catheter.

2.5.2 Magnesium

Magnesium ($MgSO_4$) (Sigma Aldrich, Australia) was administered at a dosage of 30 mg/kg, 30min post-injury using a femoral venous catheter at a total volume of 1 ml/kg.

2.5.3 EU-C-001

The NK-1 antagonist EU-C-001 (donated by Eustralis Pharmaceuticals, Brisbane, Australia) was stored at a 25°C temperature and administered at a concentration of 1 mg/kg via a femoral venous catheter at 30min post-injury in a total volume of 1 ml/kg.

2.5.4 Hypertonic Saline

Hypertonic saline (HTS) was stored at room temperature and administered via a femoral venous catheter at a concentration of 11 mOsm/kg 30min post-injury at a total volume of 1 ml/kg.

2.5.5 Mannitol

Mannitol (Sigma-Aldrich, Sydney, Australia) was stored at 25°C temperature and was administered via a femoral venous catheter at a concentration of 11 mOsm/kg at 30min post-injury in a total volume of 1 ml/kg.

2.5.6 Progesterone

Progesterone (Sigma-Aldrich, Sydney, Australia) was stored at room temperature and protected from direct heat and sunlight. Progesterone was administered at a concentration of 1667µg/kg body weight dissolved in 66.7µl ethanol over low heat, and then added to 0.33ml sesame oil vehicle. Progesterone was administered 30min post-injury at a total volume of 1ml/kg via a femoral venous catheter.

2.6 Histology and Immunohistochemistry

2.6.1 Glass-Slides Coating With Silane

For ovine experiments, glass slides (Menzel-Glaser; 100 OTG 90º 50x76 mm 1.0 mm), were coated with silane to facilitate tissue fixation onto the slide. Slides were first washed with biodegradable dishwashing detergent and dried in an incubator at 60ºC for 60-90mins. They were then rinsed in absolute ethanol (100% ethanol) for 20-30sec and dipped into silane solution (10ml 3-aminopropyl-triethoxy-silane, Sigma Cat # A-3648, in 500ml absolute ethanol) for a few seconds. After rinsing in absolute ethanol, slides were washed in deionised water and dried in and incubator at 60 ºC and subsequently stored in boxes.

2.6.2 Tissue Processing

Specimens for paraffin embedding were post-fixed in 10% neutral buffered formalin (Fronine Laboratory supplies) for a minimum of two weeks before further processing. Processing was 20 minutes in each graded ethanol bath (50%, 70%, 80%, and 95% for once and 100% for twice), followed by two xylene baths for 1.5 hours each, and four paraffin baths of increasing time (30, 60, 60, and 90 minutes). 5 mm coronal sections were embedded in paraffin wax. Serial 5µm sections were cut using a microtome (Microm, Walldorf Germany), floated onto silane (Cat. No. A-3648; Sigma-Aldrich, Sydney, Australia) coated glass-slides (Menzel-Glaser; 100 OTG 50x76 mm 1.0 mm), and dried over the heater for 15min.

2.6.3 Haematoxylin and Eosin Staining

Slides were re-hydrated with xylene and two different ethanol solutions (100% and 90%) for one minute in each. Following a 1-minute rinse in running water tap water, slides were stained in Haematoxylin (Lillie Mayers) for 5-8 minutes. Slides were then rinsed in tap water for the second time followed by a dipping in a differentiator bath of acid alcohol, and a further rinse in tap water. Slides were then placed in saturated aqueous lithium carbonate for 10-15 seconds or until sections went blue. They were rinsed in running tap water for 5-6 minutes and then 2 minutes in Young's eosin (Eosin [yellowish] 15g, Erythrosine 5mg, calcium Chloride 5g, water 2L) for 2-4 minutes. The slides were then differentiated in tap water, dehydrated in ethanol (90% for 15 seconds, and then 2 x 100% for 15 seconds) cleared in xylene (2 baths of 2 minutes) and mounted in a synthetic Gurr's Depex mountant to coverslip. Once dried and excess resin removed, the sections were viewed by light microscopy (Olympus).

2.6.4 Substance P Immunohistochemistry

From selected wax-blocked sections, 5µm sections were cut using a microtome (Microm, Walldorf Germany) and immunolabelled with Substance P (SP) primary anti-goat antibody (1:2000 in NHS; polyclonal Santa Cruz cat. No. SC-9758) by overnight incubation at room temperature. After a PBS wash, slides were incubated with an anti-goat IgG-HRP conjugated secondary antibody (1:250 in NHS; Sigma-Aldrich) for a minimum of 30 minutes at room temperature. Slides were then incubated in the tertiary streptavidin peroxidase conjugate (SPC) (1:1000 in NHS; Pierce) for about 1hour at room temperature, and the subsequent immunocomplex visualised by using diaminobenzidine tetrahydrochloride (DAB) for 7 minutes as a chromogen in a peroxidase reaction (Sigma-Aldrich, Sydney, Australia).

2.6.5 Amyloid Precursor Protein (APP) Immunohistochemistry

From selected wax blocked sections, 5µm sections were sliced using a microtome (Microm, Walldorf Germany) and immunolabelled with amyloid precursor protein (APP) primary antibody (1:2000 in NHS; monoclonal antibody 22C11, Chemicon) by overnight incubation at room temperature. After washing the slices in PBS, slices were then incubated with an anti-mouse IgG-HRP conjugated secondary antibody (1:250 in NHS, Sigma-Aldrich) for 1h at room temperature, and the subsequent immunocomplex visualised using diaminobenzidine tetrahydrochloride (DAB) as a chromogen in a peroxidase reaction (Sigma-Aldrich, Sydney, Australia).

2.6.6 Albumin Staining

From selected wax-blocks, 5µm sections were cut using a microtome (Microm, Walldorf Germany) and immunolabelled with albumin primary goat polyclonal antibody (1:20 000 in NHS; Cat No: 0113-0344, Cappel Laboratories) by overnight incubation at room temperature. After washing the slices in PBS, slices were then incubated with an anti-goat IgG (H+L) conjugated secondary antibody (1:250 in NHS, Cat No: BA-9500, Vector Laboratories) for 1h at room temperature, and the subsequent immunocomplex visualised using diaminobenzidine tetrahydrochloride (DAB) as a chromogen in a peroxidase reaction (Sigma-Aldrich, Sydney, Australia).

2.7 Statistical Analysis

Data are shown as mean ± standard error of measurement (SEM). Repeated measures analysis of variance (ANOVA) followed by Bonferroni *t*-test (or post-hoc test). In all experiments a p value of 0.05 was considered significant. Prism (Graphpad™ Software, San Diego, CA) statistics computer program was used for all analysis. Machine learning with Gaussian processes was use to analyze the data in chapter 8.

CHAPTER 3

INTRACRANIAL PRESSURE CHANGES FOLLOWING TRAUMATIC BRAIN INJURY IN RATS: LACK OF CHANGE IN THE ABSENCE OF MASS LESIONS OR HYPOXIA

Gabrielian, L., Willshire, L.W., Helps, S.C., van den Heuvel, C., Mathisa, J. & Vink, R. (2011) Intracranial pressure changes following traumatic brain injury in rats: lack of significant change in the absence of mass lesions or hypoxia.
Journal of Neurotrauma, v. 28(10), pp. 2103-2111

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This publication is included on pages 94-114 in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

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CHAPTER 4

CHARACTERIZATION OF AN OVINE MODEL OF TRAUMATIC BRAIN INJURY

4.1 Introduction

In the previous chapter, we demonstrated that TBI induced in rats, either alone or in combination with secondary hypoxia, does not result in any sustained increase in ICP unless there was a mass lesion associated with a hemorrhage. Although the reasons for the lack of ICP change are unknown, we speculated that rats may be able to compensate for the intracranial expansion associated with cerebral edema along their craniospinal axis, partially because of an underdeveloped tentorium cerebelli. We concluded that the development of neuroprotective treatments targeting increased ICP after TBI might therefore be best pursued in other species that may more consistently produced sustained increases in post-traumatic ICP. More specifically, animals with a more developed tentorium cerebelli may be more useful in this respect since they might limit any post-traumatic increase in ICP to the supratentorial compartment, similar to that seen in human TBI.

In selecting an appropriate species to pursue studies of ICP after TBI, there are several morphological aspects of brain that also need to be considered. The relative allocation of white and grey matter in brain tissue is one of the most important aspects of morphology with regards to the type of injury observed and its severity (Hagberg *et al.* 2002). Specifically, the brain tissue of rodents has relatively little white matter compared to humans, rendering it difficult to reproduce an injury with a white matter response, including DAI and contusional brain swelling. Accordingly, using animal species with large white matter more proportional to that of humans would be preferable to reproduce these aspects of injury (Hagberg et al. 2002). Surface structure of the brain is another important aspect in regards to injury mechanisms and outcome. Rodents have a lissencephalic brain, while humans have a well-developed gyrencephalic brain. This

structural difference in brain tissue changes its mechanical properties as well as the distribution of vasculature inside the brain tissue, resulting in different clinical outcomes following brain injury. Finally, the connectivity and organization of the brain is a morphological difference between small animals and humans that needs to be considered when selecting an appropriate species so as to mimic human pathophysiology after TBI.

In previous work emanating from our laboratory, an ovine (sheep) model of diffuse injury was developed that replicated a number of aspects of human blunt TBI, including diffuse axonal injury apparent in the white matter domains, plus grey matter contusions. This model of TBI is particularly relevant to humans, in part because sheep have gyrencephalic brains with large white matter domains, but also because the brain in sheep is supported by a substantial bony and fibrous tentorium that is similar to humans (Bull 1969). Thus, sheep are better suited to reproduce the post-traumatic physiological state that exists in human TBI than in rodents. Of particular relevance to the present thesis, an increase in supratentorial ICP was noted immediately after the induction of acceleration injury in this model (Lewis et al. 1996). However, these animals had the ICP probe in place before induction of injury, invariably resulting in probe-induced tissue damage that is not present in clinical TBI. Moreover, the presence of hemorrhage around a fixed ICP probe complicates interpretation of the ICP response, with the presence of a craniotomy defect altering both the response of the skull to impact and the physiological conditions experienced by the brain in what is normally a completely enclosed space.

In the present study, we have therefore focused on placing the ICP probe as quickly as possible after experimental trauma, and then monitoring ICP for 4 h after TBI. Thus injury would be induced in an enclosed skull and the craniotomies for probe placed performed after the injury. Furthermore, the present studies have included monitoring of brain oxygenation ($P_{bt}O_2$) after TBI, thus more clearly delineating the nature and degree of acute changes in both ICP and $P_{bt}O_2$ that occur following blunt trauma.

4.2 Materials and Methods

All studies were performed according to the guidelines established by the National Health and Medical Research Council (NH&MRC) of Australia, for the use of animals in experimental research and were approved by the Animal Ethics Committees of the Institute of Medical and Veterinary Science and The University of Adelaide, South Australia.

Two-year-old sterilised male Merino sheep (wethers) were anesthetised by an intravenous injection of thiopentone before intubation and ventilation (4L/min) with oxygen enriched air (30 – 35% oxygen) containing 2.5% isoflurane. A femoral arterial catheter was implanted to continuously monitor mean arterial blood pressure (MABP) using a MacLab data acquisition system (MacLab 2e), where a Statham-type pressure transducer was connected via a polyethylene tube to the animal's arterial cannula. The pressure trace was relayed from the transducer to the MacLab via a bridge amp and all data recorded and stored using a personal computer. Arterial blood was taken every hour for gas analysis and adjustment of ventilation parameters as necessary. After placement of the catheter, isofluorane was lowered to 1.0-1.5%

and an intravenous infusion of Ketamine (4mg/kg/h) was initiated. This regime provided an appropriate level of anesthesia for surgery with intact cardiovascular reflexes.

Injury was induced using a humane stunner (Lewis et al. 1996, Van Den Heuvel et al. 2004) armed with a number 17 red charge (model KML, Karl Schermer & Co., Germany) according to the detailed methodology described in Chapter 2. Briefly, the sheep were placed into a prone sphinx position, and impact injury induced at the midpoint between the left supraorbital process and the left external auditory meatus (Fig. 4.1) using the captive humane bolt stunner. After injury, animals were stabilised and the heads restrained to the operating table to facilitate insertion of both the ICP and $P_{bt}O_2$ probes as described in detail in sections 2.4.3 and 2.4.4. Briefly, the ICP burr hole was completed on the left side of the skull, 15mm lateral to midline and in front of the coronal suture, while the second burr hole for $P_{bt}O_2$ monitoring was completed on the right side of the skull, 15mm lateral to sagittal midline and just in front of the coronal suture (Fig. 4.2). Specifically modified No14-gauge intravenous cannulae was placed into the holes and fixed into place using acrylic superglue (Fig. 4.3). The ICP and $P_{bt}O_2$ probes were inserted to a depth of 15 mm and 35 mm, respectively (see Fig 4.4). To prevent CSF leakage, the gauges were sealed using bone wax (Ethicon, W810) and ICP and $P_{bt}O_2$ data was then continuously obtained for 4 hours using the MacLab data acquisition system.

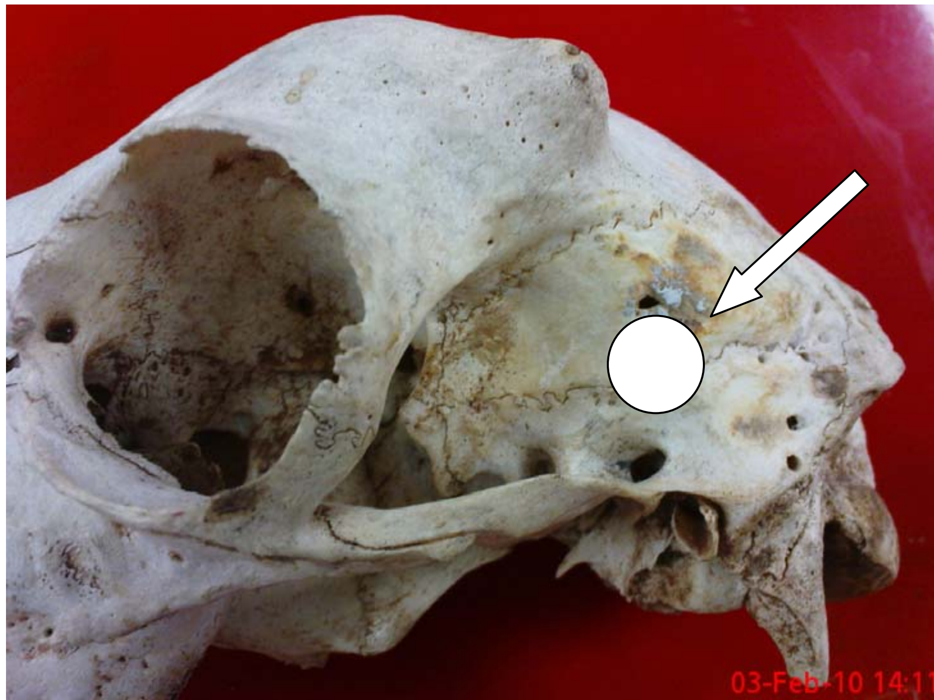


Figure 4.1 Location of a delivered injury on the skull of a sheep; temporal fossa.

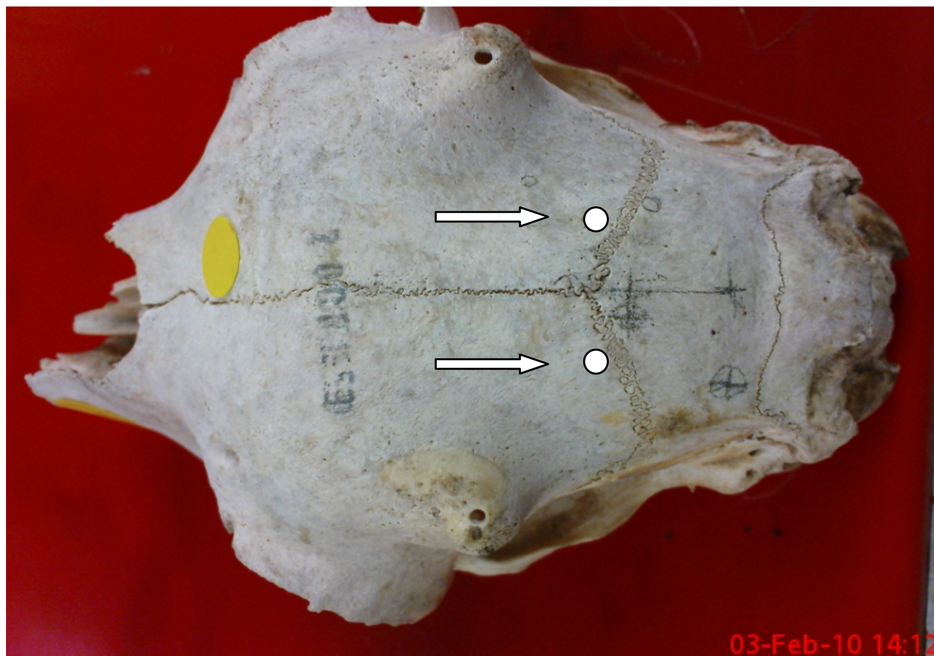
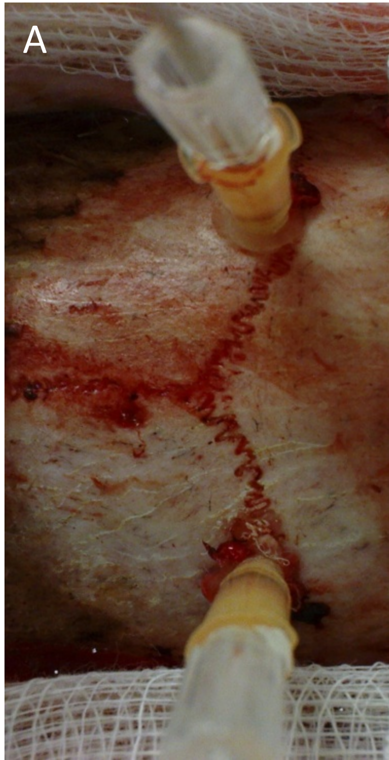


Figure 4.2 ICP and Licox probe locations at 1.5 cm lateral from sagittal suture and in front of transverse lambda suture.



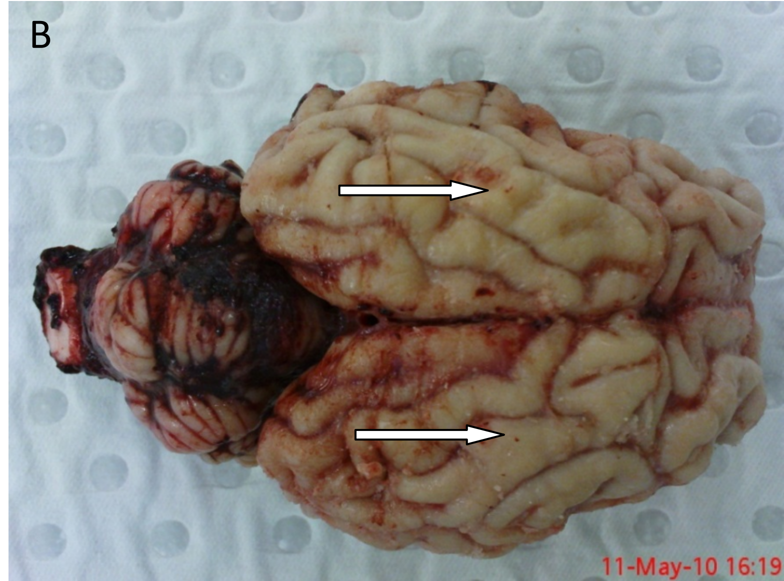


Figure 4.3 ICP and Licox probes inserted into the brain through a sheep skull; (A) during the experiment, (B) their locations on both hemispheres after the experiment.

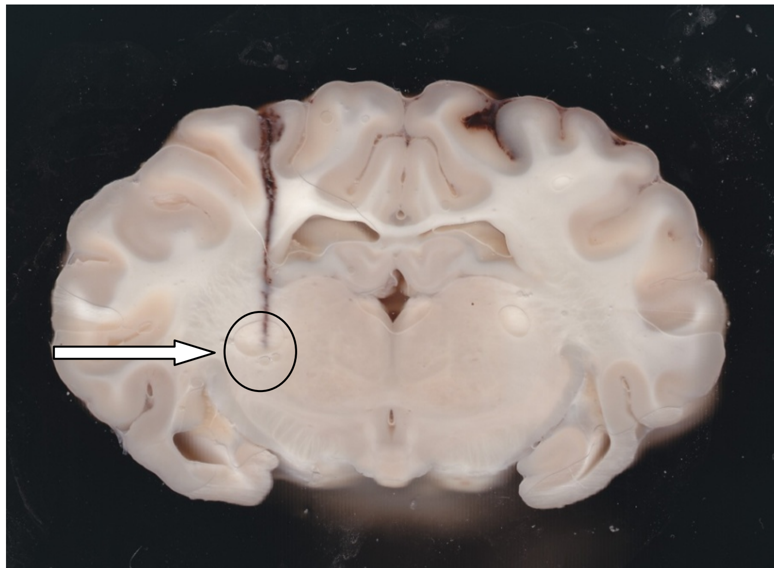


Figure 4.4 Trace of a Licox probe on sagittal section of a 5 mm coronal slice (the tip of a probe is in white matter).

Eight animals were used as sham controls, where animals were surgically prepared and monitoring initiated in the absence of any induced brain injury, to establish baseline ICP and $P_{bt}O_2$ values. Animals were euthanised by barbiturate overdose at the conclusion of the 4-hour monitoring period and the brains removed for macroscopic evaluation and microscopic confirmation of diffuse axonal injury by APP immunohistochemistry (section 2.6.5).

Physiological data (including MABP, ICP, CPP and $P_{bt}O_2$) are expressed as mean and standard error of the mean (SEM) and were analysed by one-way analysis of variance (ANOVA) followed by Duncan's tests (PRISM, Graphpad Software, San Diego). A p value of less than 0.05 was considered significant.

4.3 Results

There was a degree of variability in the initial studies that was largely accounted for by the status of the humane stunner and the charge. Specifically, initial experiments on a limited number of animals produced either few changes in physiological variables or severe injury with open skull fractures. Upon inspection, the humane stunner was defective and required servicing, while the charges were beyond their expiry date and unpredictable in their behaviour. After servicing of the stunner and replacement of the charges, the injury became very reproducible and induced the moderate injury that we expected from the previous studies in our laboratory (Lewis et al. 1996, Van Den Heuvel et al. 2004). Although the early physiological data was accordingly excluded from the analysis (e.g., ICP and $P_{bt}O_2$), it was nonetheless interesting to examine the effects of variable injury on macroscopic and microscopic brain changes to gain a better appreciation of the ovine TBI model.

Macroscopic changes following TBI were evaluated using such parameters as skull fracture, intracranial bleeding, contusion, and brain laceration. These macroscopic changes fell into three broad categories of severity, namely mild, moderate, and severe (Fig. 4.5). Animals exhibiting no skull fracture, with local, patchy intracranial (subarachnoid, subdural) haemorrhage over the left hemisphere, and a cortical contusion with no brain laceration at the site of the injury, were classified as mildly injured. The moderate injury group was defined on the basis of a closed, small skull fracture at the site of injury, more widespread intracranial subarachnoid and subdural hemorrhages, a moderate size contusion at the site of injury, and subarachnoid and subdural bleeding at the brain stem. In contrast, animals with an open skull fracture at the site of injury, widespread intracranial bleeding over both hemispheres, subarachnoid and subdural haemorrhage surrounding the brain stem, and severe contusion with significant brain laceration at the site of an injury and other areas of brain tissue, including occasional countercoup injuries, were considered as severely injured animals (Fig. 4.5).

At the microscopic level, widespread AI along the lengths of the axons as indicated by APP immunostaining as well as axonal swelling and lobulation was observed at different levels in all animals (Fig. 4.6). There was extensive high level AI in the corpus callosum and fornix compared to other areas such as the thalamus, hippocampus, and the margins of lateral ventricles. Axonal injury was more apparent in temporal sections compared to frontal sections of the brain, and in most cases the left hemisphere that was exposed to the impact injury showed more axonal injury than the right.

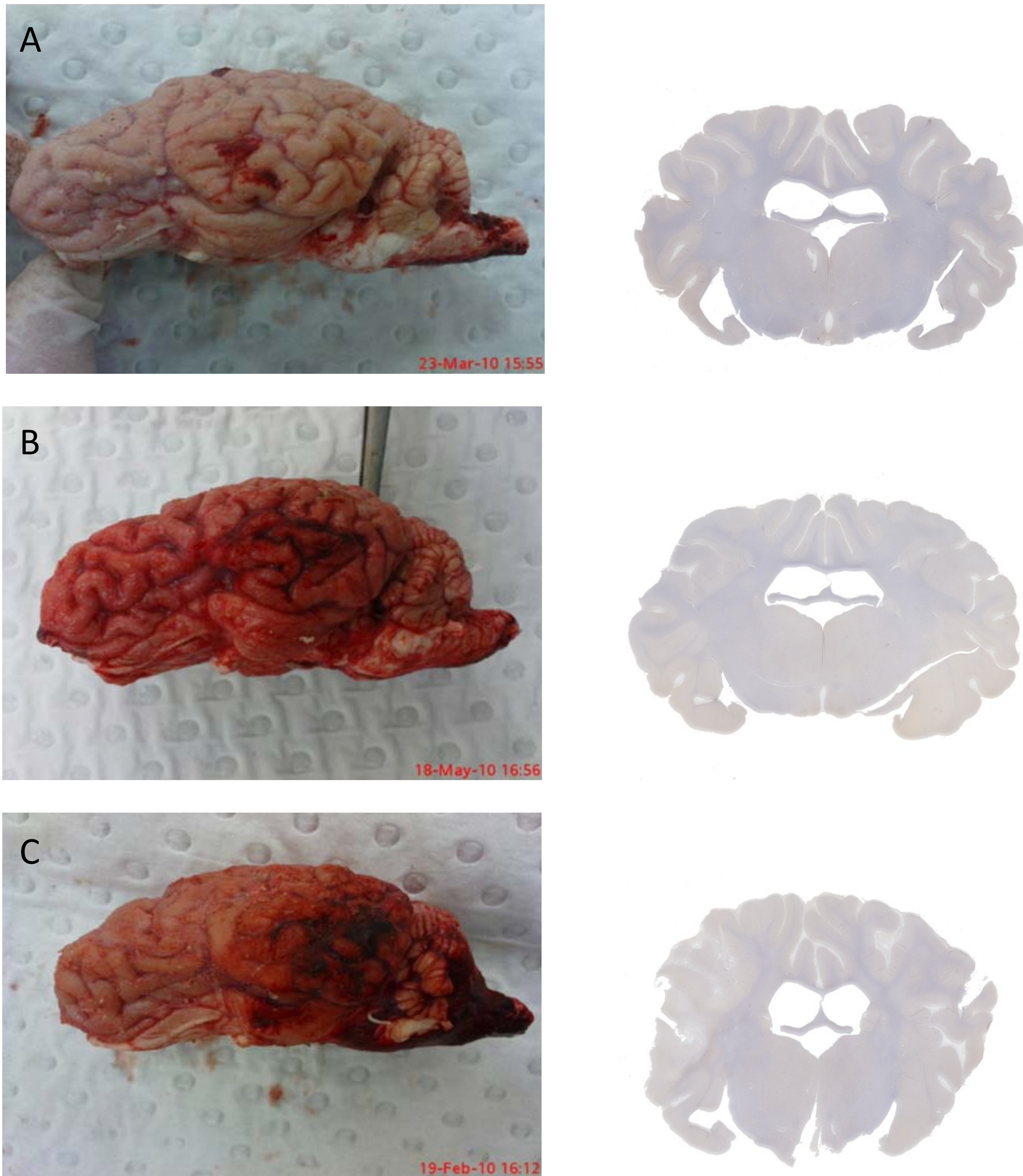


Figure 4.5 Typical examples of sheep brain after mild, moderate and severe TBI; (A) mild injury following TBI; (small SA haemorrhage over left temporal lobe and brain stem), (B) moderate injury following TBI; (SA and SD haemorrhage over left temporal lobe and around brain stem, with underlying brain contusion and laceration), (C) severe injury following TBI; (SA and SD haemorrhage over left temporal lobe and around brain stem, with underlying severe brain contusion and laceration).

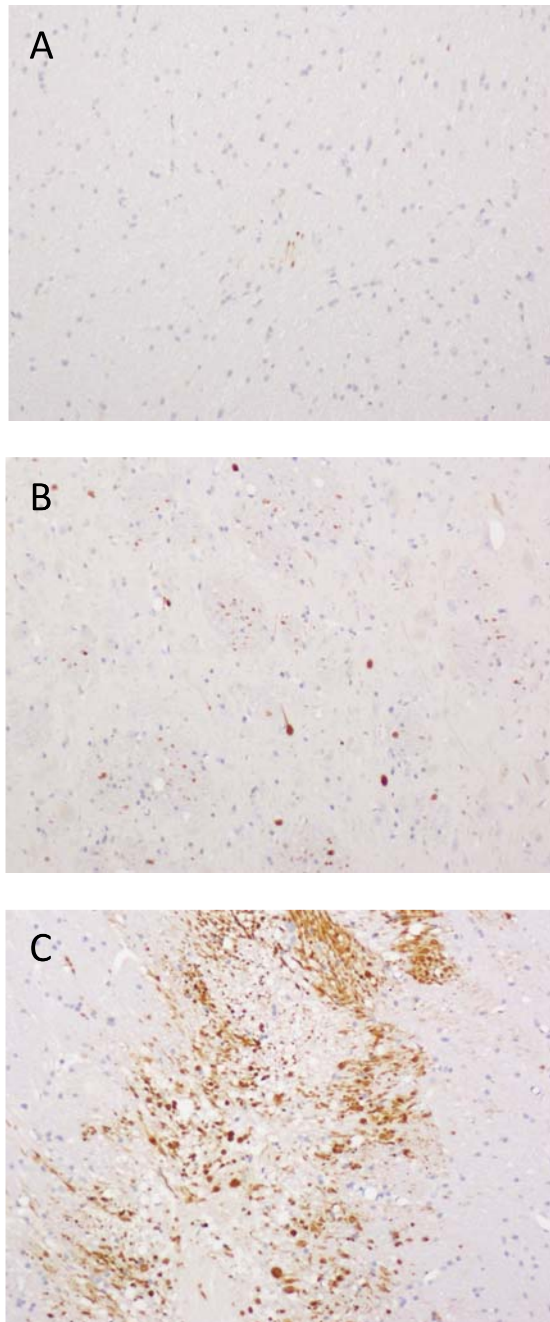


Figure 4.6 Microscopic evaluation of TBI severity using APP immunostaining; (A) typical levels of APP seen in axons (AI) related with mild TBI in white matter, (B) typical levels of APP seen in axons (AI) related with moderate TBI in white matter, (C) typical levels of APP seen in axons (AI) related with severe TBI in white matter.

With respect to physiological variables, only moderate injury animals (n=8) were analysed given that this severity was preselected for any future study of interventional pharmacotherapies. The temporal profile of the changes in physiological variables could be subdivided into the immediate profound changes occurring in the first 30 min after injury (Fig. 4.7) followed by a prolonged period of relative stability between 1 and 4 h, the end point of our data collection (Fig. 4.8).

Baseline MABP prior to injury was 109 ± 4 mm Hg (Figure 4.7). Immediately after trauma, there was a highly significant ($p < 0.001$) increase in MABP to 176 ± 10 mm Hg, followed by a decline over time with significant hypotension at 25 minutes post-trauma ($p < 0.05$). Although a transient increase in MABP occurred between 30 min and 1 h, there was a gradual development of sustained hypotension thereafter, showing a minimum of 65 ± 8 mm Hg at 4 hours after TBI ($p < 0.001$; Figure 4.8). Transient hypertension followed by sustained hypotension is typical of experimental TBI (McIntosh et al. 1989, Marmarou et al. 1994, Lewis et al. 1996).

Normal ICP in sham (non-injured) animals was 7 ± 2 mm Hg (Figure 4.7). At 10 minutes after TBI, which was the earliest time point at which the craniotomy was completed and the ICP probe successfully implanted in all injured animals, ICP was 56 ± 7 mm Hg. The ICP gradually decreased to 25 ± 2 mm Hg by 60 minutes after TBI (Figure 4.8), before again increasing to 32 ± 2 mm Hg by 4 hours after injury. The increase in ICP between 1 and 4 hours after trauma has been previously associated with ongoing edema formation (Vink *et al.* 2008, Byard *et al.* 2009). ICP was significantly greater than baseline values at all time points ($0.001 < p < 0.05$).

Cerebral perfusion pressure (CPP), as determined by the difference between MABP and ICP, was 99 ± 6 mm Hg in sham (uninjured) animals. Immediately after ICP probe insertion, CPP was calculated as less than 60 mm Hg (Figure 4.7), and did not recover over the ensuing 4 hours (Figure 4.8).

Brain tissue oxygen partial pressure ($P_{bt}O_2$) was 42 ± 2 mm Hg in sham (uninjured) animals. At 1 h post-trauma, which was the earliest time point possible for $P_{bt}O_2$ given that this probe was placed after the ICP probe and that the LICOX probe takes up to 15 min to stabilize, the $P_{bt}O_2$ had decreased to 24 ± 4 mm Hg ($p<0.001$) and did not change significantly for the remainder of the 4 h monitoring period (Fig. 4.9). The LICOX probe also has a temperature sensor that could be used to monitor brain temperature at all time points. Throughout the experiment, brain temperature did not change significantly in any group of animals from a mean of 38.3 ± 0.1 °C.

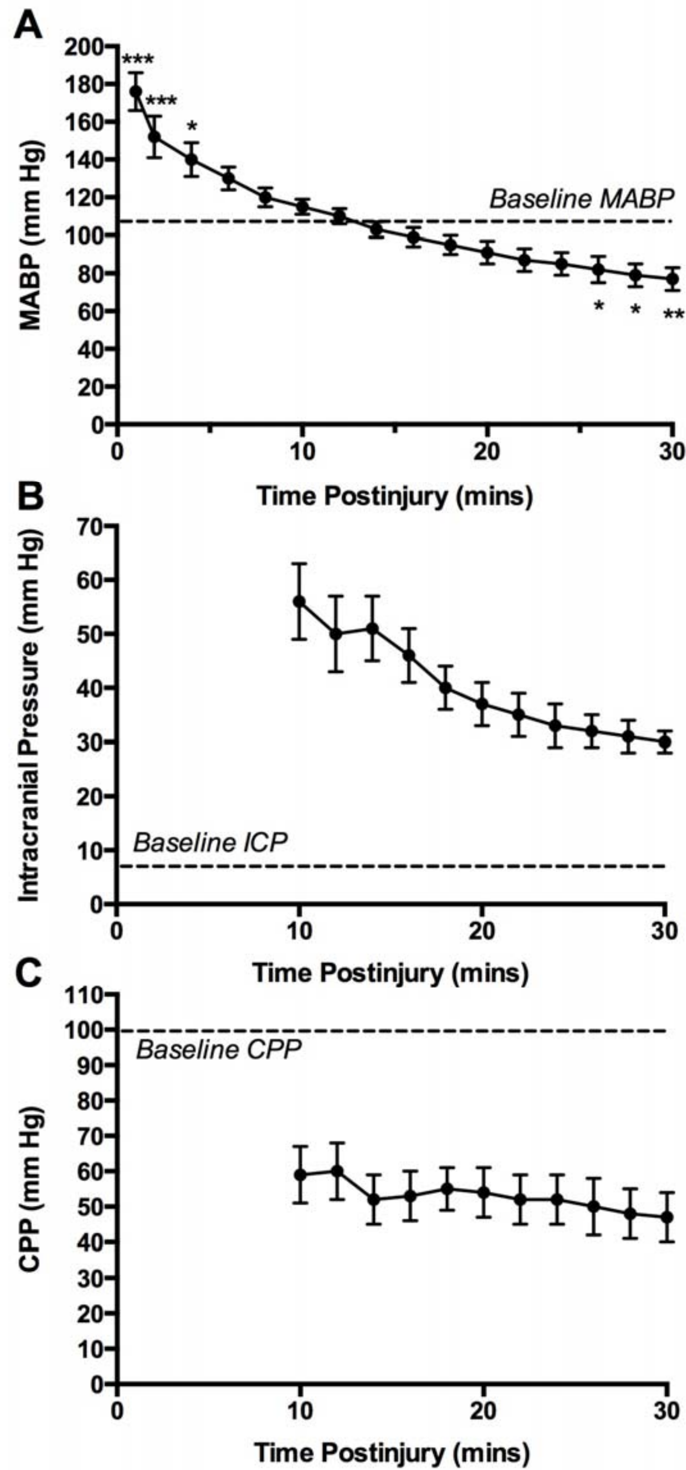


Figure 4.7 Changes in (A) MABP, (B) ICP and (C) CPP immediately following moderate TBI in sheep (n=8).

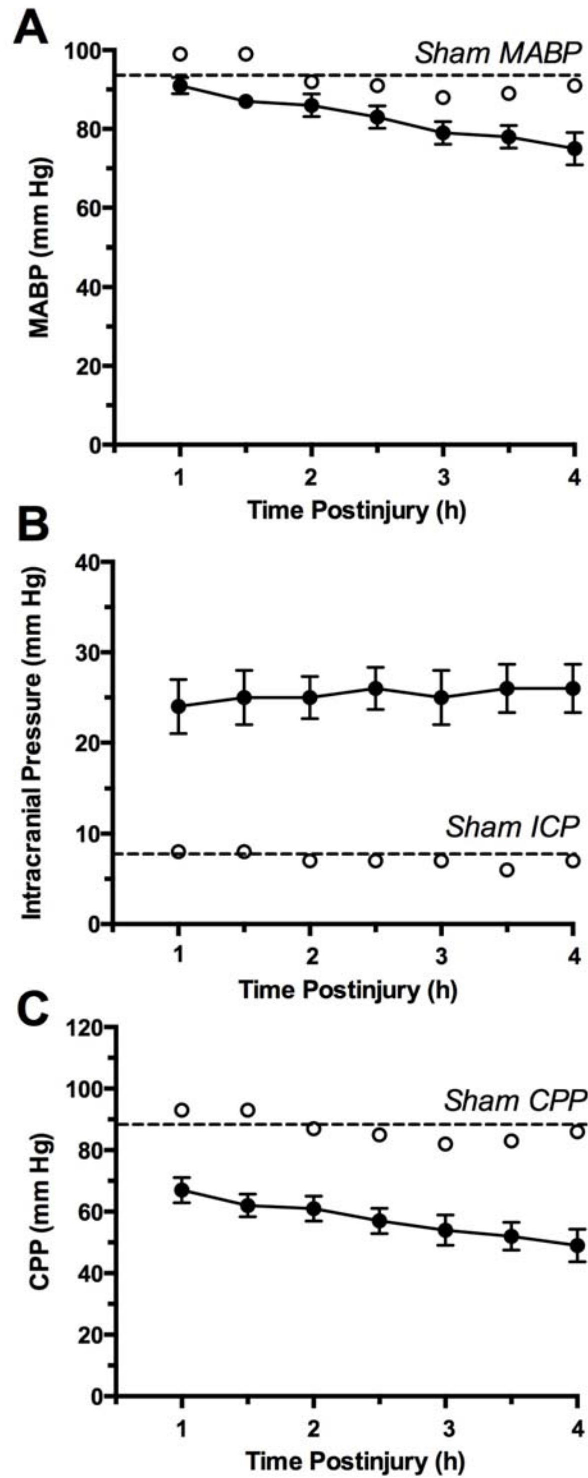


Figure 4.8 Changes in (A) MABP, (B) ICP and (C) CPP over 4 h following moderate TBI in sheep (n=8).

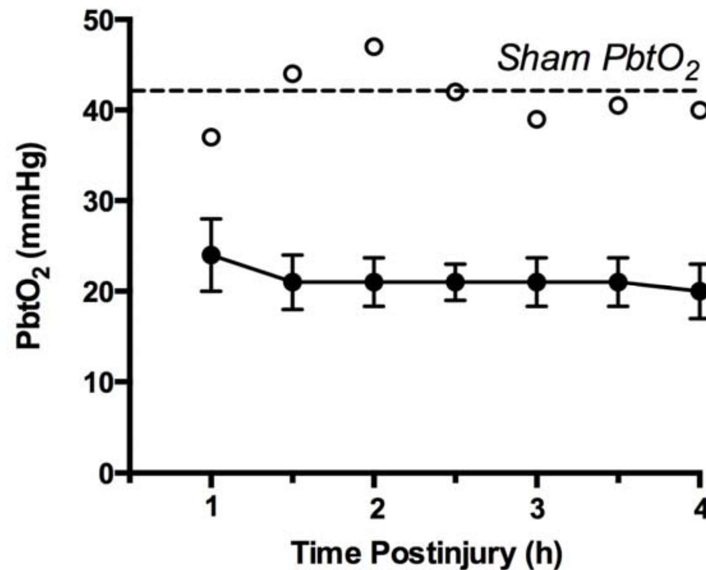


Figure 4.9 Changes in $P_{bt}O_2$ following moderate TBI in sheep (n=8). Data acquisition commenced at 1 h after injury, reflecting the time to insert the LICOX probe and for the probe to stabilise.

4.4 Discussion

In the current experiments, we have demonstrated that diffuse TBI in the sheep results in a consistent and sustained increase in ICP, which together with the previously described post-traumatic hypotension, decreases CPP and brain tissue oxygenation. In previous studies by our group, blunt cranial trauma in the sheep model produced profound increases in ICP immediately after induction of injury (Lewis et al. 1996). However, the implantation of the ICP probe prior to induction of injury, and the free movement of the head with injury, would have potentially resulted in direct mechanical disruption of brain tissue from the inserted probes, with resultant haemorrhage. This may have profoundly influenced the result. In the current study, I have placed

the probe immediately after trauma, and have focused on minimising the time required to perform the craniotomy and correctly placing the ICP probe. I managed to consistently complete the procedure in less than 10 minutes, and in so doing, have been able to demonstrate profound and highly significant increases in ICP occurring within the first 30 min after trauma that would be independent of any direct tissue damage by the probe.

The current study sheds further light on the nature of the changes in ICP immediately following blunt cranial trauma, as increases in ICP followed a biphasic pattern, with an initial sharp rise followed by a steady reduction to levels of around 25 mm Hg. Subsequently there was a steady increase in ICP over the ensuing hours. The initial very rapid increase in ICP occurred within 10 minutes and was correlated with systemic hypertension and is most likely due to reactive vasodilatation, as significant edema may take some time to develop after an injury (Barzo *et al.* 1997a, O'Connor et al. 2006). The peak of ICP due to early vasodilatation then reduced, to be followed by slowly increasing pressures. Given the previously shown positive immunohistochemical staining of albumin extravasation after sheep TBI (Byard et al. 2009), the slow rise in ICP from 1 h to 4 h is most likely associated with developing brain oedema, and more specifically over this time frame, vasogenic oedema. Throughout the entire monitoring period (from 10 min post-trauma), CPP in the injured sheep was less than 60% of normal values. Whether the immediate increase in MABP after the injury represents an attempt to maintain CPP in response to a significant increase in vasodilation-dependent ICP is unknown. Following the vasodilation-dependent increase in ICP, MABP declined and remained at hypotensive levels throughout the remainder of the observation period. Although ICP levels did reduce from an

early peak, the mean values were always greater than 25 mm Hg, a level previously shown to be associated with increased mortality (Treggiari *et al.* 2007).

The immediate increase in ICP and the decrease in CPP were associated with a reduction in cerebral oxygenation as measured using a LICOX tissue oxygenation probe. Indeed, $P_{bt}O_2$ was at approximately 50% of sham values throughout the entire 4 h monitoring period. Such a decrease may contribute to the hypometabolism previously reported in TBI (Marcoux *et al.* 2008). The results are also consistent with other TBI studies using large gyrencephalic animals. For example, in blunt impact in piglets, ICP increased significantly to 40 mm Hg, cerebral perfusion pressures fell from 85 to 40 mm Hg, and cerebral blood flow dropped from 55 to 22 ml/min/100gm (Pfenninger et al. 1989). However, in the current study, I have further shown that the temporal profile of these changes could be divided into two distinct phases: vasoactive responses occurring in the first 30 min immediately after the impact, and those occurring between 1 and 4 h after injury that are most likely associated with development of oedema.

While pressors can be used to improve MABP after TBI, there is no therapy that is universally accepted as an ideal treatment for increased ICP after acute injury. Such reduction in ICP is critical, not only for improving CPP beyond what can be achieved with pressors, and accordingly improve brain tissue oxygenation, but also for the prevention of herniations that frequently occur in the most severe forms of TBI and result in death. Having characterized the ovine model of TBI as ideal for replicating ICP and LICOX changes observed in human TBI, the following chapters will seek to characterize pharmacological interventions that hold promise in the management of post-traumatic increases in ICP.

CHAPTER 5

EFFECTS OF NK1 ANTAGONIST-NAT VERSUS MANNITOL ON ICP, MABP, CPP, AND $P_{bt}O_2$ CHANGES AFTER TRAUMATIC BRAIN INJURY IN SHEEP

5.1 Introduction

Traumatic brain injury (TBI) in developed countries is the leading cause of death and disability in people under 40 years of age (Harrison *et al.* 2008**).** While a number of injury factors have been identified as playing a role in its pathophysiology (Vink & Nimmo 2009, Loane & Faden 2010), it is widely recognized that water accumulation, or brain edema, is closely associated with patient outcome (Marmarou 2007). Indeed, up to half of all deaths following TBI having been attributed to brain edema (Feickert et al. 1999), Rowlands & Morris 2007). Brain edema often results in increased ICP, which subsequently causes local tissue compression, reduced perfusion pressure, hypoxia, ischemia, brain herniations and, in severe cases, death. While a number of treatment strategies have been introduced to reduce edema-associated brain swelling(Meyer et al. 2010), these largely target the end result of edema rather than its cause. As such, these strategies are limited in their effectiveness.

Increased ICP would require an increase in the amount of water (volume) in the cranial vault. The primary source of this water is the vasculature where water originating from the blood vessels would increase the total fluid volume and drive an increase in ICP (Simard et al. 2007, Donkin & Vink 2010). The entry of water from the vasculature into the brain tissue is termed vasogenic edema and has long been recognized as a contributor to edema formation in TBI(Marmarou 2007). Vasogenic edema is dependent on an increased permeability of the blood brain barrier (BBB). Our own recent studies have shown that BBB permeability and edema formation after TBI is linked to the release of the neuropeptide, substance P (Donkin et al. 2009). Furthermore, the administration of a substance P, NK1 receptor antagonist after acute brain injury restores BBB integrity and reduces edema formation (Donkin et al. 2009, Turner *et al.*

2011). Since restoring BBB integrity after TBI would potentially attenuate the movement of water from the vasculature to the brain parenchyma, we sought to determine the effects of an NK1 receptor antagonist on ICP after TBI.

While a number of effective animal models have been developed that recreate many of the pathophysiological responses observed clinically, rat models are the model of choice in neurotrauma research because of their cost effective and highly reproducible nature (Cernak 2005). However, in terms of their ICP response after trauma, our own studies have shown that rats do not consistently produce an increase in ICP after TBI (Chapter 3). As an alternative, we have developed a diffuse model of TBI using sheep that reproduces consistent changes in ICP and brain tissue oxygenation ($P_{bt}O_2$) that are more representative of the clinical situation than that observed in the more commonly used rodent models (Chapter 4). Accordingly, we have for the first time used the ovine TBI model to characterise the effects of the NK1 receptor antagonist, n-acetyl-L-tryptophan (NAT), on ICP and $P_{bt}O_2$, and to compare these effects against the clinically used osmotic agent, mannitol. We focus on ICP since pressor agents can be effectively used to increase MABP and CPP, although no treatment has been developed that can effectively reduce ICP. We have used $P_{bt}O_2$ as a primary outcome measure given that the goal of interventional therapy is to improve effective brain oxygenation. Finally, we have restricted monitoring to the period after 30 minutes so as the vasodilation-dependent changes would have largely subsided (Chapter 4), and the focus would be on effects of the treatment administered at 30 min after TBI.

5.2 Materials and Methods

Subjects

All experimental animal studies were approved by the local animal ethics committees representing SA Pathology and The University of Adelaide, South Australia, and were performed according to the guidelines established by the National Health and Medical Research Council (NHMRC), Australia.

5.2.1 Induction of Impact Acceleration Head Injury

Two-year-old, sterilised male Merino sheep (n=34; 52 ± 5 kg) were injured using the humane stunner as described in detail in Chapter 2. Briefly, animals were anesthetized by an intravenous injection of thiopentone before intubation and ventilation (4L/min) with oxygen enriched (30-35%) air containing 2.5% isoflurane. A femoral arterial catheter was then implanted to continuously monitor mean arterial blood pressure (MABP) using a MacLab data acquisition system (MacLab 2e). After placement of the catheter, isoflurane was lowered to 1.0-1.5% and an intravenous infusion of Ketamine (4mg/kg/h) was initiated, thus providing an adequate level of anesthesia for surgery with intact cardiovascular reflexes. Temperature was maintained using a thermostatically controlled heating pad while ventilation parameters were adjusted as necessary on the basis of arterial blood sampled at regular intervals for gas analysis.

To induce injury, sheep were placed into a prone sphinx position, and their torso restrained to the surgical table leaving the neck and head mobile relative to the body. Impact acceleration injury was induced at the midpoint between the left supraorbital process and the left external auditory meatus using a captive humane bolt stunner armed with a number 17 red charge (model KML,

Karl Schermer & Co., Germany). We have previously shown that this impact causes moderate diffuse axonal injury (Lewis et al. 1996, Van den Heuvel et al. 1999).

5.2.2 Groups and Drug Treatments

At 30 min after induction of TBI, animals were intravenously administered 20% mannitol (11 mOsm/kg; n=6), 2.5 mg/kg n-acetyl-tryptophan (NAT; n=10) or equal volume saline (n=9), with the dosage used based on previously published work (Donkin et al. 2009, Meyer et al. 2010). Animals were randomly assigned to a treatment group and drug treatment was blinded. A further 9 animals were used as controls (shams) to establish normal, baseline ICP and $P_{bt}O_2$ values in surgically prepared animals in the absence of any induced brain injury. ICP and $P_{bt}O_2$ was recorded in all animals for a period of 4 h, after which animals were euthanized by barbiturate overdose and their brains removed to confirm lack of any supratentorial mass lesions.

5.2.3 ICP, $P_{bt}O_2$, MABP, CPP Monitoring

After injury, animals were stabilized and the heads restrained to the operating table to facilitate insertion of ICP and $P_{bt}O_2$ probes between 10 and 30 min after trauma as described in detail in Chapter 2.

Intracranial pressure monitoring

Following exposure of the skull, a 2.5 mm burr hole was performed at a point 15 mm lateral to the sagittal midline on the ipsilateral side just in front of the coronal suture. A 1.73 mm diameter catheter was fixed into the burr hole, the dura matter opened and a calibrated Codman Microsensor ICP transducer inserted such that the tip of the sensor was 1.5 cm into the

parenchyma of the left parietal lobe. The probe was attached to a Codman ICP Express monitoring system (Codman and Shurtleff Inc., USA) which was linked to an AD Instruments PowerLab® system where the data was digitally recorded.

Cerebral tissue oxygenation monitoring

A second burr hole 1.5 cm lateral to the sagittal midline and over the left fronto-parietal suture allowed insertion of the distal end of a LICOX® $P_{bt}O_2$ probe to a depth of 3.5 cm. The LICOX® probe, which contained an integrated brain temperature sensor, was attached to a LICOX® brain tissue oxygen monitoring system (Integra, USA) for digital recording. After insertion of the probes, both burr holes were sealed using bone wax.

Mean arterial blood pressure monitoring

Mean arterial blood pressure was monitored with a MacLab data acquisition system (MacLab 2e). The animal femoral artery catheter was connected to a Statham-type pressure transducer and the arterial blood pressure was continuously recorded via the MacLab data acquisition unit onto a laptop computer running LabChart. The pressure trace was relayed from the transducer to the MacLab via a bridge amp. In addition data was collected manually every 15min.

Cerebral perfusion pressure monitoring

Cerebral perfusion pressure (CPP) was calculated by the equation:

$$CPP=MABP-ICP$$

CPP was monitored and recorded using the same MacLab data acquisition system, used for ICP and MABP data recording, and was linked to a personal computer.

5.2.4 ABG Analysis

Arterial blood gas analysis was conducted 5 times during each experiment: the first one being done 10min before the IA injury, and other four analyses being done every other hour after the injury. The Osmetech OPTI blood gas analyser (CCA, Helena Laboratories, Australia Pty Ltd) and Osmetech OPTI cassettes were used for arterial blood gas analysis. Arterial blood samples (0.6-0.7ml) were obtained using the femoral arterial cannula using a 3-way tap connecting the cannula with the transducer. PaO_2, $PaCO_2$, pH, SO_2, tCO_2, tHb, Hct, Na^+, K^+ and BE were measured during each arterial blood gas analysis.

5.2.5. Statistical Analysis

Data are expressed as mean and standard error of the mean (SEM) and were analysed by two-way analysis of variance (ANOVA) followed by Bonferroni correction (PRISM, Graphpad Software, San Diego). A p value of less than 0.05 was considered significant.

5.3 Results

There were no significant differences between groups with respect to MABP, blood pH and gases, or in brain temperature after TBI (Table 5.1), confirming that no treatment had any significant effect on any of these parameters in the 4 h monitoring period. Interestingly, the posttraumatic hypotension described in Chapter 4 was not as significant in the current group of injured animals, although a slow decrease in MABP from a baseline (pre-injury) level of 109 ± 4 mm Hg was again observed in all groups over time. Presumably the slight decrease in observed MABP in the sham animals was related to the effects of prolonged anaesthesia.

Table 5.1 Changes in MABP, blood PaO_2, blood $PaCO_2$, blood pH and brain temperature over 4h following traumatic brain injury in sheep (mean ± SEM).

	Sham		Vehicle		Mannitol		NAT	
	1h	4h	1h	4h	1h	4h	1h	4h
MABP (MM Hg)	99±6	91±7	112±3	104±6	117±10	93±9	112±10	97±9
PaO_2 (mm Hg)	137±10	165±3	168±2	161±5	131±8	125±6	155±7	151±5
$PaCO_2$ (mm Hg)	44±3	37±4	39±4	35±3	43±3	40±2	38±2	36±4
pH	7.47±0.03	7.51±0.02	7.52±0.02	7.52±0.02	7.50±0.03	7.48±0.01	7.49±0.02	7.49±0.01
Brain Temp (°C)	37.1±0.4	37.6±0.6	37.2±0.2	37.7±0.3	38.9±0.2	39.8±0.2	38.3±0.3	38.3±0.6

5.3.1 Effects of NAT vs. Mannitol on ICP after TBI

ICP in uninjured, control animals was 7 ± 2 mm Hg (mean ± SEM). By 30 min after TBI, and immediately prior to drug administration, ICP in all groups had increased significantly ($p<0.05$) to approximately 20 mm Hg (Fig. 5.1). The consistency in ICP values between groups at 30 min suggests that the severity of injury was similar in all groups. In vehicle (saline) treated animals, ICP continued to increase such that by 4 h post-injury, it was 26 ± 3 mm Hg ($p<0.001$). This increase in ICP is consistent with the continued oedema formation previously shown in diffuse models of TBI, including the sheep, and shown to peak between 4 and 6 h after injury (O'Connor et al. 2006, Byard et al. 2009). In contrast to the saline vehicle treated animals, administration of the NK1 antagonist at 30 min post-injury resulted in a profound and sustained reduction in ICP, which was significantly different from vehicle treated animals from 1.5 h onwards ($0.001 < p < 0.05$). Administration of mannitol similarly resulted in a decline in ICP, albeit that the decline was more variable than in the NK1 treated animals. Such variability with mannitol has also been

observed clinically (Bratton et al. 2007b, Meyer et al. 2010), with rebound increases in ICP having been noted where prolonged mannitol infusion results in mannitol crossing the more permeable BBB (Node & Nakazawa 1990). Nonetheless, in the present experiments, there were significant differences between vehicle and mannitol treated animals noted at 3 h ($p<0.05$) and 4 h ($p < 0.01$).

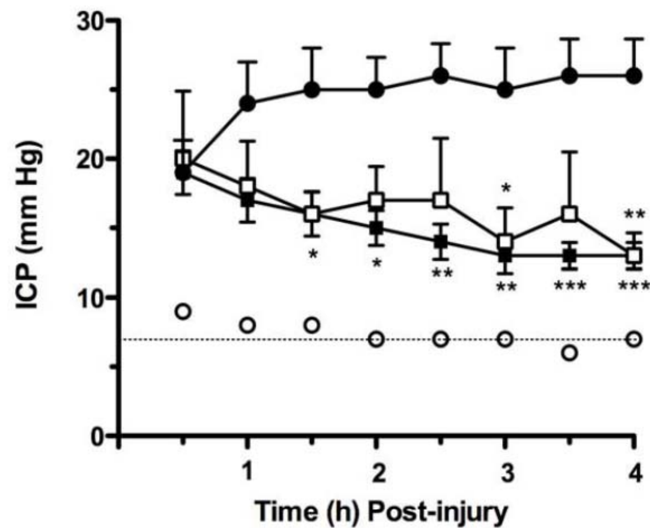


Figure 5.1 Changes in intracranial pressure (ICP) following traumatic brain injury in sheep (mean ± SEM). ○ = uninjured controls (n = 9); ● = vehicle (saline) treated (n = 9); ■ = NAT treated (n = 10); □ = mannitol treated (n = 6). * = $p < 0.01$, ** = $p < 0.01$, *** = $p < 0.001$ versus saline treated vehicles, respectively. Dotted line represents mean level in uninjured control animals.

5.3.2 Effects of NAT vs. Mannitol on $P_{bt}O_2$ after TBI

With respect to $P_{bt}O_2$ (Fig. 5.2), uninjured control animals recorded a mean value of 42 ± 4 mmHg (mean ± SEM) over the 4 h monitoring period. By 30 min after TBI, and immediately before drug administration, all groups demonstrated a significant fall in $P_{bt}O_2$ ($p<0.05$) to approximately 22 mm Hg, confirming that the severity of injury was similar for all treatment groups. Animals treated with saline vehicle did not improve their $P_{bt}O_2$, remaining at approximately 50% of normal, uninjured values over the remainder of the monitoring period. In contrast, animals treated with the NK1 antagonist resulted in a profound and sustained improvement in $P_{bt}O_2$, which was significantly different from vehicle treated animals from 2.5 h onwards ($0.01 < p < 0.05$). $P_{bt}O_2$ increased in these animals to more than 80% of normal values, which was not significantly different from the uninjured animals. Treatment with mannitol also improved $P_{bt}O_2$ levels, although the variability amongst animals was large, which is consistent with the variability previously described clinically (Bohman *et al.* 2011). Accordingly, statistical significance was never achieved when compared to vehicle treated animals.

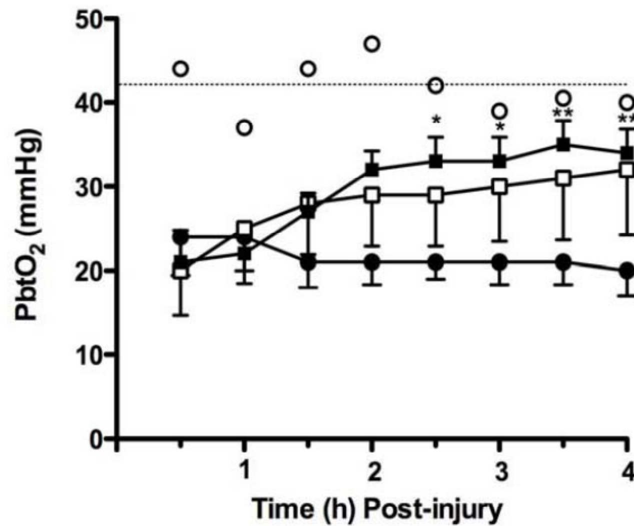


Figure 5.2 Changes in cerebral oxygenation ($P_{bt}O_2$) following traumatic brain injury in sheep (mean ± SEM). ○ = uninjured controls (n = 9); ● = vehicle (saline) treated (n = 9); ■ = NAT treated (n = 10); □ = mannitol treated (n = 6). * = $p < 0.01$, ** = $p < 0.01$, and *** = $p < 0.001$ versus saline treated vehicles, respectively. Dotted line represents mean level in uninjured control animals.

5.3.3 Effects of NAT vs. Mannitol on CPP after TBI

The CPP values at 30 min were relatively high compared to later values in the posttraumatic period, largely because of the transient hypertension observed in the initial period after TBI (see Chapter 4), which has been widely reported in the literature (Marmarou et al. 1994, Lewis et al. 1996). After 30 min, there was a decline in CPP in all groups (Fig. 5.3), although given the data variability, there was never any statistical significance detected either within or between treatment groups. Even sham (uninjured) animals demonstrated a decline in CPP, although this is most likely related to the gradual decline in MABP associated with prolonged anesthesia.

Vehicle treated animals demonstrated the largest initial fall in CPP, and this value remained depressed for the entire monitoring period. In contrast, animals treated with the NK1 antagonist consistently showed higher CPP values than the other groups, albeit that the data variability precluded any significance. Mannitol treated animals showed an increase in CPP immediately after administration of the compound, but this was transient and the CPP values in this group fell to the lowest of all the treatment groups at later time points. Despite the interesting trends, there was no statistical significance in the results. Nonetheless, the dissociation between CPP and brain oxygenation was noted, in stark contrast to the close association between ICP and $P_{bt}O_2$.

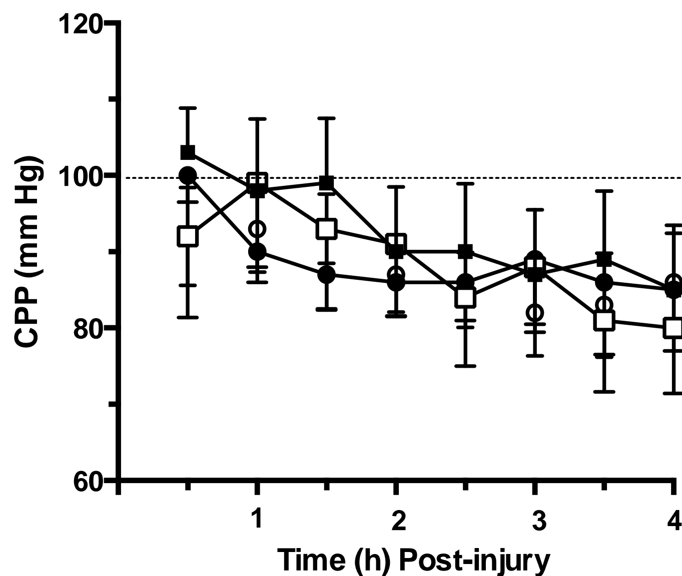


Figure 5.3 Changes in cerebral perfusion pressure (CPP) following traumatic brain injury in sheep (mean ± SEM). ○ = uninjured controls (n = 9); ● = vehicle (saline) treated (n = 9); ■ = NAT treated (n = 10); □ = mannitol treated (n = 6). * = $p < 0.01$, ** = $p < 0.01$, *** = $p < 0.001$ versus saline treated vehicles, respectively. Dotted line represents mean baseline level in uninjured control animals.

5.4 Discussion

In the current study, we have demonstrated that administration of an NK1 receptor antagonist, n-acetyl-tryptophan, significantly reduced ICP by 32% within 3.5 hours of administration whereas ICP continued to increase by a further 36% in saline (vehicle) treated animals. By 4 h after injury, ICP in the NK1 treated animals was down to just 50% of the saline treated animals, and approaching normal values. The reduction in ICP was just as effective as with mannitol administration, albeit that the effect of mannitol was less consistent with more variability in the data. While mannitol is widely used clinically as a therapeutic intervention for increased ICP after acute brain injury (Bratton et al. 2007b, Meyer et al. 2010), its mechanism of action is to draw water out of brain tissue down an osmotic gradient. Not only does this fail to address the potential mechanisms associated with development of ICP, in the presence of a more permeable BBB, the osmotic agent can actually cross into the brain parenchyma and exacerbate the movement of water from the vasculature to the brain tissue (Node & Nakazawa 1990). In contrast, the NK1 receptor antagonist was shown to be just as effective at reducing ICP as mannitol, although its mechanism of action involves reducing blood brain barrier permeability and inhibiting the development of vasogenic edema (Donkin et al. 2009, Turner et al. 2011). This mechanism of action eliminates any possibility of a reversal of osmotic gradient and a rebound increase in ICP, presumably accounting for the more consistent reduction in ICP seen in the present study.

The reoxygenation of brain tissue after TBI is an important part of effective therapeutic intervention, with restoration of energetic state facilitating recovery from tissue damage. In the present study, $P_{bt}O_2$ reduced to less than 50% of normal (Rapoport et al.) values in those animals

administered with the saline vehicle, and this reduction in $P_{bt}O_2$ persisted for the entire duration of the monitoring period. In contrast, administration of the NK1 receptor antagonist resulted in an immediate increase in $P_{bt}O_2$ such that by 4 h after TBI, these values were no longer significantly different from normal values. A similar increase in $P_{bt}O_2$ was observed in the mannitol treated animals, although as with the ICP response, this increase with mannitol was more variable and, in this instance, not as rapid. The effective reoxygenation of the brain with the NK1 antagonist may, in part, explain the neuroprotective effects previously reported in experimental studies where administration of the NK1 antagonist resulted in reduced neuronal cell death and an improved functional outcome in injured animals (Vink & van den Heuvel 2010, Turner et al. 2011). Presumably, the effective reoxygenation of the brain is a reflection of the relationship between ICP and $P_{bt}O_2$ noted in the current study. Whether there are also effects on cerebral blood flow is unknown, although this seems unlikely given the lack of any significant difference in MABP and cerebral perfusion pressure between groups in the current studies.

The use of a large animal, ovine model of TBI delivered a number of advantages that are not present in the more commonly used rodent models of TBI. First there is the presence of a gyrencephalic brain with large white matter domains as opposed to a lissencephalic brain. The sheep also has an intact tentorium cerebelli, which separates the cranial vault into supra-and infra-tentorial compartments (Klintworth 1968). These anatomical differences may account for the more consistent ICP response we achieve in the ovine model as compared to the rodent models of TBI (Chapter 3). These factors may also account for the similarity in normal ICP and $P_{bt}O_2$ values between sheep and humans, with ICP in the sheep being 7±2 mm Hg and $P_{bt}O_2$ being 42 ± 7 mmHg, and the responses after TBI. We were also able to use identical

instrumentation and neurosurgical technique to that used clinically. All these factors facilitate comparison between our experimental ovine results and human TBI, and may potentially contribute to the translation of the experimental results.

In conclusion, the present chapter has demonstrated that NK1 receptor antagonists are at least as effective as mannitol in reducing ICP and increasing $P_{bt}O_2$ following TBI, without the risk of rebound ICP increases sometimes experienced with mannitol infusions. Only clinical trials will address whether they are similarly effective in human TBI.

CHAPTER 6

EFFECTS OF MGSO4 AND PROGESTERONE ON ICP, MABP, CPP, AND $P_{bt}O_2$ CHANGES AFTER TRAUMATIC BRAIN INJURY IN SHEEP

6.1 Introduction

In the previous chapter, we demonstrated that the substance P NK1 receptor antagonist n-acetyl-tryptophan (NAT) reduced ICP and improved $P_{bt}O_2$ following TBI in sheep. Moreover, the antagonist was at least as effective as the widely used clinical compound mannitol, but with less variability and with reduced risk of rebound increases in ICP. In addition to the substance P antagonists, a number of other compounds have shown particular promise in experimental studies at reducing oedema, including magnesium and progesterone. Given that that magnesium has delivered mixed results in recent clinical TBI trials (Sen & Gulati 2010) and progesterone is currently the subject of a major phase III clinical TBI trial, we chose to investigate the efficacy of these two experimental compounds at reducing ICP and improving $P_{bt}O_2$ in our ovine model of TBI.

Previous studies have shown that magnesium plays an important role in the pathophysiology of TBI and that it may have therapeutic potential (McIntosh 1993, Vink & Nimmo 2002, Hoane 2007). Under normal physiological conditions magnesium regulates calcium influx via noncompetitive inhibition of N-methyl-D-aspartate (NMDA) receptors (Garfinkel & Garfinkel 1985). Following TBI there is a reduction of magnesium level which may lead to the reduction or loss of its homeostatic control of the NMDA receptors. This in turn leads to a massive influx of calcium, potentially resulting in neuronal cell death (van den Heuvel & Vink 2004). Experimental studies have demonstrated that parenteral administration of magnesium up to 12 h post trauma can improve neurological outcome (Vink & Nimmo 2009). While the exact mechanism are unknown, magnesium may have neuroprotective effects via inhibition of the release of presynaptic excitatory neurotransmitters, blockade of NMDA channels, and

potentiation of presynaptic adenosine (Meloni *et al.* 2006, Temkin et al. 2007). Magnesium has also been shown to attenuate such secondary injury factors as brain oedema, glutamate excitotoxicity, lipid peroxidation, and apoptosis (Hans *et al.* 2002, Sen & Gulati 2010), as well as improving cerebral blood flow by relaxing vascular smooth muscles (Altura & Altura 1981, Turlapaty *et al.* 1981). However, there is an inverted U dose response with magnesium administration, and when given at high doses, studies have shown that magnesium administration may not always result in positive outcomes following TBI (Heath & Vink 1999a, Temkin et al. 2007). So far, no study has explored the effects of magnesium on increased ICP following TBI, despite a number of studies suggesting it reduces oedema after TBI (Esen *et al.* 2003, Feldman *et al.* 1996, Imer *et al.* 2009).

Similarly, a number of experimental studies have shown that progesterone administration following TBI can decrease brain oedema, as well as attenuate free radical damage and reduce neuronal cell loss (Asbury et al. 1998, Cervantes *et al.* 2002, Shear et al. 2002, Roof et al. 1992, Roof et al. 1994, Roof *et al.* 1997). Progesterone is present in small concentrations in the brains of both men and women and is reported to have not only steroid, but also neuroactive, and neurosteroidal actions in the brain tissue (Rupprecht & Holsboer 1999, Ma *et al.* 2012). As a drug, progesterone has been in clinical use for a number of years and its pharmacokinetics is well known (Wu *et al.* 1989, Allolio *et al.* 1995, Schumacher & Baulieu 1995). Progesterone receptors are broadly present in brain tissue, and a numbers of studies have now shown that progesterone has neuroprotective effects following brain injury via a number of different mechanisms (Chen *et al.* 1999, Thomas *et al.* 1999, Kumon *et al.* 2000, O'Connor *et al.* 2007). The reduction of oedema is just one of these mechanisms (O'Connor et al. 2005). Few studies

have investigated the effects of progesterone on ICP following TBI (Shahrokhi et al. 2010), and none of them have used large animal models. The early clinical trials of progesterone in TBI have suggested that progesterone may have the potential to reduce ICP in humans following TBI (Wright et al. 2007, Xiao et al. 2008), albeit that the results were not statistically significant.

In the current study, we will investigate the effects of magnesium and progesterone on ICP, $P_{bt}O_2$, and CPP in an ovine model of TBI. The first ever testing of these compounds in a large animal model of TBI will provide additional evidence as to their potential clinical utility.

6.2 Materials and Methods

Subjects

All experimental animal studies were approved by the local animal ethics committees representing SA Pathology and The University of Adelaide, South Australia, and were performed according to the guidelines established by the National Health and Medical Research Council (NHMRC), Australia. A total of 29 2-year old male Merino sheep (weighing between 45-60kg), were used in this study.

6.2.1 Induction of Impact Acceleration Head Injury

TBI was induced in all sheep according to the detailed methodology in Chapter 2. Briefly, after induction of anaesthesia and surgical preparation, the animals were put into a prone sphinx position and injury induced on the left side of the head at the midpoint between the supraorbital process and external auditory channel using the humane stunner device. After injury, the head

was secured on the chin support and ICP and LICOX probes were inserted into the left and right hemispheres, respectively.

6.2.2 Drug Treatments

Excluded animals

Out of twenty nine animals, 3 were excluded: 2 animals died (9.1 % mortality) following TBI at different time periods during the 4 h monitoring period, and 1 animal had health problems, namely pulmonary problems discovered during the experiment and confirmed by post-mortem examination. As a result, final statistical analysis included data from 26 animals out of 29.

Drug treatments

At 30 min after TBI, animals were administered either $MgSO_4$ (30 mg/kg; n=6), progesterone (16 mg/kg; n=6), or equal volume saline vehicle (n=6) through the intravenous catheter previously inserted into the right femoral vein. Dosages were derived from previously published work from this laboratory (Heath & Vink 1999c, O'Connor et al. 2005). All three solutions were prepared by an independent researcher and coded in identical containers to ensure that the investigator was blinded to the treatment. A further 8 animals served as sham (uninjured) controls.

6.2.3 Physiological Monitoring

All physiological parameters (ICP, $P_{bt}O_2$, MABP and CPP) were continuously monitored between 30 min and 4 h after TBI as described in detail in Chapter 2.

Briefly, ICP was monitored using a Codman Microsensor ICP transducer probe, which was connected to a Codman ICP Express monitoring system (Codman and Shurtleff, Inc., DePuySpine^{TM}). Before each experiment the probe was zeroed, calibrated and checked in sterile water according to manufacturer's instructions and between experiments it was stored in normal saline solution within a calibration cylinder. The ICP probe was inserted to a depth of 15 mm through a modified No14-gauge intravenous cannula fixed to the burr hole on the left side of the skull (15mm lateral to midline and in front of the coronal suture). After insertion of the probe, the cannula was sealed using bone wax (Ethicon, W810). Similarly, a Licox oxygen catheter-microprobe (LICOX CC1.R, 0.5mm diameter, Integra Neuroscience, Plainsboro, New Jersey) was inserted to a depth of 35 mm through a modified No14-gauge intravenous cannula fixed to the burr hole on the right side of the skull (15mm lateral to midline and in front of the coronal suture). Prior to insertion, the calibration accuracy of the probe was checked in a solution of O_2 and N_2 in sterile water and in air, and between experiments the probe was stored wet in the packaging in the refrigerator. After insertion, the LICOX probe required approximately 15 min for stabilisation in cerebral tissue, consistent with the manufacturer's instructions. During this time, the cannula was sealed using bone wax (Ethicon, W810).

Mean arterial blood pressure was continuously monitored using a MacLab data acquisition system (MacLab 2e) via a Statham-type pressure transducer. The pressure trace was relayed from

the transducer to a MacLab acquisition system via a bridge amp. Cerebral perfusion pressure (CPP) was subsequently calculated by the equation:

$$CPP=MABP-ICP$$

using the same MacLab data acquisition system linked to a personal computer.

Arterial blood gas analysis was conducted 5 times during each experiment, the first being at 10min before the injury, and the other four analyses being done every hour after the injury. An Osmetech OPTI blood gas analyser (CCA, Helena Laboratories, Australia Pty Ltd) using Osmetech OPTI cassettes was used for arterial blood gas analysis. PaO_2, $PaCO_2$, pH, SO_2, tCO_2, tHb, Hct, Na^+, K^+ and BE were measured during each arterial blood gas analysis.

6.2.4 Statistical Analysis

Data are expressed as mean and standard error of the mean (SEM) and were analysed by two-way analysis of variance (ANOVA) followed by Bonferroni correction (PRISM, Graphpad Software, San Diego). A p value of less than 0.05 was considered significant.

6.3 Results

The changes in mean arterial blood pressure, blood PaO_2, blood $PaCO_2$, blood pH, or brain temperature after TBI (Table 6.1) were similar to those described previously in Chapter 5. Again, there were no significant differences between treatment groups confirming that no treatment had any significant effect on any of these parameters over the 4 h monitoring period.

Table 6.1 Changes in MABP, blood PaO2, blood PaCO2, blood pH and brain temperature over 4h following traumatic brain injury in sheep (mean ± SEM).

	Sham		Vehicle		Magnesium		Progesterone	
	1h	4h	1h	4h	1h	4h	1h	4h
MABP (MM Hg)	99±6	91±7	112±3	104±6	122±16	106±18	112±14	93±9
PaO_2 (mm Hg)	137±10	165±3	168±2	161±5	131±8	125±6	155±7	151±5
$PaCO_2$ (mm Hg)	44±3	37±4	39±4	35±3	43±3	40±2	38±2	36±4
pH	7.47±0.03	7.51±0.02	7.52±0.02	7.52±0.02	7.50±0.03	7.48±0.01	7.49±0.02	7.49±0.01
Brain Temp (°C)	37.1±0.4	37.6±0.6	37.2±0.2	37.7±0.3	38.9±0.2	39.8±0.2	38.3±0.3	38.3±0.6

6.3.1 Effects of MgSO4 and Progesterone on ICP after TBI

Intracranial pressure was continuously monitored for 4 hours in all animals although data is only shown for every 30 min for the sake of graphical clarity (Fig. 6.1). Sham animals, with no TBI, demonstrated a mean ICP of 7 ± 2 mm Hg over the 4 h monitoring period. Injured animals all demonstrated an increased ICP after TBI ($0.001<p<0.05$ versus sham values), with the 30 min value before treatment ranging from 15 to 25 mm Hg. The progesterone treated group were at the higher end of this range, although this difference was non-significant and still within the expected range for moderately injured animals (see Chapter 4). Treatment with saline vehicle did not result in any significant change in ICP with time, remaining at approximately 3 times greater than normal sham values for the duration of the monitoring period. While treatment with $MgSO_4$ tended to reduce ICP with time (down to 14 ± 3 mm Hg at 4 h), the trend was not significant compared to vehicle treated animals. Similarly, treatment with progesterone had no significant effect on ICP values after TBI, with the ICP averaging 24 mm Hg ($p < 0.001$ versus sham) for

the entire 4 h observation period. At no time point was the ICP in progesterone treated animals significantly different from values in vehicle treated animals.

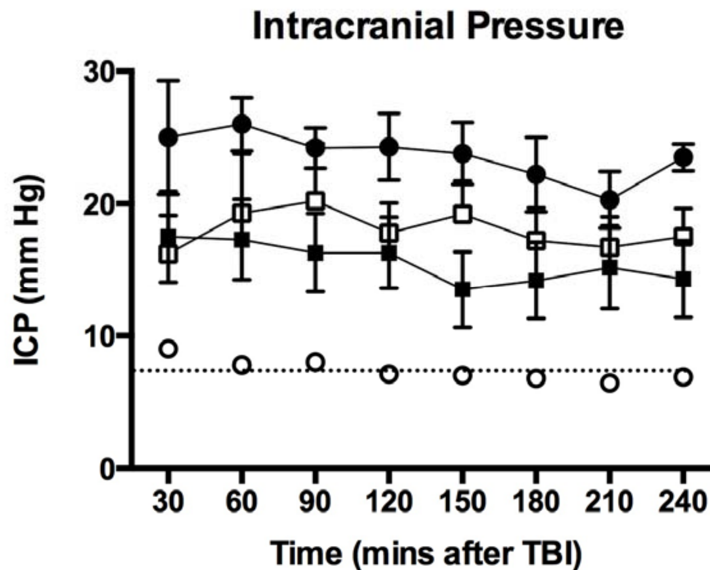


Figure 6.1 Changes in intracranial pressure (ICP) following severe traumatic brain injury in sheep (mean ± SEM). ○ = uninjured sham controls (n = 8); □ = vehicle (saline) treated (n = 6); ■ = MgSO4 treated (n = 6); ●= Progesterone treated (n = 6). Dotted line represents mean level in uninjured control animals.

6.3.2 Effects of MgSO4 and progesterone on $P_{bt}O_2$ after TBI

Brain tissue oxygenation ($P_{bt}O_2$) was continuously monitored for 4 hours post-injury animals although data is only shown for every 30 min for the sake of graphical clarity (Fig. 6.2). Sham animals, with no TBI, demonstrated a mean $P_{bt}O_2$ of 41 ± 3 mm Hg over the 4 h monitoring period. Although 30 min data is shown for $P_{bt}O_2$, there is a high degree of variability in the data,

presumably because the probe had not adequately stabilized in cerebral tissue as recommended by the manufacturer (15 min). Nonetheless, it is clear that $P_{bt}O_2$ declined significantly in all treatment groups within the first 30 min. Treatment with saline or progesterone vehicle did not result in any improvement in $P_{bt}O_2$ over the 4 h monitoring period, with values remaining at approximately 20 mm Hg for the duration. In contrast, $P_{bt}O_2$ levels in animals treated with MgSO4 showed some improvement over the 4 h monitoring period, from a value of 9 ± 2 at 1 h to 22 ± 7 mm Hg, albeit that this improvement was never statistically significant compared to the vehicle treated animals (Fig. 6.2). The low value (10 mm Hg) of the initial $P_{bt}O_2$ determinations in the magnesium treatment group does suggest moderate injury more towards the severe end of the spectrum than the other treatment groups (see Chapter 7), although this difference was not statistically significant. Indeed, there was no significant difference between any of the treatment groups at any time point.

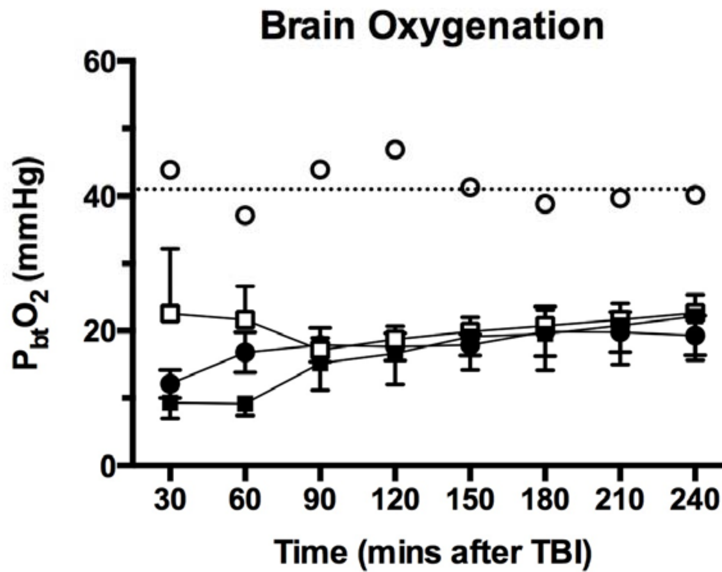


Figure 6.2 Changes in cerebral oxygenation ($P_{bt}O_2$) following traumatic brain injury in sheep (mean ± SEM). ○ = uninjured sham controls (n = 8); □ = vehicle (saline) treated (n = 6); ■ = MgSO4 treated (n = 6); ●= Progesterone treated (n = 6). Dotted line represents mean level in uninjured control animals.

6.3.3 Effects of MgSO4 and Progesterone on CPP after TBI

There were no significant differences among any of the treatment groups with regard to CPP following TBI (Fig. 6.3). As in the previous chapters, there was a gradual decline in CPP over time, even sham animals and even in the absence of ICP changes. Presumably, this was related to the developing hypotension that is observed after TBI, and which has been described for this model in chapter 4. The smallest decline in CPP occurred in the $MgSO_4$ treated animals (3%), although this was non-significant when compared to vehicle treated (12%) or progesterone treated (18%) animals. It nonetheless supported the trend seen in both the ICP and $P_{bt}O_2$ data above.

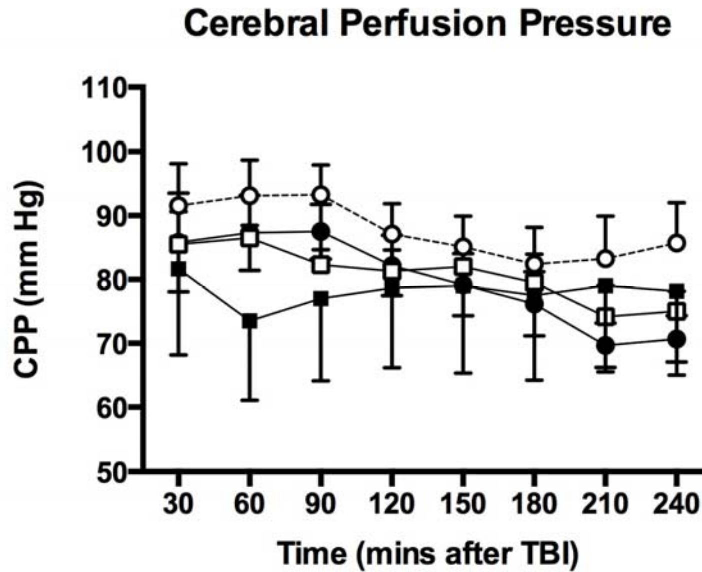


Figure 6.3 Changes in cerebral perfusion pressure (CPP) following traumatic brain injury in sheep (mean ± SEM). ○ = uninjured sham controls (n = 8); □ = vehicle (saline) treated (n = 6); ■ = MgSO4 treated (n = 6); ●= Progesterone treated (n = 6). Dotted line represents mean level in uninjured control animals.

6.4 Discussion

The present study has shown that administration of either magnesium or progesterone following TBI did not significantly reduce ICP or improve $P_{bt}O_2$ in an ovine model of moderate TBI. This is despite several previous studies having shown that both compounds significantly reduce brain water content following rodent TBI. Specifically, progesterone has been shown to attenuate oedema formation in a number of different rodent TBI models in both female and male animals (Roof et al. 1992, O'Connor et al. 2005), irrespective of oestrogen. Moreover, the reduction of oedema was significant even when progesterone treatment was delayed for up to 24h after injury

(Roof et al. 1996). These observations have since been confirmed in the bilateral medial frontal cortex injury model of trauma (Wright et al. 2001), as well as in ischaemic injury (Kumon et al. 2000). The underlying mechanisms by which progesterone may reduce oedema have not been fully elucidated, however several possible mechanisms have been proposed (Roof & Hall 2000) including inhibition of active ion uptake through the Na+/K+-ATPase, inhibition of vessel growth associated with leaky BBB function after TBI, modulation of levels of vasopressin, inhibition of neurogenic inflammation (Limmroth *et al.* 1996), actions as a free radical scavenger mediating lipid peroxidation, and finally reduction of inflammation (Pettus *et al.* 2005). Similar mechanisms of action have also been proposed to explain the ant-oedema effects of magnesium after rodent TBI that have been reported in both focal and diffuse models of rodent TBI (Esen et al. 2003, Feldman et al. 1996, Imer et al. 2009, Okiyama *et al.* 1995).

Despite the widely reported effects on oedema formation after rodent TBI, the current experiments failed to show any benefit on post-traumatic ICP, CPP or $P_{bt}O_2$ in the ovine model of TBI. While this may seem contradictory, it is consistent with our previous findings described in chapters 3 and 4 that oedema in rodents does not necessarily translate to an increased ICP in large animals. As stated in chapter 3, the lack of a substantial tentorium cerebelli, the lack of a gyriform brain and the absence of significant white matter domains significantly alter the pathophysiology of oedema formation and the development of an increased ICP in rodents. The observations described in the current chapter are consistent with recent phase II clinical trials using progesterone where both reduced mortality and improved outcome was reported, although the improvement was not related to any statistically significant effect on ICP (Xiao et al. 2008).

The trends toward improvement of ICP, CPP and $P_{bt}O_2$ after magnesium administration in the current study are intriguing, despite the lack of any statistically significant effects. Indeed, the trends were always in a positive direction. Given that the magnesium clinical trials to date have delivered mixed results (Sen & Gulati 2010), further studies of magnesium as a neuroprotective agent may be warranted. Notably, those clinical trials that were negative were thought to be so on the basis of poor central penetration by magnesium (McKee *et al.* 2005), and the early administration of magnesium in the current study, when the BBB was highly permeable, would have facilitated magnesium entry. Alternatively, it may be that the effects of magnesium on TBI in the current study were so marginal, despite excellent CNS penetration, that a positive effect in clinical trials is unlikely. The current study was limited to moderate injury with drug therapy at 30 min, and a statistically significant effect may be unlikely in a more heterogeneous clinical TBI population. In any case, the present study has highlighted the importance of testing the efficacy of prospective therapeutic ICP interventions in a large animal TBI model.

CHAPTER 7

EFFECTS OF EU-C-001 VS. HTS ON ICP, MABP, CPP, AND $P_{bt}O_2$ CHANGES AFTER TRAUMATIC BRAIN INJURY IN SHEEP

7.1 Introduction

In the previous chapter, the importance of testing the efficacy of prospective therapeutic ICP interventions in a large animal TBI model was highlighted, as was the heterogeneity of human TBI. With such heterogeneity, it seems clear that prospective therapies should be tested at both moderate and severe injury levels before being considered for entry into clinical trials. We did induce severe injury during the ovine model characterization in Chapter 4, although this was largely due to the faulty stunner and charge, and these animals were excluded from the analysis. A feature of the more severe injury was the very low $P_{bt}O_2$ (< 10 mm Hg) detectable within the first hour after injury, even though we used postmortem gross morphology to retrospectively classify the injury severity in that particular study. In attempting to consistently induce a more severe injury, the stunner could be calibrated to allow more contact between the bolt and the skull, similar to the method adopted in abattoirs for humane killing of large animals.

In chapter 5, we demonstrated that administration of the NK1 receptor antagonist, n-acetyl-tryptophan (NAT), significantly reduced increased ICP after TBI within 3.5 hours and that it was at least as effective mannitol, if not more so as reflected by the reduced variability in the data. Treatment of increased ICP with mannitol in patients with moderate TBI has been shown to be reasonably effective given that mannitol molecules are normally too large to cross the BBB (Bratton et al. 2007b). However, in patients with severe TBI, the BBB is often more permeable given the increased BBB damage induced by the injury, and administration of mannitol may have deleterious effects on ICP since the mannitol molecules may cross the damaged BBB and increase the water movement into the brain tissue. In so doing, it reverses the osmotic gradient and thus worsens edema, causing what is commonly known as a rebound increase in ICP

(Bratton et al. 2007b). This is one of the more serious reservations with repeated use of mannitol in clinical management of post-traumatic ICP. Such a rebound is not possible with the NK1 antagonist class of compounds given that they do not contribute to the osmotic gradient.

The experimental aims in the current chapter were therefore to characterize the effects of an NK1 antagonist on ICP and $P_{bt}O_2$ following severe TBI in the ovine model, to determine whether these effects on ICP and $P_{bt}O_2$ are a class effect (and not specific to NAT) by using the alternative NK1 antagonist, EU-C-001 (Eustralis Pharmaceuticals), and to compare these effects with an alternative osmotic agent that is being increasingly used clinically, hypertonic saline.

7.2 Materials and Methods

Subjects

All experimental animal studies were approved by the local animal ethics committees representing SA Pathology and The University of Adelaide, South Australia, and were performed according to the guidelines established by the National Health and Medical Research Council (NHMRC), Australia. A total of 18, 2-year old male Merino sheep (weighing between 45-60kg), were used in this study.

7.2.1 Induction of Impact Acceleration Head Injury

Injury was induced as described in detail in Chapter 2, with the exception that the stunner was adjusted to allow more contact between the bolt and the skull, thus inducing a severe injury as described in Chapter 4. Briefly, after induction of anaesthesia and placement of femoral arterial and venous lines, animals were placed into a prone sphinx position and injury induced on the left

side of the head at the midpoint between the supraorbital process and external auditory channel. The head was then secured onto the chin support and the ICP and LICOX probes inserted into the left and right hemispheres, respectively, as described in detail in Chapter 2.

7.2.2 Groups and Drug Treatments

Excluded animals

Out of 18 animals 2 were excluded. One animal had lung problems discovered during the experiment and confirmed by subsequent post-mortem investigation, while another had pus in the frontal sinuses. As a result final analysis included data only from 16 animals, plus 9 historical sham controls as described in Chapter 5.

Drug treatments

At 30 min after induction of injury, animals were administered either EU-C-001 (1 mg/kg; n = 5), 7.5% hypertonic saline (n=7), or saline vehicle (n=4) via the implanted intravenous catheter. EU-C-001 was a gift from Eustralis Pharmaceuticals (Melbourne, Australia) who also provided the optimal dosage information. The dose of hypertonic saline was obtained from the clinical guidelines for the management of severe TBI (Bratton et al. 2007b). All three solutions were prepared by an independent researcher and coded in identical containers to ensure that the investigator was blinded to the treatment. A further 9 animals served as sham (uninjured) controls.

7.2.3 Physiological Monitoring

All physiological parameters (ICP, $P_{bt}O_2$, MABP and CPP) were continuously monitored between 30 min and 4 h after TBI as described in detail in Chapter 2.

Briefly, ICP was monitored using a Codman Microsensor ICP transducer probe, which was connected to a Codman ICP Express monitoring system (Codman and Shurtleff, Inc., DePuySpine^{TM}). Before each experiment the probe was zeroed, calibrated and checked in sterile water according to manufacturer's instructions and between experiments it was stored in normal saline solution within a calibration cylinder. The ICP probe was inserted to a depth of 15 mm through a modified No14-gauge intravenous cannula fixed to the burr hole on the left side of the skull (15mm lateral to midline and in front of the coronal suture). After insertion of the probe, the cannula was sealed using bone wax (Ethicon, W810). Similarly, a Licox oxygen catheter-microprobe (LICOX CC1.R, 0.5mm diameter, Integra Neuroscience, Plainsboro, New Jersey) was inserted to a depth of 35 mm through a modified No14-gauge intravenous cannula fixed to the burr hole on the right side of the skull (15mm lateral to midline and in front of the coronal suture). Prior to insertion, the calibration accuracy of the probe was checked in a solution of O_2 and N_2 in sterile water and in air, and between experiments the probe was stored wet in the packaging in the refrigerator. After insertion, the LICOX probe required approximately 15 min for stabilisation in cerebral tissue, consistent with the manufacturer's instructions. During this time, the cannula was sealed using bone wax (Ethicon, W810).

Mean arterial blood pressure was continuously monitored using a MacLab data acquisition system (MacLab 2e) via a Statham-type pressure transducer. The pressure trace was relayed from

the transducer to a MacLab acquisition system via a bridge amp. Cerebral perfusion pressure (CPP) was subsequently calculated by the equation:

$$CPP=MABP-ICP$$

using the same MacLab data acquisition system linked to a personal computer.

Arterial blood gas analysis was conducted 5 times during each experiment, the first being at 10min before the injury, and the other four analyses being done every hour after the injury. An Osmetech OPTI blood gas analyser (CCA, Helena Laboratories, Australia Pty Ltd) using Osmetech OPTI cassettes was used for arterial blood gas analysis. PO_2, PCO_2, pH, SO_2, tCO_2, tHb, Hct, Na^+, K^+ and BE were measured during each arterial blood gas analysis.

7.2.4 Statistical Analysis

Data are expressed as mean and standard error of the mean (SEM) and were analysed by two-way analysis of variance (ANOVA) followed by Bonferroni correction (PRISM, Graphpad Software, San Diego). A p value of less than 0.05 was considered significant.

7.3 Results

7.3.1 Mortality

Mortality in animals treated with saline vehicle (n=4) was 100% within the 4 h monitoring period. These animals were clearly severely injured on the basis of their low $P_{bt}O_2$ (<10 mm Hg) within the first 30 minutes of injury, which never improved thereafter. ICP was typically above 25 mm Hg (range 25 – 38 mm Hg), while CPP was less than 50 mm Hg after injury, and

declined to below 40 mm Hg over time as MABP declined. Mortality in EU-C-001 and HTS treated animals was zero and their physiological data has been subsequently further analysed.

7.3.2 Blood Parameters and Brain pH

The changes in MABP, blood PaO_2, blood $PaCO_2$, blood pH, or brain temperature in surviving animals after TBI are shown in Table 7.1. While blood pH, gases and brain temperature were similar to those described previously in the moderately injured animals summarized in Chapter 5, there was a more profound post-traumatic hypotension in these more severely injured animals. Moreover, there was a tendency for animals treated with hypertonic saline (HTS) to have greater hypotension than animals treated with the NK1 antagonist (EU-C-001), although this difference was not statistically significant.

Table 7.1 Changes in MABP, blood PaO2, blood PaCO2, blood pH and brain temperature over 4h following traumatic brain injury in sheep (mean ± SEM).

	Sham		EU-C-001		HTS	
	1h	4h	1h	4h	1h	4h
MABP (mm Hg)	99±6	91±7	107±8	87±11	80±9	73±12
PaO_2 (mm Hg)	137±10	165±3	166±6	168±5	153±8	149±6
$PaCO_2$ (mm Hg)	44±3	37±4	44±3	44±2	39±4	38±2
pH	7.47±0.03	7.51±0.02	7.50±0.03	7.48±0.01	7.49±0.02	7.49±0.01
Brain Temp (°C)	37.1±0.4	37.6±0.6	38.0±0.3	38.1±0.5	37.8±0.4	38.9±0.4

7.3.3 Effects of EU-C-001 versus HTS on ICP after TBI

Sham animals, with no TBI, demonstrated a mean ICP of 7 ± 2 mm Hg over the 4 h monitoring period (Fig. 7.1). In injured animals, the ICP at 30 min after induction of severe injury was 34 ± 4 mm Hg ($p<0.001$ versus sham), which was the highest mean ICP observed at 30 min throughout this thesis. In both the EU-C-001 and HTS treated animals, ICP declined after drug administration ($p<0.001$), although the kinetics of decline was markedly different between the two treatment groups. In the HTS treated animals, there was a rapid decline in ICP over the first 60 min after administration, presumably since brain water loss occurs rapidly in response to the increased vascular osmotic pressure. Thereafter, there was no significant change in ICP for the remainder of the 4 h observation period, with ICP not falling over a 3 h period after treatment, and never below 20 mm Hg. In contrast, with EU-C-001 treatment, there was a steady fall in ICP over the 4 h monitoring period at approximately 5 mm Hg/h. Notably, the ICP in EU-C-001 treated animals fell to 17 ± 2 mm Hg by 3.5 h post-trauma, and the trend was for it to keep falling thereafter. Nonetheless, there was no statistical difference between treatment groups and ICP values remained significantly elevated ($p<0.001$) compared to sham values at all time points.

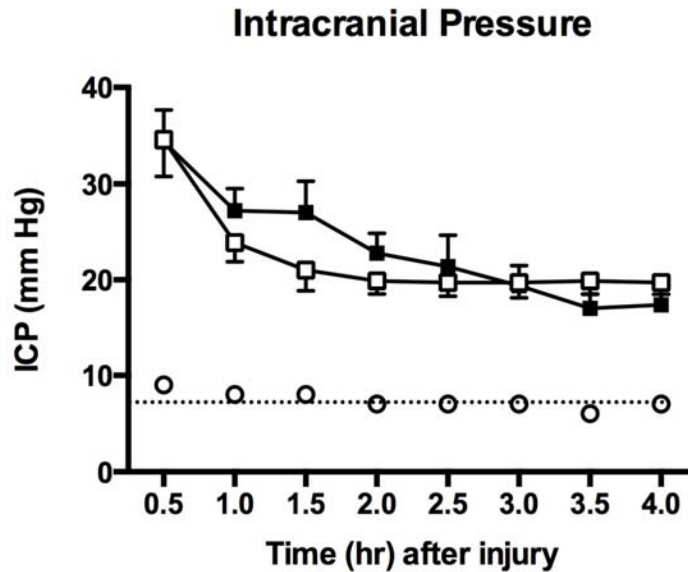


Figure 7.1 Changes in intracranial pressure (ICP) following severe traumatic brain injury in sheep (mean ± SEM). ○ = uninjured (Rapoport et al.) controls (n = 9); □ = HTS treated (n = 7); ■ = EU-C-001 treated (n = 5). Dotted line represents mean level in uninjured (Rapoport et al.) control animals.

7.3.4 Effects of EU-C-001 versus HTS on PbtO2 after TBI

Brain tissue oxygenation ($P_{bt}O_2$) was continuously monitored for 4 hours post-injury animals although data is only shown for every 30 min for the sake of graphical clarity (Fig. 7.2). Sham animals, with no TBI, demonstrated a mean $P_{bt}O_2$ of 42 ± 4 mm Hg over the 4 h monitoring period. At 30 min after injury, mean $P_{bt}O_2$ typically dropped to less than 10 mm Hg ($p<0.001$). Such a profound drop in $P_{bt}O_2$ seems to be a feature of severe injury in this ovine TBI model, which clearly raises implications for severe human TBI. Measurement variability is low at 30 min despite the requirement to allow the probe to stabilize, presumably because the values of

$P_{bt}O_2$ are so low. Treatment with HTS did not result in any significant improvement in $P_{bt}O_2$ over the 4 h monitoring period, with values remaining at < 13 mm Hg for the duration. In contrast, $P_{bt}O_2$ levels in animals treated with EU-C-001 showed significant improvement ($p<0.05$) over the 4 h monitoring period, from a value of 8 ± 1 at 30 min to 22 ± 3 mm Hg at 4 h, albeit that this improvement was never statistically significant compared to the HTS treated animals (Fig. 7.2).

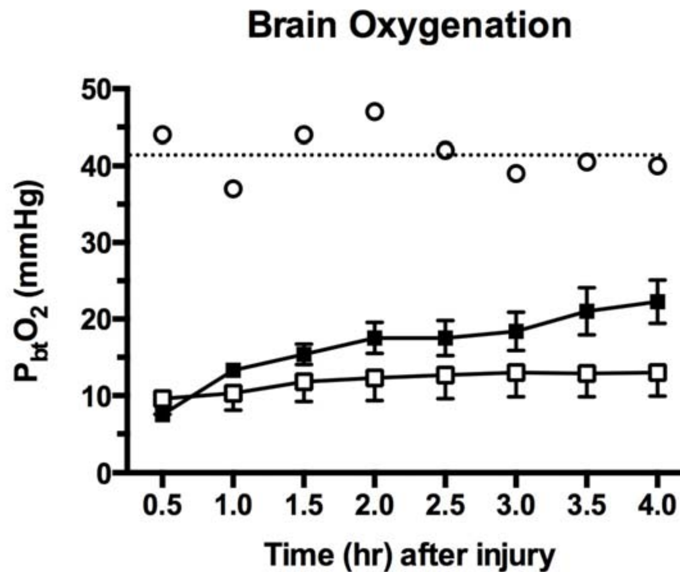


Figure 7.2 Changes in brain tissue oxygenation ($P_{bt}O_2$) following severe traumatic brain injury in sheep (mean ± SEM). ○ = uninjured (Rapoport et al.) controls (n = 9); □ = HTS treated (n = 7); ■ = EU-C-001 treated (n = 5). Dotted line represents mean level in uninjured (Rapoport et al.) control animals.

7.3.5. Effects of EU-C-001 versus HTS on CPP after TBI

The mean sham (uninjured) CPP was 88 ± 7 mm Hg over the entire monitoring period. The mean CPP values in injury survivors ranged between 50 and 61 mm Hg ($p<0.001$ versus shams) at 30 min after injury in both treatment groups (Fig. 7.3). Administration of HTS at this time did not result in any significant change in CPP, with values remaining at 54 ± 11 mm Hg. This lack of improvement correlated with the lack of improvement in $P_{br}O_2$ values with HTS administration seen above. In contrast, administration of EU-C-001 improved CPP to the point that it was no longer different from sham values. Presumably, this was related both to the decrease in ICP noted above, as well as the improvement in MABP relative to the HTS treatment group (see Table 1).

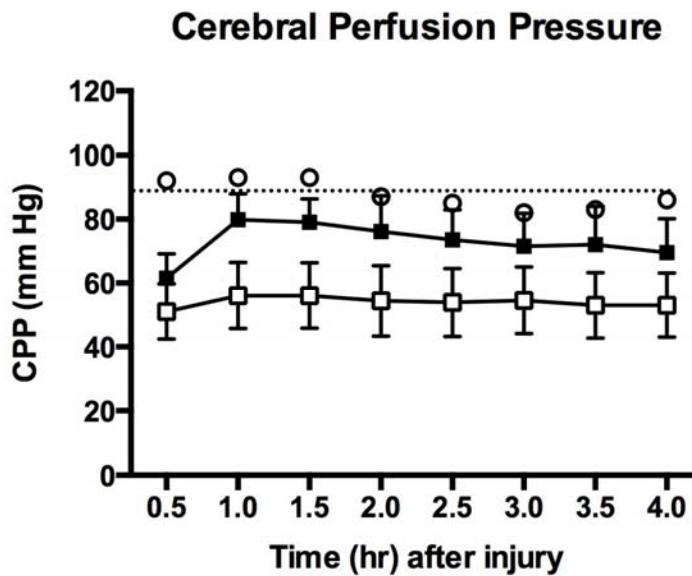


Figure 7.3 Changes in cerebral perfusion pressure (CPP) following severe traumatic brain injury in sheep (mean ± SEM). ○ = uninjured (Rapoport et al.) controls (n = 9); □ = HTS treated (n =

7); ■ = EU-C-001 treated (n = 5). Dotted line represents mean level in uninjured (Rapoport et al.) control animals.

7.4 Discussion

The present study has shown that a substance P NK1 antagonist successfully reduces ICP after severe TBI in sheep, and that this ICP lowering effect was equal to hypertonic saline. In contrast to the very rapid (< 60 min) effect of HTS on ICP, and the lack of further benefit thereafter, the NK1 antagonist steadily reduced ICP (5 mm Hg/h) to below what could be achieved using HTS. Moreover, in contrast to HTS, the NK1 antagonist improved CPP and brain oxygenation. Given that the ultimate goal of ICP management is to improve brain oxygenation, this would suggest that the use of NK1 antagonists for reduction of post-traumatic ICP is superior to that of HTS. While the current study was performed in severely injured animals, the data is consistent with that shown in Chapter 5 where an NK1 antagonist was demonstrated to be superior to the osmotic agent, mannitol, in moderate TBI.

Another important point demonstrated in the current chapter is that the effect of the NK1 antagonists on ICP and $P_{br}O_2$ is a drug class effect, and not just specific to a particular compound. The studies in moderate injury used the compound n-acetyl-tryptophan, which has been previously shown by us to be effective at reducing BBB permeability, oedema and neuronal cell death while improving functional outcome in rodent TBI (Donkin et al. 2009). These beneficial effects of NAT on these parameters are present in both male and female animals (Corrigan *et al.* 2012) and are apparent even when the drug is administered as late as 12 h after injury (Donkin *et al.* 2011). Similarly, preliminary studies by our group have shown that EU-C-001 is a potent NK1 receptor antagonist, which similarly improves BBB permeability, oedema

and functional outcome following both focal and diffuse TBI (Donkin *et al.* 2007). The fact that both compounds are also able to reduce ICP and improve $P_{bt}O_2$ after both moderate and severe TBI induced in a large animals (ovine) model augers well for the potential translation of the class into the clinical arena.

Of interest in the current study was the observation that CPP was linked to improved brain oxygenation at severe injury levels, but not ICP. Indeed, both HTS and EU-C-001 reduced ICP to similar levels, but only EU-C-001 improved both CPP and $P_{bt}O_2$. In contrast, there was no link between CPP and $P_{bt}O_2$ at the moderate levels of injury used in Chapter 5, with NAT having no effect on CPP but a pronounced effect on both ICP and $P_{bt}O_2$. Whether there are differences in the regulation of $P_{bt}O_2$ at different levels of injury severity is the subject of the next chapter.

CHAPTER 8

THE DYNAMICS OF INTRACRANIAL PRESSURE, MEAN ARTERIAL BLOOD PRESSURE, CEREBRAL PERFUSION PRESSURE AND CEREBRAL OXYGENATION AFTER RAUMATIC BRAIN INJURY IN SHEEP: RECONSIDERING CRITICAL THRESHOLDS OF ICP AND THE CPP FORMULA

8.1 Introduction

In the preceding chapters, we have observed associations between cerebral oxygenation and ICP, MABP, or with CPP, and while they seemed to depend on injury severity, it was unclear as to what the true associations were and whether they were indeed dependent on injury severity. To understand the entire dynamic relationship between $P_{bt}O_2$, ICP, MABP, and CPP at different injury severities, we have therefore chosen to analyse the correlation between all of these parameters simultaneously. To do so requires a sophisticated computer program capable of performing the required interconnected 3D analysis; Gaussian Processes (GPs) for machine learning is such a method and has been utilised in the present study.

Understanding the relationship between these parameters after TBI is critical to improving patient outcome. Indeed, both clinical and experimental studies have shown that cerebral hypoxia is closely associated with poor outcome after moderate to severe traumatic brain injury (TBI), emphasising the importance of optimal $P_{bt}O_2$ for TBI outcome (Ang *et al.* 2007, Narotam *et al.* 2006, Narotam *et al.* 2009, Nangunoori *et al.* 2012). In many cases, brain oedema plays a vital role in the development of cerebral hypoxia since it increases ICP and decreases CPP and $P_{bt}O_2$ by expanding brain tissue volume (McIntosh et al. 1990, Marmarou 2007). Therefore, the basic approach to maintaining adequate $P_{bt}O_2$ in neurocritical care is to control increased ICP and maintain adequate CPP (Ang et al. 2007, Figaji *et al.* 2009).

On the other hand it is well known that increased ICP is an independent predictor of outcome in patients with severe TBI and uncontrolled elevations in ICP is the primary cause of death in more than a half of all TBI patients (Marmarou 1992, Marmarou et al. 2005, Resnick *et al.*

1997). Clinical and experimental studies have shown that ICP demonstrates two distinctive thresholds, that is 20-25mm Hg (Reithmeier *et al.* 2005, Morris *et al.* 2006, Steiner & Andrews 2006, Grinkeviciute *et al.* 2008) and 35-40mm Hg, (Rangel-Castilla et al. 2008, Singhi & Tiwari 2009, Signorini *et al.* 1999), which are both considered as critical in determining a patient's good or poor outcome or survival, respectively and hasn't been fully understood so far (Struchen *et al.* 2001, Czosnyka *et al.* 2005, Hiler *et al.* 2006, Helmy et al. 2007, Sankhyan et al. 2010). Furthermore, ICP measurement is important because it allows both for an early detection of evolving mass lesions, especially in those patients who are paralyzed and sedated and in whom neurological examination is limited to pupillary size and responsiveness, and it allows for the calculation of CPP.

CPP is calculated as the difference between MABP and ICP, CPP=MABP-ICP. Although maintaining adequate CPP is important to ensure sufficient $P_{bt}O_2$, it does not reflect the actual values of ICP and MABP, which partially explains how TBI patients with the same CPP can have profoundly different outcomes (Struchen et al. 2001, Howells *et al.* 2005, Cremer *et al.* 2005). Specifically, equivalent changes in ICP and MABP, either in a positive or negative direction, will not be reflected in CPP values, and hence a focus on CPP may overlook critical changes in ICP and MABP. It is therefore not surprising that retrospective data analysis showed no benefit in maintaining CPP>70mm Hg after TBI (Bratton et al. 2007c), and that the optimal CPP after TBI accordingly remains unknown (Rosner *et al.* 1995, Howells et al. 2005). In contrast, $P_{bt}O_2$ based therapy has been associated with more favorable outcomes and, so far, clinical studies suggest that combined ICP/CPP and $P_{bt}O_2$ based therapy is associated with better outcome after TBI than ICP/CPP based therapy alone (Stiefel et al. 2005, Stiefel *et al.* 2006b,

Nangunoori et al. 2012). In terms of what the $P_{bt}O_2$ values represent, values between 20-50 mm Hg are regarded as normal; $P_{bt}O_2$ values below 20 but above 15 mm Hg is considered moderately hypoxic, $P_{bt}O_2$ below 10-15 mm Hg is considered severely hypoxic and associated with worse outcome, while $P_{bt}O_2 \leq 6$ mm Hg is critical and life threatening (Narotam et al. 2006, Stiefel et al. 2005, Sarrafzadeh *et al.* 2003, van den Brink et al. 2000, van Santbrink et al. 2003). Unfortunately, the nature and effectiveness of most commonly used interventions to correct compromised $P_{bt}O_2$ after TBI still remains unclear (Pascual *et al.* 2011).

Autoregulation is an important neuroprotective mechanism that varies arteriolar calibre with changes in MABP to maintain a relatively constant CBF. Normally, autoregulation is active within a MABP range of 50–150 mm Hg (Panerai *et al.* 2004), and responds within seconds of the blood pressure changing (Panerai 1998). When the CPP formula was initially proposed, it was based on the assumption that autoregulation had essentially failed after TBI, and that cerebral blood flow (CBF) was accordingly linearly related to MABP. However, later studies have shown that autoregulation is intact in up to 75% of patients with severe TBI, and even more in patients with moderate TBI (Peterson & Chesnut 2009, Czosnyka et al. 2005). When autoregulation is intact, increases in CPP leads to compensatory vasoconstriction to maintain a stable CBF and so decreases cerebral blood volume and therefore lowers ICP (Rosner et al. 1995). However, outside of the limits of autoregulation, increases in CPP lead to vasodilatation, an increased blood volume and so rises in ICP. Lowering CPP under conditions of failed autoregulation may increase the secondary cerebral hypoxia within a range of MABP that normally would have been regarded as acceptable (Figaji et al. 2009). Therefore, depending on whether cerebral autoregulation is preserved or not, an increase in CPP may result in either

raised or lowered ICP. It follows that the status of autoregulation will influence the choice of whether CPP or ICP directed management should be pursued (Hlatky *et al.* 2005).

In order to improve patient outcome, it is therefore critical to understand the interrelationships between MABP, ICP, CPP, and $P_{bt}O_2$ under varying conditions following moderate to severe TBI. The aim of this study is therefore to investigate the dynamics of these interrelationships using machine learning 3D analysis by a Gaussian processes, so as to investigate the two critical thresholds of ICP observed clinically and to critically assess the formula for CPP.

8.2 Materials and Methods

8.2.1 Animal Studies

All ICP, MABP, CPP and $P_{bt}O_2$ data from the ovine TBI studies in chapters 4 to 7 was included in the current analysis. This totaled 57 animals.

8.2.2 Machine Learning via Gaussian Processes

Gaussian processes (GPs) is a statistical machine learning tool which provides a means for building automated systems that can accumulate and fuse information from different sources, build concise models of the underlying hidden properties of the data and provide an estimation with corresponding uncertainties for new situations (Rasmussen & Williams 2006). GPs is a non-parametric statistical kernel machine method and uses a covariance function for non-linear data modeling.

GPs are non-parametric models where a Gaussian process prior over function values is directly applied. In a more formal way, in a Gaussian process the function outputs $f(\mathbf{x}_i)$ are a collection of random variables indexed by the inputs $\mathbf{x}_i$. Any finite subset of outputs has a joint multivariate Gaussian distribution. Given a set of training inputs $\{\mathbf{x}_i\},\{y_i\}$, the joint prior distribution of the corresponding function outputs $\{f_i\}$ is Gaussian with zero mean and covariance matrix K. The GP is entirely determined by the covariance function $K(x,x')$ and its hyper-parameters θ. A popular choice for the covariance function $K(x,x')$ is the squared exponential covariance function defined as

$$K(x,x') = \sigma_0^2 \exp\left(-\frac{(x-x')^T \Sigma (x-x')}{2}\right) \tag{1}$$

where Σ and σ_0 are the hyper-parameters of the covariance function. Here Σ is a $D \times D$ matrix and σ_0 is a constant, where D stands for the dimensionality of the data. To reduce the computational cost, one can consider the case when Σ is diagonal:

$$\Sigma = \operatorname{diag}\left(\frac{1}{l_1^2}, \frac{1}{l_2^2}, \ldots, \frac{1}{l_D^2}\right) \tag{2}$$

In the case of Eq. (2) the hyper-parameters $l_1, l_2, \ldots, l_D$ are the characteristic length-scales of the covariance function. In the expression (1) the hyper-parameter σ_0 characterizes the amplitude and $l_1, l_2, \ldots, l_D$ characterize the typical lengths of correlation of the functions generated by the GPs.

The choice of the hyper-parameters θ requires a non-linear optimization step. The optimization is conducted by maximizing the log of the marginal likelihood of the data:

$$\text{lml} = -\frac{\mathbf{y}^T K_y^{-1} \mathbf{y}}{2} - \frac{\log\left|K_y\right|}{2} - \frac{N}{2}\log 2\pi \tag{3}$$

where $K_y = K(X, X) + \sigma^2 I$ is the covariance matrix for the targets $\mathbf{y}$. The log of the marginal likelihood has three terms: data fit, complexity penalty (based on the Occam's Razor principle) and normalization constant. Once the optimal values for all the hyper-parameters θ are determined, the GPs model allows predicting the values of the function $f(\mathbf{x})$ at new locations $\{\mathbf{x}_{*i}\}$. This is done by conditioning the joint Gaussian distribution for the observed points $\{\mathbf{x}_i\}$ on the data $\{y_i\}$ available in the given dataset. The result is a new Gaussian multivariate distribution $N(m, S)$ with mean m and variance S where

$$m = K(X_*, X)\left[K(X, X) + \sigma^2 I\right]^{-1}\mathbf{y} \tag{4}$$

$$S = K(X_*, X_*) - K(X_*, X)\left[K(X, X) + \sigma^2 I\right]^{-1} K(X, X_*) + \sigma^2 I \tag{5}$$

GPs can be used for both regression and classification tasks and can successfully model datasets of different complex systems (Su & Ye 2004; Gregorcic & Lightbody 2009; Azman & Kocijan 2008). In the case of regression, the Gaussian process is provided with a set of input-output pairs (the training dataset) which is used to learn the hyper-parameters of the covariance function. In the case of classification, an input pattern is being assigned to one of multiple classes. Contrary to other methods which provide only a guess at the class label, GPs make predictions for classes and also provide corresponding probabilities (and therefore confidence levels) for this guess. In a practical application, one may well seek a class guess, which can be obtained as the solution to a

decision problem, involving the predictive probabilities as well as a specification of the consequences of making specific predictions (the loss function). A GPs is also a best unbiased linear estimator. For more information on Gaussian processes and covariance function, see Rasmussen and Williams (2006).

8.2.3 Statistical Analysis via Gaussian Processes

The data was analysed using Gaussian processes for machine learning. The values of $P_{bt}O_2$ are represented as Gaussian predictive distributions with mean and standard deviation automatically identified from the experimental data. Log marginal likelihood is used to determine the hyper-parameters of the Gaussian process. The mean prediction represents the general trend of $P_{bt}O_2$ while the uncertainty of the prediction is expressed by the standard deviation. An error histogram is calculated to evaluate the qualities of the predictive model.

8.3 Results

8.3.1 $P_{bt}O_2$ and ICP Correlation

The interrelationship between ICP and $P_{bt}O_2$ was analysed using Gaussian processes with an inclusion rate of 95% (Fig. 8.1). In terms of the interrelationship between ICP and $P_{bt}O_2$, when ICP was less than 20 mm Hg, the mean $P_{bt}O_2$ was between 20 and 35 mm Hg. When the mean ICP was between 20 and 30 mm Hg, $P_{bt}O_2$ mean values were between 15 and 20 mm Hg. Finally, when ICP was above 30 mm Hg, the mean $P_{bt}O_2$ was already below 15 mm Hg. The correlation between $P_{bt}O_2$ and ICP was different when ICP was less than 20 mm Hg compared to the correlation when ICP was above 20 mm Hg. Specifically, the effect of ICP on $P_{bt}O_2$ was much

less significant when ICP ≤ 20 mm Hg compared to the effect when ICP ≥ 20 mm Hg. The other parameter that had a significant impact on $P_{bt}O_2$ when ICP was less than 20 mm Hg was MABP.

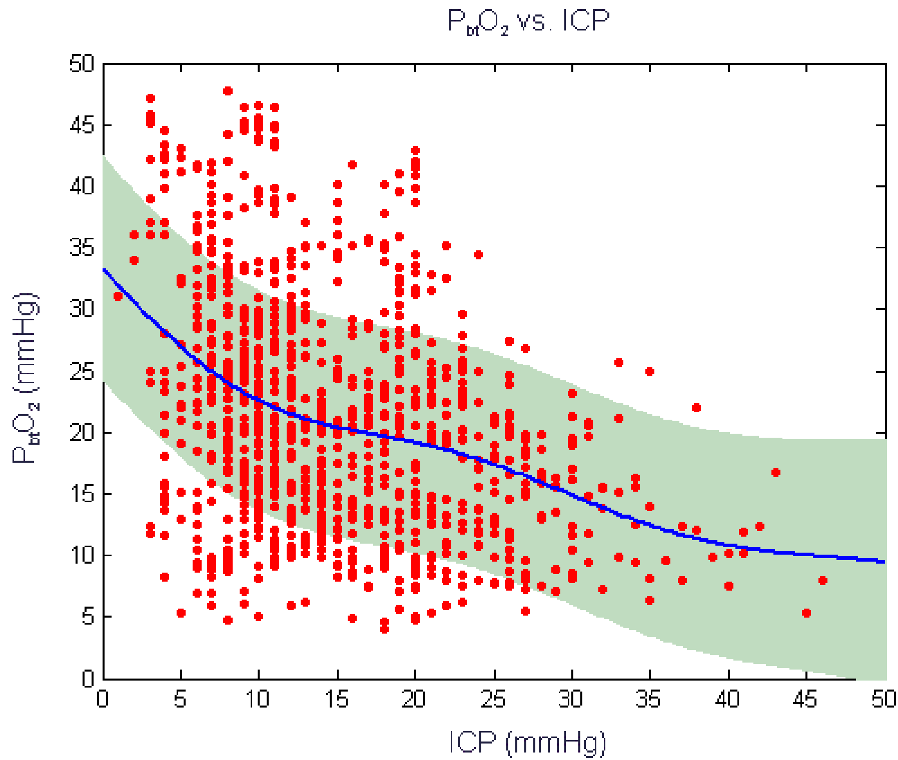


Figure 8.1 A scattergram showing the correlation between cerebral oxygenation ($P_{bt}O_2$) and intracranial pressure (ICP). The blue line represents the predicted mean values of $P_{bt}O_2$ depending on ICP, while the green zone represents the SD of the predicted distribution for $P_{bt}O_2$ (R^2=0.14). Red dots represent the individual data points (each dot can be the projection of one or more data points).

8.3.2 $P_{bt}O_2$ and MABP Correlation

The interrelationship between MABP and $P_{bt}O_2$ was analysed using Gaussian processes with an inclusion rate of 95% (Fig. 8.2). When MABP was below 80 ± 5 mm Hg mm Hg , $P_{bt}O_2$ values were below 20 mm Hg. When MABP was above 80 ± 5 mm Hg mm Hg, $P_{bt}O_2$ was above 20 mm Hg. The mean $P_{bt}O_2$ was positively correlated to MABP when MABP ≤ 100 mm Hg. However, the $P_{bt}O_2$ correlation with MABP was insignificant when MABP ≥ 100 mm Hg.

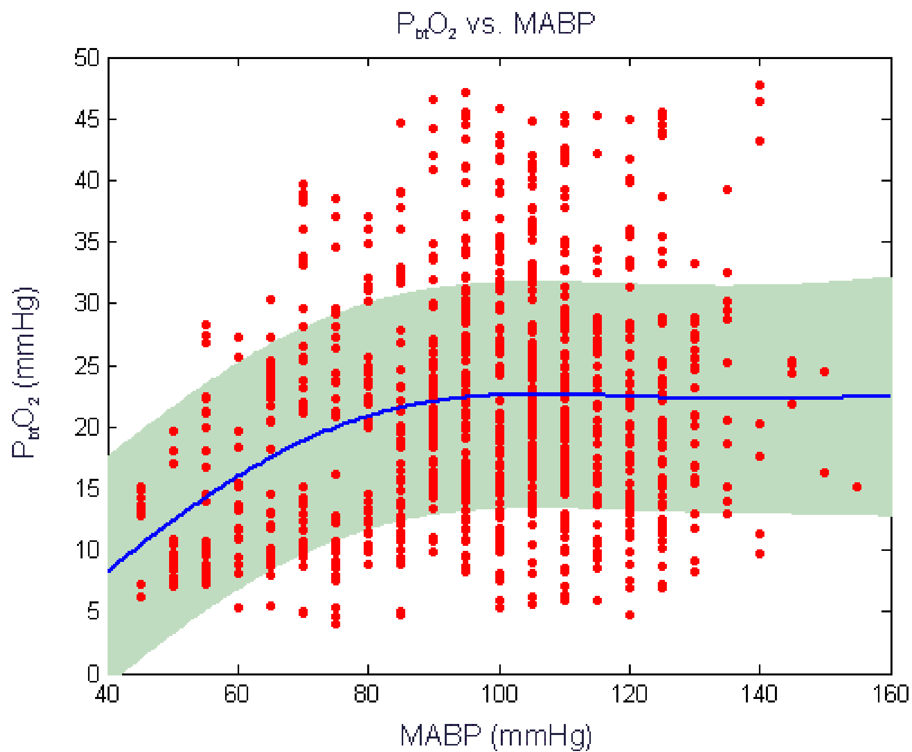


Figure 8.2 Correlation between cerebral oxygenation ($P_{bt}O_2$) and mean arterial blood pressure (MABP). The blue line represents the predicted mean values of $P_{bt}O_2$ depending on MABP, while the green zone represents the SD of the predicted distribution for $P_{bt}O_2$ (R^2=0.14). Red dots represent the individual data points (each dot can be the projection of one or more data points).

8.3.3 $P_{bt}O_2$ and CPP Correlation

The interrelationship between $P_{bt}O_2$ and CPP was analysed using Gaussian processes with an inclusion rate of 95% (Fig. 8.3). When CPP was below 70 mm Hg, $P_{bt}O_2$ values were below 20 mm Hg. When CPP was between 70 and 125 mm Hg, $P_{bt}O_2$ was above 20 mm Hg. Finally, when CPP ≥ 125 mm Hg, $P_{bt}O_2$ values were slightly decreased. The correlation between $P_{bt}O_2$ and CPP was significantly positive when CPP ≤ 100 ± 5 mm Hg while the correlation was not significantly changed when CPP ≥ 100 ± 5 mm Hg.

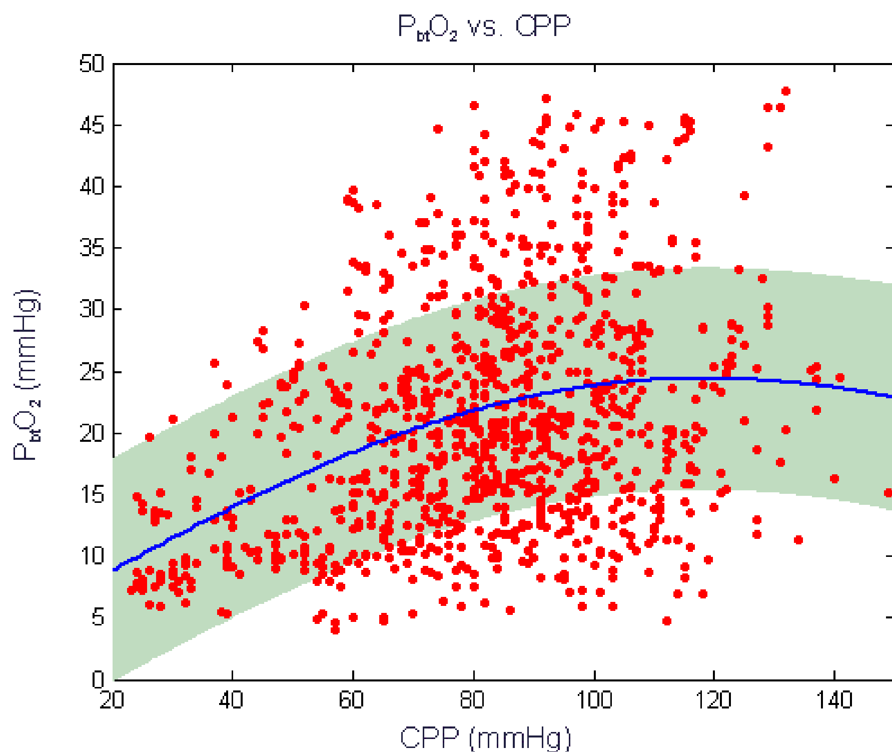


Figure 8.3 Correlation between cerebral oxygenation ($P_{bt}O_2$) and cerebral perfusion pressure (CPP). The blue line represents the predicted mean value of $P_{bt}O_2$ depending on CPP, while the green zone represents the SD of the predicted distribution for $P_{bt}O_2$ (R^2=0.14). Red dots represent the individual data points (each dot can be the projection of one or more data points).

8.3.4 Dynamic Correlation between $P_{bt}O_2$, ICP and MABP

The simultaneous 3-D analysis of the dynamic correlation between the values of $P_{bt}O_2$, ICP and MABP (Fig. 8.4) demonstrated several features. $P_{bt}O_2$ was within the normal range when ICP was below 5 mm Hg and MABP was within the range of 50-160 mm Hg. Similarly, $P_{bt}O_2$ was within normal range when ICP was between 5-10 mm Hg, and MABP was within the range of 60-160 mm Hg. When ICP was between 10-20 mm Hg, $P_{bt}O_2$ was normal when MABP was between 80-150 mm Hg. When ICP was between 20-25 mm hg, $P_{bt}O_2$ was borderline normal only when MABP was within the most optimal range, which was 100 - 110 ± 5 mm Hg. When the MABP was below or above this optimal range, the brain tissue was already hypoxic with $P_{bt}O_2$ values below 20 mm Hg. $P_{bt}O_2$ became critical when ICP was between 30-40 mm Hg, and life threatening when ICP was above 40-45 mm Hg, regardless of MABP values. Fig 8.5 shows a contour plot of the same data but expressed in a simplified manner thus facilitating the management of $P_{bt}O_2$ during critical care monitoring.

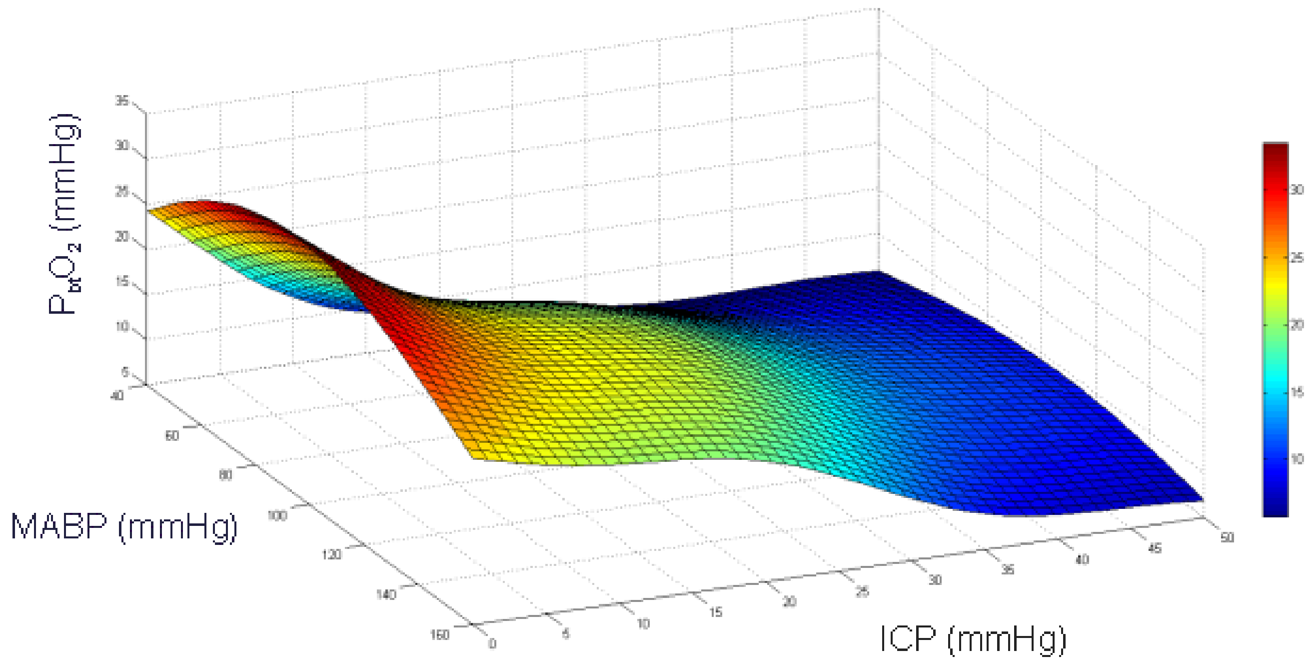


Figure 8.4 3D plot of the dynamic interrelationship between $P_{bt}O_2$, ICP and MABP throughout the monitoring process showing mean predicted values of $P_{bt}O_2$ as a function of ICP and MABP. The colour output reflects $P_{bt}O_2$ in mm Hg. This plot allows for the prediction of specific $P_{bt}O_2$ values for any given ICP and MABP.

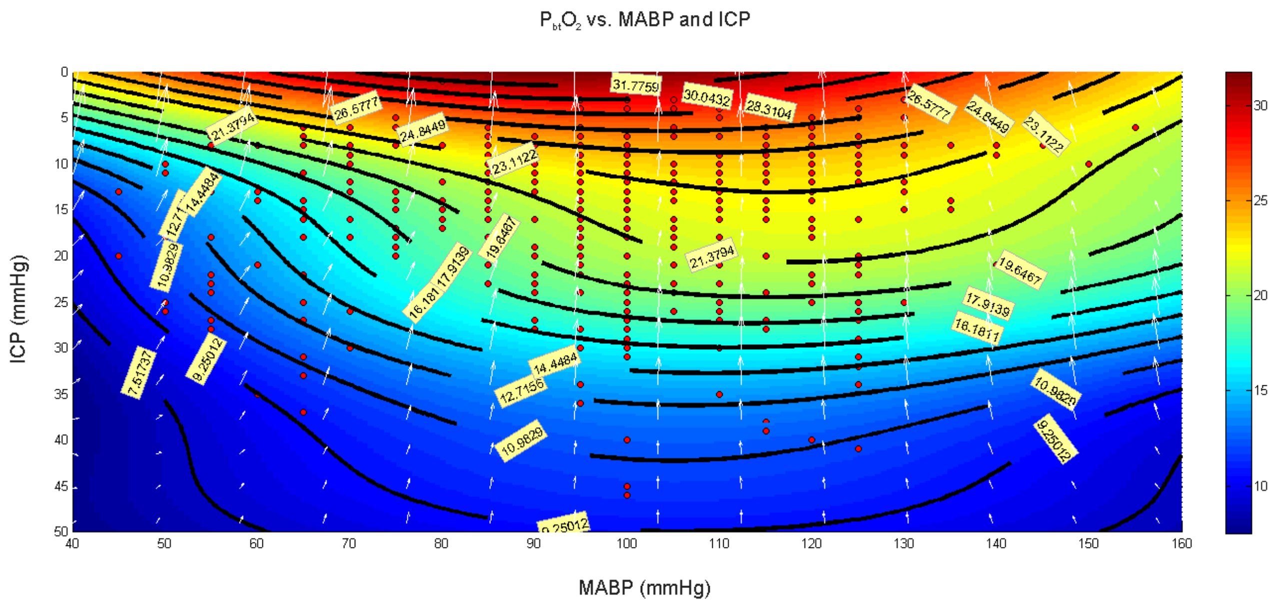


Figure 8.5 Contour plot of the dynamic interrelationship between $P_{bt}O_2$, ICP and MABP shown in Fig. 8.4. Black curves represent different constant $P_{bt}O_2$ values for varying values of ICP and MABP. This plot facilitates the prediction of mean $P_{bt}O_2$ values for any given ICP and MABP during monitoring time.

8.3.5 Dynamic Correlation between $P_{bt}O_2$, ICP and CPP

The simultaneous 3-D analysis of the dynamic correlation between the values of $P_{bt}O_2$, ICP and CPP (Fig. 8.6) demonstrated that $P_{bt}O_2$ was within normal range when ICP was below 5 mm Hg and CPP was within the range of 50-150 mm Hg. $P_{bt}O_2$ was within normal range when ICP was between 5-10 mm Hg, and CPP was within the range of 50-140 mm Hg. When ICP was between 10-20 mm Hg, $P_{bt}O_2$ was normal when CPP was between 65-130 mm Hg. When ICP was between 20-25 mm Hg, $P_{bt}O_2$ was borderline normal only when CPP was within the most optimal range, which is 80-110 ± 5 mm Hg. When CPP was below or above this range, brain tissue was already hypoxic, and $P_{bt}O_2$ was below 20 mm Hg. $P_{bt}O_2$ became critical when ICP was between 30-40 mm Hg, and life threatening when ICP was above 40-45 mm Hg, regardless of CPP values. Fig 8.7 shows a contour plot of the same data but expressed in a simplified manner thus facilitating the management of $P_{bt}O_2$ during critical care monitoring.

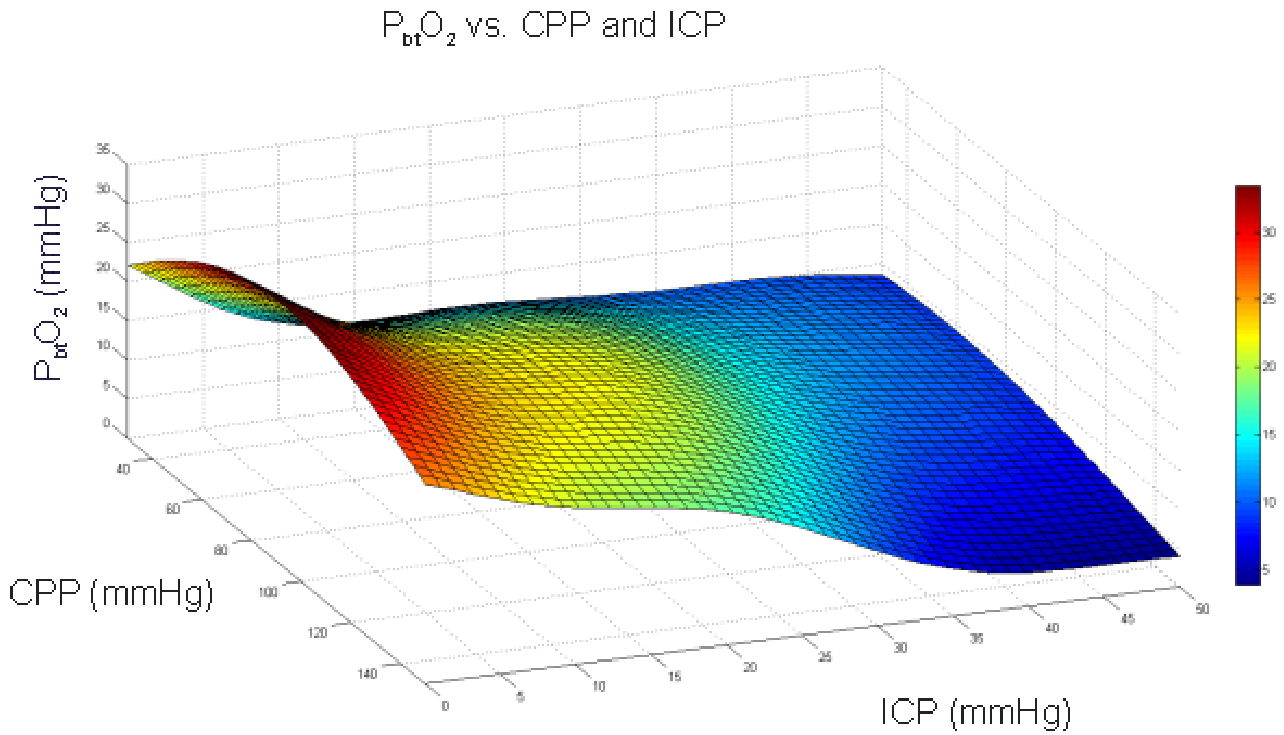


Figure 8.6 3D plot of the dynamic interrelationship between $P_{bt}O_2$, ICP and CPP throughout the monitoring process showing mean predicted values of $P_{bt}O_2$ as a function of ICP and CPP. This plot allows for the prediction of specific $P_{bt}O_2$ values for any given ICP and CPP. The colour output reflects $P_{bt}O_2$ in mm Hg.

Figure 8.7 Contour plot of the dynamic interrelationship between $P_{bt}O_2$, ICP, and CPP shown in Fig. 8.6. Black curves represent different constant $P_{bt}O_2$ values for varying values of ICP and CPP. This plot facilitates the prediction of mean $P_{bt}O_2$ values for any given ICP and CPP during monitoring time.

8.3.6 Significance of ICP versus MABP for $P_{bt}O_2$

The experimental data analysis by Gaussian processes showed that unit changes in the value of ICP and MABP have different effects on $P_{bt}O_2$. To evaluate the relative significance of changes of ICP and MABP on $P_{bt}O_2$ values, another contour plot (Fig. 8.8) was computed on the basis of the 3D data analysis presented in the previous section. The colour scale of the plot represents the significance of ICP changes over MABP changes at different ICP and MABP values on $P_{bt}O_2$. Note the inflection points in this graph at ICP values of 20 ± 5 mm Hg and 40 ± 5 mm Hg (Fig. 8.8), which we propose correspond to capillary intravenous hydrostatic pressure and pre-capillary small arteriole hydrostatic pressure, respectively.

8.3.7 Significance of ICP versus CPP for $P_{bt}O_2$

The experimental data analysis by Gaussian processes showed that similar to the case of ICP and MABP, unit changes of the values of ICP and CPP have different effects on the change of $P_{bt}O_2$. Another contour plot (Fig. 8.9) of their relative significance was computed on the basis of the 3D data analysis presented in the previous section. The colour scale of the plot represents the significance of ICP changes over CPP changes at different ICP and CPP values on $P_{bt}O_2$. Note again the inflection points in this graph at ICP values of 20 ± 5 mm Hg and 40 ± 5 mm Hg (Fig. 8.9), which we propose correspond to capillary intravenous hydrostatic pressure and pre-capillary small arteriole hydrostatic pressure, respectively. It is also apparent from a comparison of Figs. 8.8 and 8.9 that the significance of ICP over CPP is distinctively larger than that over MABP.

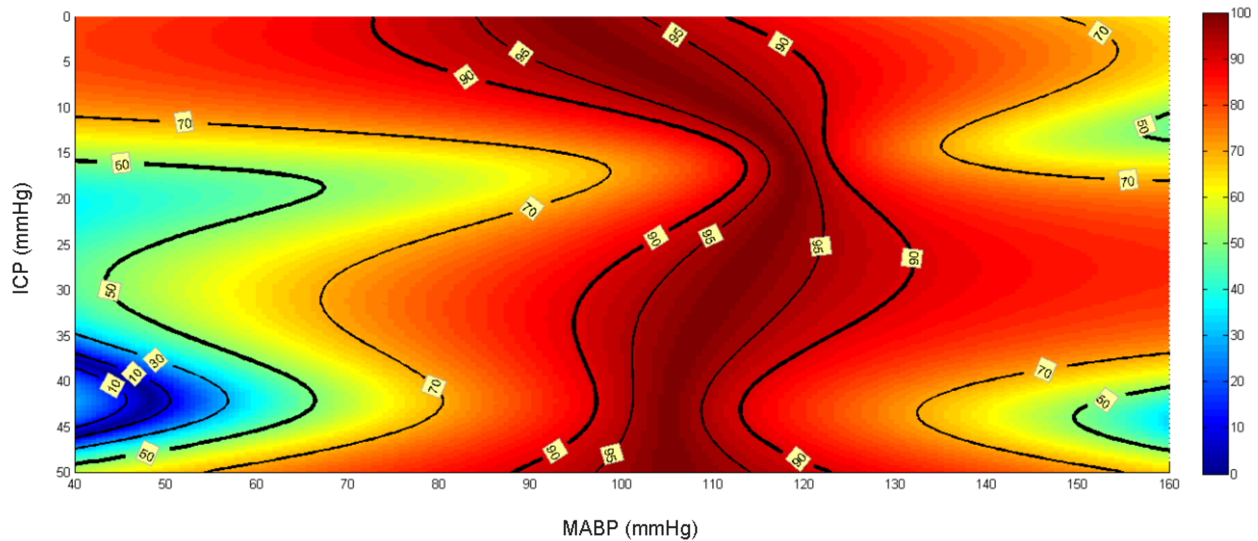


Figure 8.8 Contour plot of the significance of ICP changes over MABP changes at different ICP and MABP ranges on $P_{bt}O_2$. Black curves represent different constant values of the significance of ICP changes over MABP changes on $P_{bt}O_2$ values. The two distinct ranges of ICP, namely 20 ± 5 mm Hg and 40 ± 5 mm Hg, are clearly shown inflection points in this graph. We propose that these ranges correspond to capillary intravenous hydrostatic pressure and pre-capillary small arteriole hydrostatic pressure, respectively.

Significance of ICP over CPP for $P_{bt}O_2$

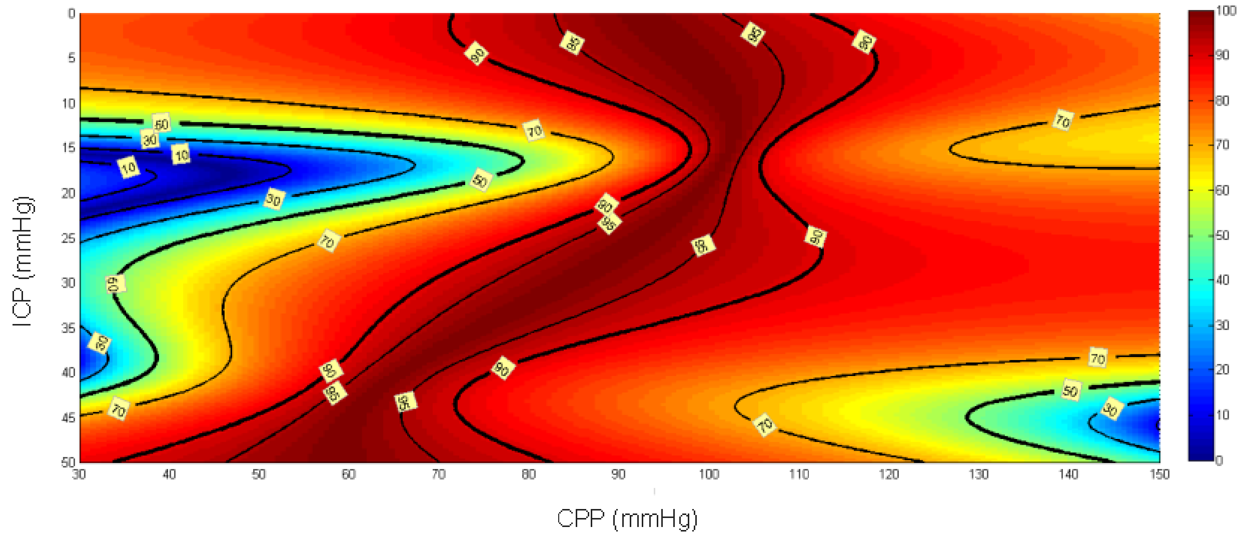


Figure 8.9 Contour plot of the significance of ICP changes over CPP changes at different ICP and CPP ranges on $P_{bt}O_2$. Black curves represent different constant values of the significance of ICP changes over CPP changes on $P_{bt}O_2$ values. The two distinct ranges of ICP, namely 20 ± 5 mm Hg and 40 ± 5 mm Hg, are clearly seen as inflection points in this graph. We propose that these ranges correspond to capillary intravenous hydrostatic pressure and pre-capillary small arteriole hydrostatic pressure, respectively.

8.3.8 Mathematical Analysis of Dynamic Interrelationship between ICP, MABP, and $P_{bt}O_2$

Since we had over 1000 data points, it allowed us to conduct a reliable simultaneous analysis of the dynamic interrelationship of ICP, MABP and $P_{bt}O_2$ and construct a 3D plot of their dynamic interaction. In an attempt to construct a closed form formulae representing the dynamic interdependence of ICP, MABP and $P_{bt}O_2$, the Gaussian processes predictive model was theoretically extended into negative values of ICP as show in Fig. 8.10. In Fig. 8.10 each red dot represents a projection of one or more data points/measurements, while black curves represent different constant values of $P_{bt}O_2$, which are defined by the colour scale shown next to the plot.

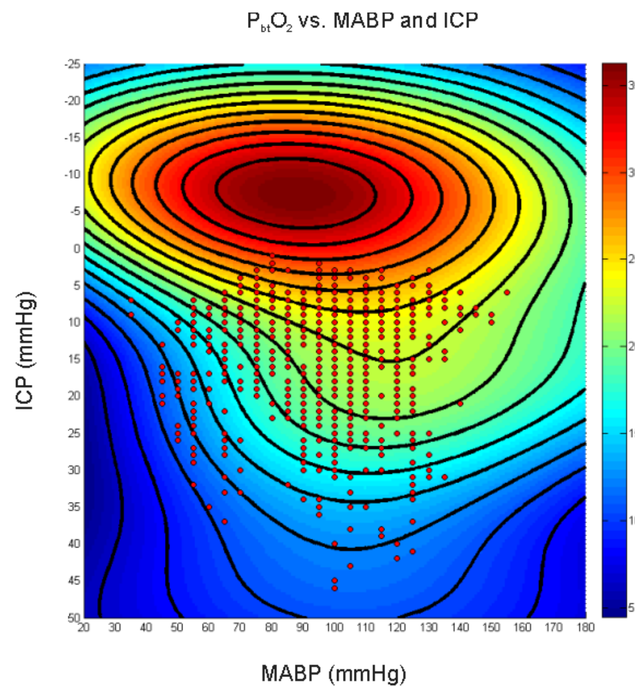


Figure 8.10 Contour plot of the interrelationship between ICP, MABP, and $P_{bt}O_2$. The ICP values below zero have been theoretically considered for the purpose of deriving the mathematical formula describing the dynamic correlation between $P_{bt}O_2$, ICP and MABP.

Three distinct regions relating ICP, MABP and $P_{bt}O_2$ can be seen in the 3D Gaussian predictive model (Fig. 8.10). Accordingly, a corresponding triple combination of Gaussian functions $F(x, y, a, b) = \exp\left(-(x/a)^2 - (y/b)^2\right)$ was employed to derive a closed form expression of the interrelationship between ICP, MABP and $P_{bt}O_2$. The derived final formula of the interrelationship between ICP, MABP and $P_{bt}O_2$ is:

$$\begin{aligned} \text{PO2} = &\ 39.6269\ F\left(\text{MABP} - \frac{\text{ICP}}{2} - 100,\ \text{ICP} + 5.4271,\ 80.6063,\ 16.6469\right) \\ &+ 16.7603\ F\left(\text{MABP} - \frac{\text{ICP}}{2} - 100,\ \text{ICP} - 22.3116,\ 80.6803,\ 12.8017\right) \\ &+ 9.5714\ F\left(\text{MABP} - \frac{\text{ICP}}{2} - 100,\ \text{ICP} - 44.4221,\ 175.3910,\ 14.0174\right) \end{aligned} \qquad (6)$$

where

$$F(x, y, a, b) = \exp\left(-(x/a)^2 - (y/b)^2\right).$$

The first, second and third Gaussian functions in formula (6) are related to the above mentioned three different regions of correlation. The coefficients 39.6269, 16.7603 and 9.5714 represent the magnitudes of the first, second and third Gaussian functions in the formula corresponding to different levels of $P_{bt}O_2$. In the formula, x and y are related to MABP-ICP/2 and ICP, respectively. The ratio *a*/*b* represents the relative impact of the changes of MABP-ICP/2 and ICP on the change of $P_{bt}O_2$ in different parts of the formula.

To evaluate the accuracy of the derived formula (6), the whole range of interest for ICP and MABP was used to construct its contour plot shown in Fig. 8.11. Visual comparison shows significant concurrences between the contour plots of the derived closed form formula (Fig.

8.11) and the Gaussian processes predictive model (Fig. 8.10). The derived formula (6) was used to calculate the value of $P_{bt}O_2$ for the measured values of ICP and MABP when ICP > 20 mm Hg and a histogram of the difference between the calculated and experimentally measured values of $P_{bt}O_2$ was constructed (Fig. 8.12). The histogram in Fig. 8.12 demonstrates that the difference between the values of $P_{bt}O_2$ computed using the derived formula and experimental measurements are concentrated around zero, with a standard deviation of 5.48. The interrelationship between ICP, MABP and $P_{bt}O_2$ given by the derived formula when ICP > 20 mm Hg was statistically significant ($p < 0.05$).

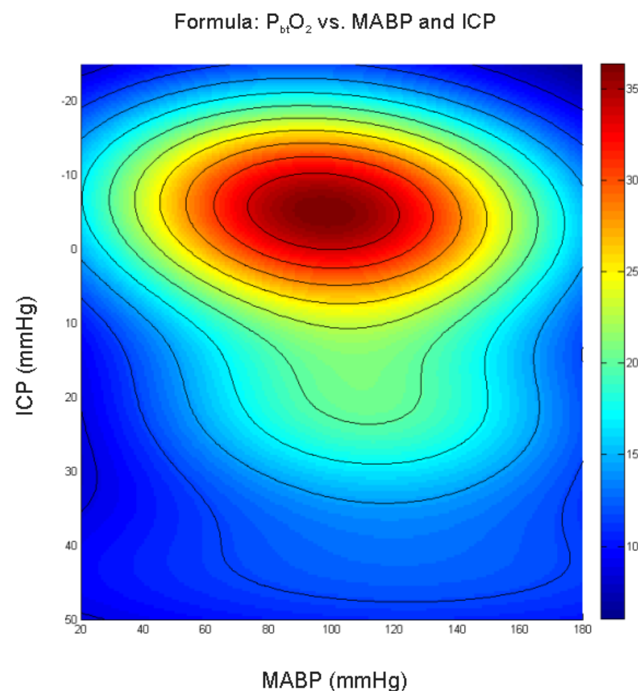


Figure 8.11 Contour plot of the interrelationship between ICP, MABP, and $P_{bt}O_2$ based on the formula. The ICP values below zero have been considered for the purpose of deriving the mathematical formula of the dynamic correlation between $P_{bt}O_2$, ICP and MABP.

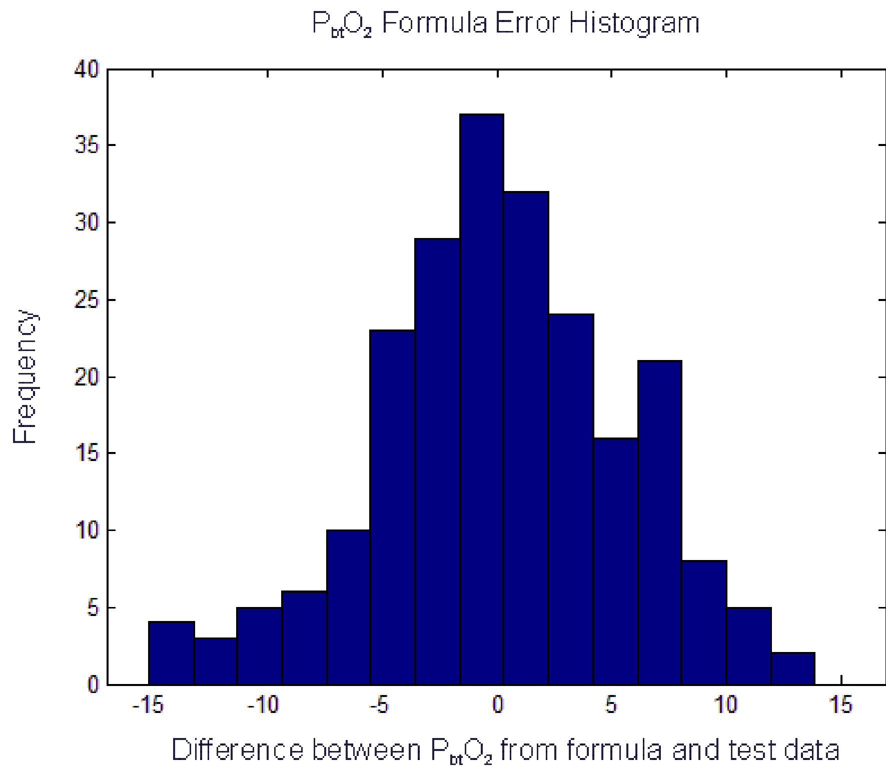


Figure 8.12 The histogram of the difference between the $P_{bt}O_2$ values computed via the derived formula and experimental measurements when ICP > 20 mm Hg. SD = 5.48.

8.3.9 Critical Thresholds of ICP

Both clinical and experimental studies have shown two distinct thresholds for ICP, that is 20-25 mm Hg and 35-40 mm Hg, which are strongly correlated to critical and life-threatening levels of brain tissue oxygenation, respectively (Steiner & Andrews 2006, Grinkeviciute et al. 2008, Rangel-Castilla et al. 2008, Singhi & Tiwari 2009). Previous studies have demonstrated that the ovine model of TBI replicates many features of human TBI (Vink et al. 2008), including correlations between ICP and $P_{bt}O_2$, and the previous chapters of the current thesis have suggested that similar thresholds for ICP exist in this ovine TBI model. Using the data of this

thesis, we therefore tried to elucidate the critical thresholds of ICP and the correlation between ICP thresholds and $P_{bt}O_2$.

It is well known that mean intravascular hydrostatic pressure in post-capillary venules is 10-15 mm Hg, within capillaries is 20-25 mm Hg, and in pre-capillary arterioles is 35-40 mm Hg (Table 8.1; Despopoulos & Silbernagl, 1991). Normal ICP values are considered to be less than 10 to 15 mm Hg for adults and an ICP greater than 15 mm Hg is considered to be abnormal (Mazzola & Adelson 2002, Rangel-Castilla et al. 2008, Smith 2008). Our study has shown that $P_{bt}O_2$ was within normal limits (>20 mm Hg) when ICP was less than 20 mm Hg, $P_{bt}O_2$ was critically low when ICP was above 20-25 mm, and $P_{bt}O_2$ was life-threatening when ICP was above 35-40 mm Hg (Fig. 8.1). We speculate that $P_{bt}O_2$ changes from normal (> 20 mm Hg) to critical (< 10-15 mm Hg) to life-threatening (<10 mm Hg) when ICP changes from normal (< 15 mm Hg) to critical (20-25 mm Hg) to life-threatening (>35-40 mm Hg), are reflecting the different intravascular hydrostatic pressures in different segments of the cerebral vasculature. These thresholds would be related to the successive compression of post-capillary venules, capillaries and precapillary met-arterioles by increasing ICP.

Table 8.1 Mean Hydrostatic pressure values in cerebral post-capillary venules, capillaries, and pre-capillary met-arterioles:

Post capillary venules	15-20 mm Hg
Capillaries	20-25 mm Hg
Pre-capillary met-arterioles	35-40 mm Hg

The first ICP threshold of 20-25 mm Hg is critical because at this level, ICP can already compress capillaries and compromise blood flow. The second ICP threshold of 35-40 mm Hg would be considered life-threatening because at this level ICP can also compress and even close the precapillary met-arterioles and dramatically decrease blood flow.

Therefore, as ICP increases after TBI, it would sequentially compress and significantly constrict post-capillary venules (when ICP is 15-20 mm Hg), then capillaries (when ICP is 20-25 mm Hg), and finally as it goes above 35-40 mm Hg, it would compress pre-capillary met-arterioles and small arterioles (Table 8.1). The three step pattern of the graph (Fig. 8.1) clearly reflects these pathophysiological changes occurring as ICP increases, with the thresholds corresponding to intravascular hydrostatic pressure differences in the above mentioned segments of the cerebral vascular bed.

8.3.10 Reconsidering the Formula for Calculation of CPP

CPP is the pressure gradient acting across the cerebrovascular bed and is regarded as a main determinant of CBF. To date, the question of what the optimal CPP is following TBI remains unanswered (Howells et al. 2005, Bratton et al. 2007c). Indeed, when CPP is vigorously maintained above 70 mm Hg, there is no significant benefit to patients and cardiovascular and respiratory complications frequently occur (Bratton et al. 2007c). Paradoxically, a level of CPP below 60 mm Hg is considered by some to be detrimental (Clifton et al. 2002). When the formula for CPP was first proposed, it was based on the assumption that autoregulation was disrupted and cerebral blood flow became linearly related to MABP; later studies, however, have

revealed that autoregulation is functioning in more than 75% of patients with severe TBI (Peterson & Chesnut 2009, Czosnyka et al. 2005).

Normally, autoregulation is active within a MABP range of 50–160 mm Hg, responds within seconds of the MABP change (Adamides et al. 2006, Panerai et al. 2004, Panerai 1998), and maintains constant cerebral post-arteriolar hydrostatic pressure at 35 ± 5mm Hg. When ICP is not increased (ICP = 5±5 mm Hg), the real cerebral perfusion pressure will actually be the difference between post-arteriolar pressure (PAP) and ICP (CPP=PAP-ICP) and would normally be maintained at 30 ± 5 mm Hg. Since autoregulation is functioning in more than 75% of patients with severe TBI, this means that CPP values are the difference between PAP and ICP, rather than between MABP and ICP. This may explain why, to date, it has been unclear how the same CPP values in different TBI patients maintain different $P_{bt}O_2$ values, and why increases in CPP only improve $P_{bt}O_2$ in some patients and not in others.

Data analysis in the present chapter shows that increasing CPP to 70-80 mm Hg will improve $P_{bt}O_2$ only when ICP is below its critical threshold level (< 25-30 mm Hg) and MABP is below 100-110 mm Hg. When ICP is above 35-40 mm Hg, there is no improvement in $P_{bt}O_2$ when CPP is increased. We speculate that regardless of MABP/CPP values, intravascular hydrostatic pressure in post-arterioles cannot be increased significantly above 35-40 mm Hg. Therefore, ICP values above 35-40 mm Hg are critical as they can overcome the intravascular pressure and compress capillaries and pre-capillary met-arterioles. In this scenario, even though CPP values calculated by currently used formulae can be maintained at normal levels (70-80 mm Hg) with

the help of increased MABP, $P_{bt}O_2$ values will still be critically low since the real cerebral perfusion, that is CPP=PAP-ICP, is closer to zero.

It is therefore clear that CPP values calculated with the currently used formula under conditions when ICP is above critical thresholds do not reflect the real value of CPP. $P_{bt}O_2$ is still critically low under these conditions. In contrast, if CPP values are calculated as the difference between PAP and ICP, the value of the revised CPP would clearly reflect the real state of cerebral perfusion and of $P_{bt}O_2$. I therefore propose that we cease application of the currently used formula for CPP and instead apply the following modification;

$$CPP=PAP-ICP$$

8.4 Discussion

This is the first time that ICP, MABP, CPP and $P_{bt}O_2$ have been analysed simultaneously with machine learning using Gaussian processes. To the best of our knowledge, it is also the first time that a chart has been developed describing the dynamic interrelationship between ICP, MABP, CPP and $P_{bt}O_2$, which has the potential to have clinical application in the management of patients with TBI by targeting ICP and MABP to achieve an optimal $P_{bt}O_2$. The similarity in responses between our large animal, ovine model of TBI and human TBI has been critical to this development, and again underscores the importance of large animal models of TBI for translational research. The outcome of the analysis by Gaussian processes can be summarised as follows:

1. Two critical thresholds for ICP after TBI, namely 20-25mm Hg and 35-40mm Hg, were identified in the analysis of over 1000 data points obtained during the ovine TBI experiments.
2. These critical ICP thresholds after TBI may relate to the different intravascular hydrostatic pressure in two different sections of cerebral vasculature, namely cerebral capillaries and cerebral pre-capillary small met-arterioles. The intravascular hydrostatic pressure of these sections of the cerebral vasculature has been shown to be 20-25mm Hg and 35-40mm Hg, respectively.
3. Simultaneous 3D analysis of the dynamic interrelationship between ICP, MABP, CPP and $P_{bt}O_2$ is a better tool to investigate these interrelationships as opposed to the more common, 2-parameter analyses that have been used previously, namely $P_{bt}O_2$ versus ICP or MABP or CPP.
4. By utilizing 3D analysis, we have developed a contour plot of the dynamic interrelationship between $P_{bt}O_2$, ICP and MABP that can be used to manage both ICP and MABP to achieve an optimal $P_{bt}O_2$ after TBI.
5. The current formula used to calculate CPP is not accurate since it overlooks the real values of ICP and MABP, and does not reflect the actual $P_{bt}O_2$ values. I propose that the formula be modified to CPP= PAP – ICP, where PAP is post-arteriolar pressure.

The importance of the simultaneous 3D analysis of the interrelationship between CPP, $P_{bt}O_2$, and ICP cannot be overstated given that increases in CPP to 70-75 mm Hg improved $P_{bt}O_2$, whereas increases of CPP above 70-75 mm Hg did not improve $P_{bt}O_2$. In contrast, separate 2-parameter analyses of the correlation between CPP and $P_{bt}O_2$ alone showed that $P_{bt}O_2$ would have

improved with an increase in CPP up to 100 mm Hg. The reason of this difference between 3D and 2-parameter analysis is that 3D analysis considers the correlation between CPP and ICP (CPP being a derivative of ICP) as well as revealing the simultaneous correlation of $P_{bt}O_2$ independently with both CPP and ICP. On the other hand, analysis of only 2 parameters overlooks the impact of ICP on both CPP and $P_{bt}O_2$. Indeed, the 2-parameter analysis, in showing that CPP values between 70 and 100 mm Hg increase $P_{bt}O_2$, ignore the fact that this would only apply for ICP values less than 15-20 mm Hg. The 3D analysis clearly showed that $P_{bt}O_2$ values depend on ICP more than on CPP when ICP increases above a critical threshold (20 mm Hg), and that increases in CPP above 70-75 mm Hg are ineffective for improvement of $P_{bt}O_2$ when ICP is above these levels.

Finally, the results of the 3D analysis showed that CPP values calculated using the current definition, CPP = MABP – ICP, does not reflect the real correlation between CPP and $P_{bt}O_2$, or CPP and ICP, and overlooks the impact of ICP on CPP and $P_{bt}O_2$. This may explain why patients with the same CPP values can often have different $P_{bt}O_2$ values and outcomes. I propose a modified formula, which considers the impact of ICP on both CPP and $P_{bt}O_2$, and so reveals the real dynamic interrelationship between these three parameters, that is;

$$CPP = PAP - ICP$$

where PAP is post-arteriolar pressure.

CHAPTER 9

GENERAL DISCUSSION

In the current thesis, I have characterized a number of features of traumatic brain injury including:

1. The effects of injury on ICP and CPP in two different models of rodent TBI. We noted that TBI does not consistently produce increases in ICP unless a haemorrhagic mass lesion is present, and therefore concluded that this species was not ideal for the development of ICP targeted pharmacotherapies.
2. The effects of TBI on ICP, CPP and $P_{bt}O_2$ in an ovine, large animal model. We noted that the sheep model of injury produces similar changes in these variables to clinical (human) TBI, and is therefore well suited to the development of ICP targeted pharmacotherapies.
3. The effects of two different substance P, NK1 receptor antagonists on ICP, $P_{bt}O_2$ and CPP in an ovine model of TBI at both moderate and severe injury levels, and compared the effects to those of the osmotic agents, mannitol and hypertonic saline. We noted that in contrast to the osmotic agents, the NK1 antagonist consistently reduced ICP and improved $P_{bt}O_2$ irrespective of the severity of injury.
4. The effects of the putative neuroprotective compounds magnesium and progesterone on ICP and $P_{bt}O_2$ following ovine, moderate TBI. We noted that neither compound significantly improved either of these physiological variables.
5. The dynamic interrelationship between $P_{bt}O_2$, ICP, and MABP and CPP with the application of Gaussian processes for data analyses. This facilitated the development of a contour plot describing these dynamic interrelationships and enabling the prediction of mean $P_{bt}O_2$ values for any given ICP and MABP, the identification of critical thresholds in ICP and elucidation of the physiological basis, and the refinement of the CPP formula.

In clinical scenarios, TBI is frequently accompanied by a reduction in the oxygen concentration of arterial blood (Bratton et al. 2007b, Bratton et al. 2007c, Bratton et al. 2007e, Bratton et al. 2007f). Hence; in chapter three we investigated ICP changes in rodent models of TBI, with and without hypoxia. Hypoxaemia following TBI results from the immediate apnea and subsequent respiratory insufficiency that accompanies the traumatic event (Atkinson et al. 1998). Hypoxemia leads to hypoxic or ischemic brain injury, which is known to significantly contribute to morbidity and mortality following TBI (Stocchetti et al. 1996, Marmarou 2007). Our results have shown that TBI, either alone or in combination with secondary hypoxia, does not result in any sustained increase in ICP in the rat unless there is a mass lesion associated with a hemorrhage. In the absence of ICP changes, reductions in CPP that were noted in TBI were closely associated with changes in MABP. Though there was some ICP increase in animals exposed to TBI with secondary hypoxia, it was nonetheless statistically insignificant.

As was discussed in chapter 3, a number of previous studies have reported ICP values in rodents after TBI, with some reporting increases after TBI (Liu et al. 2002, Wei et al. 2010, Zweckberger & Plesnila 2009) and others reporting no increase (Goren et al. 2001, Kahveci et al. 2001, Rogatsky et al. 2003, Thomas et al. 1998). Remarkably, those that report increases have usually been associated with a severe focal or penetrating injury that causes extensive haemorrhage. Although the reasons for such contradictions are unclear, the findings may well be explained by anatomical and post-injury developing pathophysiological differences, specifically the absence of a gyriform brain, large white matter domains and a tentorium cerebelli in rats compared to other mammals such as humans, sheep, pigs, cats or dogs, and intracranial hemorrhage developed following TBI and not being treated in rat models of TBI. The tentorium cerebelli is

an arched lamina of dura mater separating the supratentorial compartment (containing the cerebral hemispheres) from the infratentorial compartment, which contains the cerebellar hemispheres and brainstem (Klintworth 1968). In small animals such as mice, rats, hamsters, and gerbils, it consists of delicate meningeal folds that only separate the lateral portions of the cerebellum and cerebral hemispheres. In larger animals, such as mammals including goats, sheep, cats, dogs, and humans, the bilateral folds are joined and form a diaphragm, a structure sufficient to prevent expansion or collapse of the supratentorial matter into the infratentorial compartment (Bull 1969). Such movement through the tentorial aperture is a feature of transtentorial herniation. Given that the tentorium cerebelli of the rat is quite rudimentary and underdeveloped; any additional pressure associated with brain swelling could potentially be readily transferred to the infratentorial compartment and beyond, thus reducing the supratentorial ICP. No post-traumatic increase in ICP would be noted. In regards of pathophysiological differences, such as the presence of intracranial hemorrhage, the mass lesion will overcome the volume expansion allowed by the absence of the tentorium (reduced compliance) and an increase in ICP will result. Therefore the inconsistencies in the post-traumatic ICP reported in rodents may well be related to the presence or absence of intracranial hemorrhage. Moreover, the presence or absence of increased ICP would have a critical impact on the development of effective neuroprotective therapies designed for translation into the human condition where increases in ICP are common.

Given the difficulties in pursuing further ICP studies in rodents, chapter 4 sought to characterize ICP, MABP, CPP and $P_{bt}O_2$ changes following TBI in what could potentially be a more appropriate model for the development of ICP targeted pharmacotherapies, namely the ovine

impact model of TBI (Lewis et al. 1996, Van Den Heuvel et al. 2004). Sheep brains share many of the characteristic features of the human brain, including a highly developed gyriform structure, large white matter domains and a substantial tentorium cerebelli. After immediate changes in ICP, MABP, CPP and $P_{bt}O_2$ within the first 30 min that were largely associated with vascular responses, ICP increased more than 4-fold in the subsequent 3 h after moderate TBI, while $P_{bt}O_2$ had decreased to less than 50% of normal values within the first 60 min and remained depressed for the rest of the 4 h monitoring period. CPP also significantly declined, as a function of both the increased ICP and the systemic hypotension that developed after injury. These changes are similar to what has been previously described in human TBI studies (Bratton *et al.* 2007a, Bratton et al. 2007b, Bratton et al. 2007e), confirming that the absolute and temporal changes in ICP, MABP, CPP and $P_{bt}O_2$ in the ovine model were representative of human TBI, and that the model was ideal to further pursue the development of neuroprotective compounds, and specifically their effects on these important physiological parameters.

In chapter five, it was demonstrated that administration of an NK1 receptor antagonist, n-acetyl-tryptophan, significantly reduced ICP by 50% within 4 h, and that ICP in these treated animals was approaching normal values by this time point. The reduction in ICP was just as effective as with mannitol administration, albeit that the effect of mannitol was less consistent with more variability in the data. While mannitol is widely used clinically as a therapeutic intervention for increased ICP after acute brain injury (Bratton et al. 2007b, Meyer et al. 2010), its mechanism of action is to draw water out of brain tissue down an osmotic gradient. Not only does this fail to address the potential mechanisms associated with development of ICP, in the presence of a more permeable BBB, the osmotic agent can actually cross into the brain parenchyma and exacerbate

the movement of water from the vasculature to the brain tissue (Node & Nakazawa 1990). In contrast, the NK1 receptor antagonist mechanism of action involves reducing blood brain barrier permeability and inhibiting the development of vasogenic oedema (Donkin et al. 2009, Turner et al. 2011). This mechanism of action eliminates any possibility of a reversal of osmotic gradient and a rebound increase in ICP, presumably accounting for the more consistent reduction in ICP seen in the present study.

The reoxygenation of brain tissue after TBI is one of the critical aims of effective therapeutic intervention, with restoration of energetic state facilitating recovery from tissue damage. In the present study, $P_{bt}O_2$ reduced to less than 50% of normal values in those animals administered with the saline vehicle, and this reduction in $P_{bt}O_2$ persisted for the entire duration of the monitoring period. In contrast, administration of the NK1 receptor antagonist resulted in an immediate increase in $P_{bt}O_2$ such that by 4 h after TBI, $P_{bt}O_2$ was no longer significantly different from normal values. A similar increase in $P_{bt}O_2$ was observed in the mannitol treated animals, although as with the ICP response, this increase with mannitol was more variable. The effective reoxygenation of the brain with the NK1 antagonist may, in part, explain the neuroprotective effects previously reported in experimental studies where administration of the NK1 antagonist resulted in reduced neuronal cell death and an improved functional outcome in injured animals (Vink & van den Heuvel 2010, Turner et al. 2011). Presumably, the effective reoxygenation of the brain is a reflection of the reduced ICP, with no significant effects of the NK1 antagonist on MABP or brain temperature noted.

In chapter six, the effects of the experimental neuroprotective agents, magnesium and progesterone, on ICP, $P_{bt}O_2$, and CPP following ovine TBI was examined. These were of particular interest given that magnesium has shown mixed results in clinical trials (Sen & Gulati 2010) while progesterone has recently been entered into a large phase III clinical TBI trial. Our results demonstrated that administration of either compound after TBI did not significantly reduce increased ICP, although there were some trends with magnesium that were interesting and should be pursued, but were beyond the scope of the current thesis. The negative result with progesterone is consistent with the results of a recent phase II clinical trial, where it was shown that administration of progesterone had no apparent effects on increased ICP in patients with TBI (Wright et al. 2007, Xiao et al. 2008). Similar negative effects were observed on both CPP and $P_{bt}O_2$. Given that the focus of the progesterone preclinical work was on reduction of oedema, the failure of the compound to have any positive effects in the current large animal study does not instill confidence that the phase III clinical trial will be successful.

In chapter seven, the effects of another NK1 receptor antagonist, EU-C-001, on ICP, $P_{bt}O_2$, and CPP following ovine TBI was investigated, largely to ensure that the previous NAT results were class related and not compound specific. However, the study was also performed at a more severe level of injury to determine whether the effects of NK1 antagonists were also transferable to other levels of injury, and the results were compared with an alternative osmotic agent, hypertonic saline (HTS), which is receiving increasing attention as an osmotic intervention given the shortcomings of repeated mannitol administration. Our results demonstrated that administration of the NK1 receptor antagonist EU-C-001 was highly effective at reducing posttraumatic ICP, and increasing both CPP and $P_{bt}O_2$ following severe TBI. Moreover, while

HTS reduced ICP within 60 min of administration and did not cause any further decline, administration of the NK1 antagonist resulted in a consistent decline in ICP at 5 mm Hg/h to values below those recorded with HTS. The NK1 antagonist also significantly improved CPP and $P_{bt}O_2$, in contrast to HTS, which did not affect these parameters. Thus, the beneficial effects of both EU-C-001 and NAT in the current thesis were a drug class effect, were superior to the commonly used osmotic agents, and were apparent irrespective of the injury severity.

One limitation with the severe injury study was that all severely injured animals that served as vehicle treated controls died within the 4 h monitoring period. We reported the typical ICP, CPP and $P_{bt}O_2$ values in these animals in the text, and chose not to continue inducing severe injury for the sake of creating this subgroup for statistical analysis. This decision was made on ethical grounds considering the large number of animals that would have to be injured (and potentially die) to obtain sufficient survivors for statistical analysis. Moreover, the data acquired with EU-C-001 and HTS could be compared to both sham values (included in the graphical analysis) and the moderate injury data from chapter 4. In either case, the significant advantage of EU-C-001 in the management of ICP, CPP and $P_{bt}O_2$ was clearly apparent.

Chapter 8 used all the ovine data acquired in the previous chapters for the first time to perform Gaussian Processes for machine learning so as to understand the dynamics of the simultaneous interrelationships between $P_{bt}O_2$, ICP, MABP and CPP. More simply stated, this analysis was directed toward understanding the relative importance of ICP, MABP and CPP on $P_{bt}O_2$ given that the approaches to patient management vary between ICP directed and CPP directed depending on local protocols. Thus while maintenance of $P_{bt}O_2$ after TBI is the clear goal of

neurointensive care, the ideal approach to this has not been universally agreed. There was a similar lack of clarity in the current sheep data with instances of close association between either ICP or CPP with $P_{bt}O_2$, but also instances of no association between these parameters.

By conducting the advanced 3D computer analysis in chapter 8, a novel contour chart summarizing the dynamic interrelationship between ICP, MABP, CPP and $P_{bt}O_2$ was created, which has the potential for clinical application in the management of patients with TBI. Specifically, for any combination of ICP and MABP, $P_{bt}O_2$ can be determined from the plot. Thus, ICP and MABP can be manipulated to achieve a desired $P_{bt}O_2$. Further analysis also revealed two critical thresholds for ICP after TBI, namely 20-25mm Hg and 35-40mm Hg, which we posit is related to the different intravascular pressures in cerebral capillaries and pre-capillary small met-arterioles, respectively. Accordingly, as ICP increases after trauma, there is sequential compression of different vascular elements as their pressure is exceeded, namely post-capillary venules (15-20 mm Hg), capillaries (20-25 mm Hg) and precapillary met-arterioles (35-40 mm Hg). This sequential compression results in the thresholds noted and marks the fall of $P_{bt}O_2$ from mild hypoxic levels to the life-threatening lack of tissue oxygen. It also emphasizes that any increase in ICP, no matter how small, impacts on the brain's ability to recover from injury and that an intervention that can be applied at all levels of ICP would be most desirable.

The 3D computer analysis in chapter 8 also elucidated the deficiencies in the conventional interpretation of CPP as MABP – ICP. Indeed, patients with the same CPP values can have profoundly different $P_{bt}O_2$ values and different outcomes, largely because the conventional CPP formula does not take into account the simultaneous impact of ICP on both CPP and $P_{bt}O_2$. The

discussion above of the different vascular elements and the effects of ICP highlights this possibility. Therefore the modified formula of CPP = PAP – ICP has been proposed, which assumes that real cerebral perfusion depends upon the pressure within precapillary arterioles, and whether their flow has been restricted by ICP.

Conclusion

The current thesis has demonstrated that the large animal, ovine model of impact TBI is superior to rodent TBI models for the analysis of intracranial fluid dynamics after TBI. Moreover, given the similarities in intracranial fluid dynamics between the sheep and humans, the ovine TBI model is well suited for the characterization of dynamic interrelationships between ICP, CPP, MABP and $P_{bt}O_2$, and their translation to the clinical arena. In addition to developing a novel contour plot for the targeted management of MABP and ICP to achieve a desired $P_{bt}O_2$, the current thesis has also identified critical thresholds in ICP that significantly impact on $P_{bt}O_2$, and potentially TBI outcome. These thresholds appear to be caused by and coincide with the two different intravascular hydrostatic pressures of the cerebral vasculature, namely pre-capillary arterioles and capillaries. This has direct implications on CPP and a modified CPP formula has been proposed (CPP = PAP – ICP) to account for the simultaneous interrelationships between CPP, ICP and $P_{bt}O_2$. In using the ovine TBI model, the studies in this thesis have shown that NK1 antagonists are more effective than osmotic agents for the reduction of ICP and improvement in $P_{bt}O_2$ after TBI, irrespective of the injury severity. While other experimental compounds such as magnesium and progesterone have been proposed as potential neuroprotective compounds for the management of clinical TBI, they were ineffective at either decreasing elevated ICP after injury or improving $P_{bt}O_2$ status in this ovine model of TBI. The further investigation of NK1 antagonists as a potential clinical intervention is warranted.

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