The investigation of histopathological changes after the administration of Vaccinia Virus Complement Control Protein in brain injured rats

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University of Cape Town

TABLE OF CONTENTS

Glossary

Abstract

Chapter 1: Introduction 1

1.1. Background and significance 1

1.2. Hypothesis 3

1.3. Purpose of the study 3

1.4. Overview of the thesis 4

Chapter 2: Literature review 5

2.1. Introduction 5

2.2. Traumatic brain injury 5

2.2.1. Definition 5

2.3. Other criteria for defining traumatic brain injury 6

2.3.1. Mild TBI 6

2.3.2. Moderate TBI 7

2.3.3. Severe TBI 7

2.4. Background 7

2.5. Morphological and physiological changes following TBI in patient and animal models 9

2.6. Degenerative disorders 12

2.7. Experimental models of traumatic brain injury 14

2.8. Traumatic brain injury and diffuse axonal injury 17

2.8.1. Diffuse axonal injury 17

2.8.2. Penetrating head injuries 18

2.9. Cellular injury 20

2.9.1. Cellular adaptation 20

2.9.2. Acute inflammation 26

2.9.3. Chronic inflammation 27

2.10. Cellular changes 28

2.11. Complement system 29

2.12. Therapeutic approaches to brain injury 33

University of Cape Town

2.13. Vaccinia Virus Complement Control Protein 35
2.14. Conclusion 37

Chapter 3: Methodology 39
3.1. Ethics 39
3.2. Rats 39
3.3. Experimental Design 39
3.4. Procedures 40
3.4.1. Anaesthesia 40
3.4.2. FPI induction 41
3.4.3. Experimental conditions 43
3.4.4. Euthanasia 44
3.5. Neuropathology and immunocytochemical staining 46
3.5.1. Processing 46
3.5.2. Staining 47
3.5.3. Indicators for analysis 47
3.5.4. Additional investigations 51
3.6. Data analysis 53
3.6.1. Histological assessment 53
3.6.2. Statistical analysis 53
3.7. Conclusion 55

Chapter 4: Results 56
4.1. Diffuse axonal injury 56
4.1.1. Acute group (2 weeks) 56
4.1.2. Chronic group (3 months) 57
4.1.3. Other comments 57
4.2. Specific morphological findings of the two week acutely injured group 58
4.3. Specific morphological findings of the three month low grade injured group 61
4.4. Negative findings 63
4.5. Other observations 63
4.6. Conclusion 64

University of Cape Town

Chapter 5: Discussion **65**

5.1. Discussion of findings 65

5.1.1. Location and distribution of changes 65

5.1.2. The implications of time and TBI 67

5.1.3. The effects of VCP 67

5.1.4. Clinical implications 70

5.2. Discussion of methodology 71

5.2.1. Methods 71

5.2.2. Limitations 71

5.2.3. Future directions 72

5.3. Implications of the study 73

5.4. Conclusion 73

References **75**

Appendix A: Diagrammatic representation of the events following trauma, complement initiation and the possible therapeutic effects following administration of VCP.

Appendix B: Reagents and chemicals used in study design

Appendix C: Perl's Prussian blue reaction for ferrous iron

Appendix D: PTAH stain for astrocytes

Appendix E: Cresyl fast violet stain

Appendix F: Immunocytochemical detection of GFAP, CD68 and β-amyloid

Appendix G: Alkaline Congo red technique

Appendix H: Spreadsheet of raw data

Appendix I: Diagrammatic representation of location of injury

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List of Plates

Plate 1 ....60a
Plate 2 ....60b
Plate 3 ....60c
Plate 4 ....62a
Plate 5 ....62b
Plate 6 ....62c

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Glossary

AD	Alzheimer's disease
APP	Amyloid precursor protein
ATM	Atmosphere
ATP	Adenosine triphosphate
Aβ	Amyloid beta
BBB	Blood brain barrier
CBF	Cerebral blood flow
CCI	Controlled cortical impact injury
CD68	Cluster of differentiation 68
CNS	Central nervous system
CR 1	Complement receptor 1
CT SCAN	Computed tomography
DA	D-Aspartate
DAB	3,3'-Diaminobenzidine tetrahydrochloride
DAF	Decay accelerating factor
DAI	Diffuse axonal injury
DNA	Deoxyribonucleic acid
EAAs	Excitatory amino acids
ECM	Extra-cellular matrix
EDTA	Ethylenediamine tetra acetic acid
FPD	Fluid percussion device
FPI	Fluid percussion injury
GCS	Glasgow coma scale
GFAP	Glial fibrillary acidic protein
HIER	Heat induced epitope retrieval
H+E	Haematoxylin and Eosin
HRP	Horseradish peroxidase
ICP	Intra-cranial pressure
IL-1	Interleukin 1
IL-6	Interleukin 6
LOC	Loss of consciousness
MAC	Membrane attack complex

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MCP	Membrane co-factor protein
MRI	Magnetic resonance imaging
MWM	Morris water maze
NBM	Nucleus basalis of Meynert
NF	Necrosis factor
NKC	Natural killer cell
NMDA	N-Methyl-D-Aspartate
NSAID	Non-steroidal anti-inflammatory drugs
PCD	Programmed cell death
PMN	Polymorphonuclear leucocytes
PTA	Post traumatic amnesia
PTAH	Phosphotungstic acid haematoxylin
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
SCR	Short consensus repeats
TBI	Traumatic brain injury
TNF	Tumour necrosis factor
VCP	Vaccinia virus complement control protein
VEGF	Vascular epithelial growth factor
WKY	Wistar Kyoto

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ABSTRACT

Traumatic Brain Injury (TBI) leads to temporary or permanent impairments, functional disability, or psycho-social maladjustment. Injuries which occur at the moment of trauma are believed to initiate a secondary injury. This occurs immediately after trauma and produces effects via multiple molecular mechanisms, contributing to the pathogenesis of TBI.

The mechanism by which TBI causes delayed cognitive deficits and increased risk of dementia remains largely unknown. In conditions such as TBI, pathological inflammation is caused by the prolonged or inappropriate activation of the complement system. The complement system plays a pivotal role in the mediation of inflammation and is activated in both humans and, following experimental TBI, in rats. This prolonged inflammation is a major cause of tissue destruction.

Vaccinia virus complement control protein (VCP), a virus-derived complement inhibitor, inhibits both the classical and alternate complement pathways. VCP is structurally similar to the family of human complement control proteins. Containing four short consensus repeats and functionally similar to the first complement receptor, it is able to bind the third and fourth components of the complement system. Complement components C3 and C5 have previously been shown to play a critical role in secondary damage caused by inflammation in TBI.

This study was undertaken to evaluate the therapeutic role of VCP in both a mild and a severe TBI rat model following standardised fluid percussion brain injury (FPI).

A total of 48 male Wistar rats were divided into 2 treatment groups, n=24 (3 month group) n=24 (2 week treatment group.) Rats were subjected to mild (1.0-1.1 atmospheres) or severe (2.7-3.0 atmospheres) lateral FPI, 3.0 mm lateral to the sagittal suture and 4.5mm posterior to bregma. Sham animals were included in the experiment. 10µl of VCP (1.7µg/µl) was injected into the cortical injury site immediately after FPI. After euthanasia at 2 week and 3 month intervals, fixed brains underwent routine processing, sectioning and screening as a means of evaluating tissue damage.

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After screening, the data obtained was analysed using inferential statistics by means of a one-way ANOVA. Significant findings were ascertained by using Bonferroni's test for post-hoc comparison. Comparisons of specific morphologic findings of the injury groups were examined by using t-tests for independent samples, which was confirmed by non-parametric analysis, in this case, the Kruskal-Wallis test.

The results across both groups of injury severity revealed a significant reduction in neuronal change, haemorrhage and oedema, and a significant increase in neo-vascularisation in the animals that had received VCP. The evidence of haemorrhage within both cortical and subcortical structures was restricted to the ipsilateral hemisphere receiving the impact. The data supports the notion that the cell loss and damage are significantly reduced, in varying degrees of severity, in both time periods in the animals who received VCP.

These results suggest that using VCP at a lower dose when compared to previously published results may play a neuro-protective role in traumatic brain injured rats. VCP may further assist in conceptualising a therapeutic regime for neurologic impairments caused by TBI.

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CHAPTER 1: INTRODUCTION

1.1 Background and significance

In order to understand the complex mechanisms of head injury, a good experimental model of traumatic brain injury (TBI) should closely mimic the clinical progression of human TBI. This study uses the fluid percussion technique in rats, which mimics focal head injury in humans.

"Traumatic Brain Injury is the result of an external mechanical force applied to the cranium and intracranial contents, leading to temporary or permanent impairments, functional disability, or psychosocial maladjustment. TBI can manifest clinically from concussion to coma and death" (Silver et al., 2005; Baskin et al., 2003; Van der Staay et al., 1996).

Injuries can be divided into 2 subcategories: (1) primary injury, which occurs at the moment of trauma, and (2) an initiated secondary injury, which occurs immediately after trauma and produces effects via multiple molecular mechanisms, which may contribute to the pathogenesis of TBI (Silver et al., 2005; Ray et al., 2002; Laurer et al., 2000).

Unfortunately, TBI is ubiquitous and remains a major cause of considerable morbidity, neuropsychological sequelae and death. The ethical and legal issues in the treatment and management of the TBI patient are both challenging and complex. Information is key to understanding TBI and implementing the support that people with TBI need.

Traumatic brain injury produces combinations of contusion, laceration, intraparenchymal haemorrhage and diffuse axonal damage. There is increasing belief that what separates categories of injury is not so much the nature of the brain lesions as their multiplicity, amount and distribution (Silver et al., 2005). If correct, then there is likely to be a continuum from mild to severe brain damage, the structural basis of which can be inferred from postmortem studies of patients who have died with varying degrees of disability after brain injury. Such injury to the central

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nervous system (CNS) causes glial reactions, which eventually lead to the formation of a glial scar and inhibit axonal regeneration (Hayashi et al., 2004).

Statistics in the USA show that approximately 52,000 deaths per year result from TBI, which accounts for approximately 40% of all deaths from acute injuries in the United States. Annually, 200,000 victims of TBI need hospitalization, and 1.74 million persons sustain mild TBI, requiring a visit to a physician or resulting in temporary disability of at least 1 day. The financial cost is estimated at approximately $48 billion per year (Silver et al., 2005). This estimate includes loss of potential income and medical expenses such as continuous ambulatory and rehabilitation care. Patients may have difficulties in several areas of functioning that would involve family, interpersonal relations, career and recreational activities. Unfortunately the psychiatric impairments and sequelae of TBI are often unrecognized as a major health problem due to the lack of sufficient and appropriate education in this area for psychiatrists and other health care professionals, and for those involved in the rehabilitation of persons with TBI (Silver et al., 2005).

A common symptom that results from brain injury is post traumatic amnesia (PTA). PTA is characterized by disorientation, impaired attention, and memory failure in daily events, illusions, and misidentification of family, friends and medical staff (Brooke et al., 1992). PTA may vary in its duration from an hour to days and this is used as a guide to the extensiveness of brain injury. The longer the duration of PTA, the stronger the possibility of extensive damage (Walsh, 1987). TBI is also one of the few known risk factors for Alzheimer's disease (AD), (Fratiglioni, 1993; Graves et al., 1990) and yet the mechanisms by which trauma causes these delayed cognitive deficits for as long as one year post injury, and an increased risk of dementia, remain largely unknown. Although the pathophysiological mechanisms are complex, a common characteristic of TBI is cellular swelling and cytotoxic oedema (Nemetz et al., 1999).

It would be ideal if cognitive impairments, psychosis, depression, anxiety, aggression and agitation after TBI could be controlled without medication. When appropriately administered, medications may significantly alleviate these symptoms and improve rehabilitation. However, there have been few controlled clinical trials to assess the

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effects of medication in patients with brain injury, and if left untreated, some of these problems may not only endanger the patient, but others too (Silver et al., 2005).

It has been shown that activation of the complement system is implicated in both TBI and AD (Halliday et al., 2000; Keeling et al., 2000; Webster et al., 2000). Previous studies have shown that the vaccinia virus complement control protein (VCP) binds C3b and C4 of the complement system, thereby rendering it capable of blocking complement activation initiated by the beta peptide in *in-vitro* studies. This inhibits the formation of chemotactic factors C3a, C4a and C5a, which mediate inflammatory response, thus preventing tissue destruction (Kotwal et al., 1990). Results of these studies suggest that VCP may be a suitable candidate in blocking the detrimental effects caused by inflammation after head injury and slows down the potential progression to AD. VCP will be introduced in this study as a potential agent to enhance functional recovery subsequent to traumatic brain injury.

This study will evaluate the effects of the VCP on the morphological progression of TBI.

1.2 Hypothesis

Traumatic brain injury induced morphological changes in rats can be attenuated by means of the VCP administration.

1.3 Purpose of study

The purpose of this study is to determine whether the administration of VCP has an effect on the outcome of traumatic brain injury in rats that has been induced by lateral fluid percussion. More specifically, the experiments are aimed at determining whether complement regulation by VCP attenuates the long-term effects of experimental TBI in rats. This would be done by means of histological comparisons.

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1.4 Overview of thesis

Chapter 2 reviews pertinent literature on the background of TBI. The criteria for defining this entity, degenerative disorders, cellular changes including inflammation and the possible therapeutic approaches to TBI will also be discussed. The role of the complement system and the potential role of VCP as a therapeutic agent in TBI will also be covered in Chapter 2.

In Chapter 3, the experimental design and methods by which the study was executed are described in detail. The findings are reported in Chapter 4 with a focus on indicators of inflammation and injury. The implications of the findings are discussed in Chapter 5, which will conclude with suggestions for further research.

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CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Traumatic brain injury is a major public health problem that often receives little support or attention from the media or policy makers (Fuller, 1998). This chapter reviews the literature on TBI in order to arrive at an accurate definition of TBI and its sequelae. This includes a brief background on TBI as well as the criteria applied in defining TBI.

After a discussion of the neurochemical changes that occur in TBI, the chapter reviews the association of TBI with the pathophysiology of the degenerative disorder of AD. This is followed by a discussion and review of the models of experimental TBI, cellular injury and adaptation. This includes a section on the inflammatory process and the brain's response to injury. This leads to further discussion on the role of complement components and their role in the inflammatory response, as well as the factors that mediate this response.

The chapter concludes with a discussion on VCP and support for its role in mimicking the human complement, as well as introducing VCP as a potential novel attenuator of TBI.

2.2 Traumatic brain injury

2.2.1 Definition

Traumatic brain injury is one of the most devastating diseases in our society, accounting for a high percentage of mortality and disability (Smith et al., 2003). TBI is a non-congenital insult to the brain from an external mechanical force applied to the cranium and intracranial contents, possibly leading to permanent or temporary impairments of cognitive and physical functions, and psychosocial adjustments associated with a diminished or altered state of consciousness (Silver et al., 2005; Baskin et al., 2003; Van der Staay et al., 1996).

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Kumar et al (2005) reported that when the head sustains a blow, the skull, which is of lighter mass and first hit, accelerates and decelerates more rapidly than the brain. Thus the brain, with its greater inertia, is slammed against the walls of the skull. Unless the blow passes directly through the centre of gravity, the head rotates to some degree. The skull rotates more than the brain, while the outer layers of the brain rotate more than the deeper layers of the brain. This in turn, contributes a shearing force to the injury. Where the skull normally affords the brain a great deal of protection, it in some ways constitutes a liability.

Traumatic brain injury can manifest clinically in a range from concussion and contusion to coma and death (Silver et al., 2005; Baskin et al., 2003; Van der Staay et al., 1996). The Head Injury Interdisciplinary Special Interest Group of the American Congress of Rehabilitation Medicine (1996) defines mild head injury as "a traumatically induced physiologic disruption of brain function, as manifested by one of the following:

- Any period of loss of consciousness (LOC),
- Any loss of memory for events immediately before or after the accident,
- Any alteration in mental state at the time of the accident,
- Focal neurological deficits, which may or may not be transient."

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2.3 Other criteria for defining TBI

2.3.1 Mild TBI

The following criteria define mild TBI:

- A Glasgow Coma Scale (GCS) score greater than 12. This is an assessment of eye-opening and motor and verbal responses (Laurer et al., 2000).
- No abnormalities on a CT scan.
- No operative lesions.
- Lengths of hospital stay less than 48 hours.

2.3.2 Moderate TBI

The following criteria define moderate TBI:

- Lengths of hospital stay of at least 48 hours.
- GCS score of 9-12.
- Operative intracranial lesion.
- Abnormal CT scan findings.

2.3.3 Severe TBI

Severe TBI is indicated when the GCS score is below 9 within 48 hours of the injury.

2.4 Background

The brain is not just a physical structure with 3 dimensions occupying space, but a living organ, which performs cognitive, visuo-motor, emotional, psycho-social and behavioural functions through burning oxygen and glucose, firing electric impulses down axons and secreting excitatory or inhibitory neurotransmitters and neuro-modulators (McIntosh, 1994; Toulmond et al., 1993). Although the brain occupies only 2% of the volume of the body, it burns 20% of the body's oxygen and 20% of its glucose to do this mental work (Miller et al., 1997).

The definition of TBI has been problematic and inconsistent. It is often used synonymously with "head injury", and tends to vary according to medical specialties and circumstances, with variations in inclusion criteria that may or may not be associated with neurological deficits. In addition to the inconsistencies in defining and classifying TBI, discrepancies in data collection have made the epidemiology of TBI difficult to describe accurately. The vast majority of TBI's are closed head injuries, and thus inaccessible to direct visual inspection (Miller et al., 1997).

Objective visualization of functional damage is more meaningful in evaluating TBI than having a static picture or snapshot of the physical appearance of the brain structure. Functional brain disturbance, which can cause disorders of behaviour(s),

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often does not manifest on structural imaging. It is important to understand that even the most powerful magnetic resonance imaging (MRI) scanners of today's technology cannot penetrate to the cellular level of the brain or show diffuse cellular brain damage from shear-strain injury. Therefore, the great majority of patients diagnosed with mild TBI continue to have negative MRI's. While many of these patients may present with clear concussion, which may be manifested by dazing, confusion and a very brief period of PTA following closed head trauma, many do not lose consciousness. As today's MRI technology stands, a negative MRI is not very informative with regard to the extent of injury. Thus, millions of potentially damaged brain cells are hidden from view. A closed head trauma may cause many separate islands of cellular damage smaller than that which is detectable by MRI (Silver et al., 2005; Miller et al., 1997).

The term acute neuro-degeneration describes clinical conditions in which neurons are rapidly damaged and usually die in response to a sudden insult (Allan & Rothwell, 2001). This description encompasses injuries from stroke, head injury, cerebral or sub-arachnoid haemorrhage and ischaemic brain damage. Although the type of insult and onset of neuronal injury is acute, subsequent neuronal loss can occur hours or days after the initial event. This delayed cell damage results from endogenous factors that are released in response to the primary injury.

Inflammatory processes have been implicated in both acute and chronic neurodegenerative conditions. The central nervous system (CNS) differs from other tissues in its inflammatory response (Perry et al., 1995). In general terms, cellular infiltration in the brain in response to inflammation, infection and injury is weaker and delayed in comparison with other organs, yet many inflammatory responses can be induced rapidly. These include the activation of microglia, and the expression and release of classical inflammatory mediators, such as acute phase proteins, eicosanoids, complements and cytokines (Barone & Feuerstein, 1999).

Damage to the brain is one of the most feared consequences of violence (Hogg et al., 1997). Traumatic brain injury accounts for approximately 40% of all deaths from acute injuries in the United States. The majority of these individuals (75-90%) have relatively benign acute symptoms and thus their injuries are classified as mild to

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moderate (Kraus & Nourjah, 1988; Levin, 1985; Langfitt & Gennarelli, 1982; Jennet & MacMillan, 1981).

Nearly three quarters of victims of head injuries are males, most between the ages of 10 and 40 years (Silver et al., 2005). The lesions that are produced are variable, depending on the location, type and force of the blow. It should however be remembered that in most cases more than one lesion may occur (Kumar et al., 2005; Silver et al., 2005). The financial cost of treatment for TBI is estimated at approximately $48 billion per year (Silver et al., 2005). This estimate includes loss of potential income of the patient and their relatives who may need to become caregivers, cost of acute care, and other medical expenses such as continuous ambulatory and rehabilitation care.

A certain portion of these people die, whilst those that are likely to survive suffer from any of a large number of physical or behavioural disabilities, such as motor in-coordination or paralysis, as well as speech and cognitive impairments (Hogg et al., 1997). However, the long-term consequences of these mild-moderate injuries are poorly understood with frequent sequelae including hemiparesis, aphasia, post-traumatic epilepsy and post-concussive syndrome (McIntosh, 1994).

There is therefore a great need for effective therapeutic intervention, either neuro-protective or neuro-regenerative.

2.5 Morphological and physiological changes following TBI in patients and animal models

Traumatic injury of the CNS results in neuronal damage by at least two mechanisms. There is a direct, mechanical lesion, which is surrounded by an area in which indirect damage occurs. In this penumbral region, a cascade of events occurs, which are not dissimilar to those observed in classical ischaemic lesions, and include haematoma, increased release of neurotransmitters and alterations in cell membrane permeability (McIntosh, 1994).

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These secondary injuries are attributable to further cellular damage from effects of the primary injuries, which may develop over a period of hours or days following the initial traumatic assault and are mediated through the neurochemical mediators presented in Figure 2.1, which schematically illustrates the processes of inflammation (McIntosh, 1994; Jenkins et al., 1988).

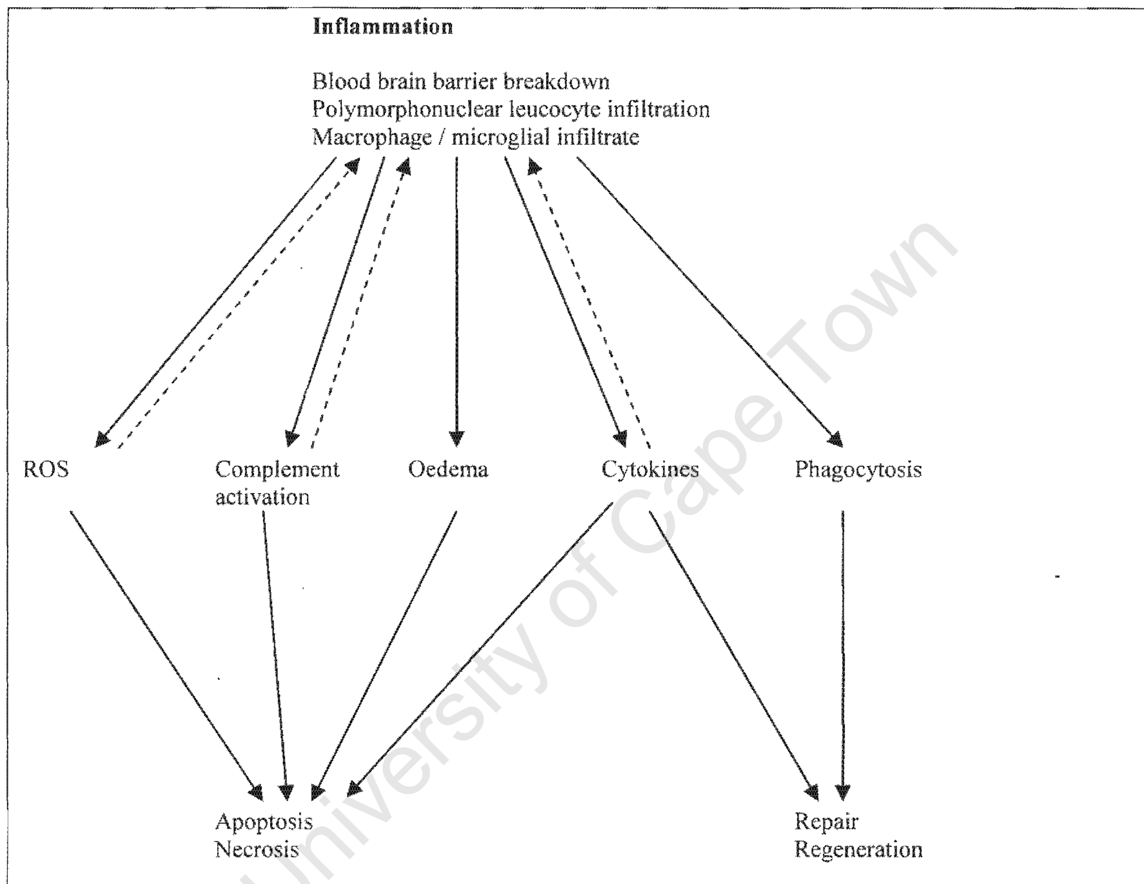

Figure 2.1. Schematic representation of the inflammatory process.

* ROS: Reactive Oxygen Species.

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Excitatory amino acids (EAA), including glutamate and aspartate, are elevated significantly after TBI (Hogg et al., 1997; Jenkins et al., 1988).

EAAs can cause cell swelling, vacuolization and neuronal death, as well as causing an influx of chloride and sodium, leading to acute neuronal swelling. EAAs can also cause an influx of calcium that is linked to delayed damage. Along with N-methyl-D-aspartate receptor agonists, which also contribute to increased calcium influx, EAAs may decrease high-energy phosphate stores (Adenosine triphosphate or ATP) or increase free radical production (Kumar et al., 2005; Jenkins et al., 1988).

Endogenous opioid peptides may contribute to exacerbation of regional neurological damage during the acute posttraumatic period by modulating the pre-synaptic release of EAA neurotransmitters. Activation of the muscarinic cholinergic systems in the rostral pons mediates behavioural suppression, which is often observed in TBI, as well as LOC (Jiang et al., 1994). Heightened metabolism in the injured brain is stimulated by an increase in circulating levels of catecholamines from TBI-induced stimulation of the sympatho-adrenomedullary axis and the serotonergic system, with associated depression in glucose utilisation, which can contribute to further brain injury (McIntosh, 1994). Other biochemical processes leading to an increased severity of injury include increased extracellular potassium leading to oedema, increased cytokines contributing to inflammation, and decreased intracellular magnesium contributing to calcium influx (McIntosh, 1994; Toulmond et al., 1993).

Among the unique anatomic and physiologic features of the CNS, the fixed and restricted volume of the cranial vault is particularly important. It renders the brain susceptible to a group of pathophysiological complications. These complications include increased intracranial pressure (ICP), cerebral oedema and ischaemia, hydrocephalus, and even brain herniation. Each complication, in turn, occurs with different pathologic processes. Severity of injury tends to increase especially when ICP exceeds 40 mmHg.

Oedema may be caused by the effects of the above-mentioned neurochemical transmitters and by increased ICP. Disruption of the blood brain barrier (BBB), with impairment of vasomotor auto-regulation leading to dilatation of cerebral blood vessels, also contributes to cellular damage (Raghupathi et al., 1995).

The rigid confines of the skull provide little room for brain parenchymal expansion during TBI, resulting in an upset of the delicate balance between brain volume and the fixed boundaries of the intracranial vault. This may result in conditions which include generalised oedema, hydrocephalus and focally extending mass lesions.

A major consequence of TBI is the rapid and long-term accumulation of proteins (Smith et al., 2003). This process largely reflects the interruption of axonal transport

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as a result of extensive axonal injury. Although many proteins are found to accumulate after TBI, three have received particular attention: beta-amyloid precursor protein and its proteolytic products (i.e. amyloid-beta (Aβ) peptides), neurofilament proteins, and synuclein proteins. Massive co-accumulations of all of these proteins are found in damaged axons throughout the white matter after TBI. Additionally, these proteins form aggregates in other neuronal compartments and in brain parenchyma after brain trauma. Interestingly, TBI is also an epigenetic risk factor for developing neurodegenerative disorders such as AD and Parkinson's disease (Smith et al., 2003).

2.6 Degenerative disorders

The degenerative diseases of the central nervous system are characterised by a slow progressive loss of neurons without known cause. To some extent, the degenerative process tends to be selective for certain regions of the nervous system. Thus, one set of disorders may predominantly involve the cerebral cortex. Other disorders may involve basal ganglia; and a third set of disorders may involve the cerebellum and spinal cord. Each of these disorders produces a fairly distinct symptom complex, for example, cortical and basal ganglia diseases tend to manifest as dementias and extrapyramidal movement disorders respectively. However, they can also produce a mixed picture, as one entity may affect more than one anatomic level (Kumar et al., 2005).

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The major cortical degenerative diseases are Alzheimer's and Pick's disease (Silver et al., 2005; Carpenter et al., 1993). Their principal manifestation and symptom complex is dementia. By convention they are divided into pre-senile and senile dementia. While the disorder may go unrecognized (pre-senile dementia), there is a gradual onset of memory loss as the full blown syndrome develops into senile dementia. Pre-senile dementia has been defined as a progressive mental deterioration leading to dementia, starting before the age of 65. Despite the widespread use of this definition, the division is not diagnostically or therapeutically helpful and has been derived from an old idea that dementia is an age related disease (Silver et al., 2005).

Further insight into a potential link between head injury and neurodegenerative change has come from the study of boxers with dementia pugilistica, or 'punch-drunk' syndrome. In this case, the association is between repetitive sub-clinical head injury experienced during the course of a boxing career and the emergence of a pre-senile dementing syndrome in later life. At a pathological level it has been known for some time that neurofibrillary tangles, similar to those seen in AD, can be found in the brains of boxers with this syndrome (Gentleman et al., 2004), and more recently the molecular composition of the tangles has been shown to be the same as that seen in AD (Carpenter et al., 1993).

However, recent accumulating epidemiological evidence implicates TBI as a risk factor for the subsequent development of AD (Gottlieb, 2000; Lye & Shores, 2000), while others reported no such association (Launer et al., 1999; Mehta et al., 1999). After experimental brain trauma the long-term accumulation of Aβ peptide suggests that neurodegeneration is influenced by apolipoprotein E epsilon 4 (ApoEε4), and after human brain injury both Aβ peptide deposition and tau protein deposition are seen, even in younger patients (Jellinger, 2004).

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Amyloid beta peptide levels in the cerebrospinal fluid and the production of beta amyloid precursor protein in humans and animals are increased after TBI. Repeated mild head trauma in both animals and humans accelerates Aβ peptide accumulation and cognitive impairment. Retrospective autopsy data support clinical studies, suggesting that severe TBI with long-lasting morphological residuals are a risk factor for the development of dementia/AD (Jellinger, 2004).

A recent study of 1776 US World War II navy veterans showed that moderate and severe TBI in early adulthood, rated by the duration of loss of consciousness or post-traumatic amnesia, was associated with increased risk of AD and dementia in later life (Gentleman et al., 2004). In the MIRAGE study, head injury has been shown to appear as a greater risk factor for AD among subjects lacking ApoEε4 (Smith et al., 2003). In addition, longitudinal studies (Plassman et al., 2000; Katzman et al, 1989) reported a significantly increased risk of developing AD in subjects with a previous history of TBI.

Despite these studies/findings, the link between head injury and dementia/AD remains controversial (Jellinger, 2004; Iwata et al., 2002).

2.7 Experimental Models of TBI

Although the understanding of TBI has been greatly advanced by the use of physical, computer and cell culture models, it has been necessary to provide biological validation of them by means of parallel animal models in which the studies are designed to replicate certain aspects of human brain injury (Silver et al., 2005).

In order to understand the complex mechanisms of head injury, various models to mimic TBI have been and are being developed. A good experimental model of TBI should be able to mimic the close clinical progression of human TBI. Furthermore, as is expected of all experiments, the model should be reliably reproducible and incorporate the appropriate and/or necessary controls, and should include sham controls, i.e. uninjured animals who would have been subjected to the identical surgical procedures as the experimental animals.

This would ensure that surgical techniques, anaesthesia and administration thereof, maintenance of body and or brain temperature, and brain damage due to placement of intracranial probes or head restraints are adequately controlled for. In addition, the reproducibility of the experimental design would need to incorporate well defined mechanical parameters for the induction of such an injury to the exact location in the brain (Dietrich et al., 1994). It is well established that several clinically relevant experimental TBI models mimic brain injury (Laurer et al., 2000). There are usually three types of animal injury models employed to mimic the induction of human TBI:

1 weight drop (Shapira et al., 1988; Feeney et al., 1981),
2 fluid percussion (Toulmond et al., 1993; Dixon et al., 1991; McIntosh et al., 1989) and
3 rigid indentation (Smith et al., 1995; Soares et al., 1992; Dixon et al., 1991).

In all three models the head is held rigidly in one position during the experimental procedure. Focal damage to the brain may include contusions that leave the pia mater

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intact, or lacerations that tear the pia mater. This can sometimes be associated with either a skull fracture or the formation of a haematoma (Dietrich et al., 1994).

In diffuse injury to the brain, swelling, concussion and ischaemic changes may occur with possible diffuse axonal pathology. This type of injury can result from searing of tissue as a result of the inertial force present at the time of impact to the brain (Laurer et al., 2000).

Devices that use gravitational, rotational acceleration forces, pressurized air and trephination all mimic focal injury to the brain. All three models, viz. weight drop closed head injury, rigid indentation injury and fluid percussion injury, similarly mimic focal damage to the brain, and these injuries are also associated with concussive events (Laurer et al., 2000).

The weight drop injury device is an easy model using gravitational force which employs a free falling weight device. Variation of the height and mass of the weight allows one to vary the severity of the injury. Energy delivered to the skull is directly proportional to the severity of the lesion. However, a major drawback is the lack of control of the number of skull fractures which are produced to the thin vertex of the rat skull during severe injury. The main advantage of this model is that when mild injury is induced, brain damage is diffuse and no local lesions or contusion in the brain is observed (Tang et al., 1997b). Additionally, the use of this model in rodents to mimic concussive-like brain injury in humans has shown injury characteristics similar to humans. These include neurobehavioral depressions, acute brain oedema and chronic learning and memory deficits without motor disability (Tang et al., 1997a).

Controlled cortical impact injury (CCI) is a pneumatic impact, which uses pressurized air as the source of mechanical energy to induce head injury (Smith et al., 1995; Lighthall, 1988). This technique provides for greater control over time, velocity and depth of brain deformation than the free-falling weight (Lauer et al., 2000). This model is thus preferred for inducing focal type damage. By increasing the velocity of air and hence the degree of brain distortion, cortical contusions with intra

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parenchymal petechial haemorrhage, which may be accompanied by epidural and or sub-dural haematoma, are formed (Lighthall, 1988).

The FPI model is currently the most widely used method for producing TBI. Injuries induced by lateral (parasagittal) fluid-percussion in the rat closely resemble the features of head injury in humans, and it is best suited to the study of the pathophysiology of TBI. The FPI device produces a pulse of increased ICP with displacement and deformation of neural tissue. This model has been shown to produce mild to severe injury that is both reproducible and quantifiable (McIntosh et al., 1989).

Furthermore, when moderate injury in rats is induced, both focal cortical contusions as well as diffuse subcortical neuronal injury are demonstrated. This makes the extent of injury comparable to that of human head injury. It has been shown that in these regions the loss of neurons is evident in the ipsilateral cortex and hippocampus 12 hours after injury. This time period therefore provides opportunity for therapeutic intervention, in which the progression of pathological effects may be blocked or reduced and the regenerative process promoted (McIntosh et al., 1989).

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The fluid percussion device (FPD) consists of a closed hydraulic system that is activated by a falling pendulum. The pendulum striking force is adjustable by either altering the angle through which the pendulum falls, or by adding weights to the pendulum. The applied pressure is accurately measured by means of a calibrated transducer (mV/psi0), coupled to an amplifier and oscilloscope to register the pressure signal. An event trigger switch activated by the swinging of the pendulum is coupled to a remote triggering device and oscilloscope, which is set to trigger on a positive or rising pulse. Once the parameters for the desired severity of the percussive injury have been obtained, these reference settings for the FPI can be used to create the same degree of injury repetitively and comparatively in as many animals as required.

2.8 TBI and diffuse axonal injury

2.8.1 Diffuse axonal injury

Neuropathological investigations have classified TBI as either focal or diffuse (Graham, 1996). Although focal injuries most often involve contusions and lacerations, which may be accompanied by haematoma (Gennarelli, 1994), diffuse brain swelling, ischaemic brain damage, and diffuse axonal injury (DAI) are also considered to be major components of the diffuse injury profile (Graham et al., 1995; Adams, 1984).

Injuries can be further stratified into 2 subcategories:

1 primary injury, which occurs at the moment of trauma, encompassing the immediate non-reversible mechanical damage to the brain, and
2 an initiated secondary or delayed injury, which represents a potentially reversible process with a time of onset ranging from hours to days after injury that progresses for weeks or months (Graham et al., 1995).

This secondary injury process is a complex and poorly understood cascade of interacting structural, cellular and molecular changes, which includes, but is not limited to, impairment of energy metabolism, ionic deregulation, breakdown of the BBB, oedema formation, activation and/or release of auto-destructive neurochemicals and enzymes, changes in cerebral perfusion, ICP, inflammation and pathologic/protective changes in intracellular genes and proteins (Silver et al., 2005; Ray et al., 2002; Laurer et al., 2000).

Although this cascade of molecular events may lead to delayed cell death, and/or neurological dysfunction, the delayed onset and reversibility of the secondary damages offers a unique window of opportunity for targeted therapeutic pharmacological intervention as a means of attenuating cellular damage and functional recovery during the chronic phase of the injury (McIntosh et al., 1998).

Traumatic brain injuries are known to produce combinations of contusion, laceration, intra-parenchymal haemorrhage and DAI (Silver et al., 2005).

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Diffuse axonal injury is characterized by extensive generalized damage to the white matter of the brain. Strains of tentorium and falx cerebri during high-speed acceleration/deceleration produced in lateral motions of the head may cause these injuries. Diffuse axonal injury can also occur as a result of ischaemia.

Neuropathologic findings in patients with DAI were graded by Gennarelli (1996) as follows:

Grade 1: Axonal injury mainly in the parasagittal white matter of the cerebral hemispheres.

Grade 2: As in Grade 1, plus lesions in the corpus callosum.

Grade 3: As in Grade 2, plus a focal lesion in the cerebral peduncle.

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2.8.2 Penetrating head injuries

Postmortem studies on humans with head injury have led neuropathologists to conclude that DAI is not only a hallmark of TBI, but is also associated with the most serious consequences regarding survival and recovery (Graham, 1996). Furthermore, hippocampal damage following TBI was significantly increased in transgenic mice that over-express amyloid precursor protein (APP) (Nakagawa et al., 1999; Smith et al., 1998). Given these findings, it is possible that DAI secondary to TBI leads to the formation of amyloidal proteins, which in turn lead to chronic activation of the complement system. Daly & Kotwal (1999) demonstrated that by adding Aβ to normal horse serum, a soluble complement protein complex, SC5b-9, was formed. This demonstrated that Aβ can activate the complement system *in vitro* (Harkany et al., 2000). However, Yamada et al. (1999) have demonstrated that Aβ injections into the rat brain are neurotoxic, which suggests an up-regulation of the inflammatory response and subsequently the complement system.

Events leading to both neurodegeneration and functional recovery after TBI can be generalized into four categories, namely:

1 a primary injury that disrupts brain tissues,

2 secondary injuries that cause pathology in the brain,

3 an inflammatory response that adds to neurodegeneration, and

4 'repair-regeneration', which may contribute to neuronal repair and regeneration to some extent, following TBI (Ray et al., 2002; Laurer et al., 2000).

However, destructive multiple mediators and a cascade of molecular events, including immune complexes, proteases and polynucleotides of the secondary injury process, ultimately dominate over a few intrinsic protective measures, resulting in an intensely neurotoxic environment, damage to neurons, and cell death, which destroys local tissue (Ray et al., 2002; Grammas & Ovase, 2001; Grammas, 2000).

Inflammatory processes may also be involved in the secondary injuries associated with TBI. Indeed, signs of inflammation were present for as long as 3 months following human and experimental brain trauma (Brooks et al., 2000; Holmin & Mathiesen, 1999). Recent evidence also suggests that there is a robust up-regulation of glial fibrillary acidic protein (GFAP) after CNS insult (Otani et al., 2003). In the presence of necrosis, where reactive astrocytes, which perform a variety of functions in the adult CNS are observed, there is also an up-regulation of collagen VIII. This suggests that type VIII collagen plays an important role in glial scar formation during the repair process by astrocytes (Hirano et al., 2004; Otani et al., 2003).

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Despite immense advances in the management of clinical TBI injury, no treatment exists to date that can reverse the molecular and cellular mechanisms leading to post traumatic death or the potential onset of AD. Currently, there is still no precise therapeutic strategy for the prevention of neurodegeneration following TBI in humans.

Understanding the pathophysiology of TBI is pivotal to halting and reversing the devastating effects of secondary brain injury. Humans owe their ability to contain injuries and heal defects to inflammation and repair. Without inflammation, infections would go unchecked, wounds would never heal, and injured organs might remain permanently scarred (Kumar et al., 2005).

2.9 Cellular injury

2.9.1 Cellular adaptation

Genetic programming for differentiation and specialization confine the normal cell to a fairly narrow range of functions and structure. This includes the finite capacity of its primary and alternate metabolic pathways.

It can be said that the normal cell is in homeostasis, that is, a steady state which renders it capable of handling physiologic demands and stresses (Silver et al., 2005). Excessive external stimuli and stress may bring about a number of cellular adaptations that are within the limits of the cell. In the event of these cellular adaptations being exceeded, a sequence of events follow, loosely known as cellular injury. This may be mild or lethal. Cellular injury is reversible up to a point. However, should stimuli persist, or should the stimulus be sufficiently severe from the outset, the cell reaches a point of irreversible cell injury, and consequently death (Silver et al., 2005).

Cellular injury is one of the most common cell responses to disease and other stimuli. However, large gaps still exist in our understanding of how much injury a cell can sustain and especially what the biochemical mechanisms are which are responsible for the transition from reversible to irreversible injury. It was previously believed that intervention was not possible in preventing cell death, but later experimental research on animals has shown that intervention by means of pharmacologic agents and metabolites can now protect ischaemic myocardium and decrease infarct size (Majno, 1998). Although this mechanism may remain unclear, any knowledge that can be gathered may improve our ability to intervene and therefore protect sub-lethally damaged cells from irreversible injury. Appendix A provides a schematic representation of the events following brain trauma.

While the causes of reversible injury and cell death are varied, unravelling the precise biochemical mechanisms responsible for cell injury proves to be very complex. However, most adverse influences on the cell can be grouped into broad categories of injury. These changes include hypoxia, physical agents, chemical agents and drugs,

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biologic agents, immunologic reactions, genetic derangements and nutritional imbalances of the cell (Li et al., 2004; Laurer et al., 2000).

Although it is not always possible to determine the precise biochemical site of action of injurious agents, there are four intracellular systems that are particularly vulnerable (Kumar et al., 2005). These are: firstly, aerobic respiration, which involves oxidative phosphorylation and production of ATP; secondly, maintenance of cell membrane integrity, on which osmotic and ionic homeostasis of the cell is dependent; thirdly, synthesis of enzymatic and structural proteins; and fourthly, the preservation of the integrity of the genetic apparatus of the cell. For example, impairment of aerobic respiration and the production of ATP rapidly disrupt an energy dependent sodium pump that maintains the ionic and fluid balances of the cell. This results in alterations of the intracellular content of ions and water (Laurer et al., 2000).

Cells that are not mechanically damaged following injury experience metabolic and ionic changes that alter their extra- and intracellular environment, which in turn causes undesirable long-term consequences (Hovda, et al., 1995; Hovda et al., 1990), an increase in extracellular potassium (Katayama et al., 1991) and an increase in glucose metabolism (Hovda et al., 1990), resulting in an ionic flux that induces an increase in glycolysis with consequent lactic acid accumulation.

Due to the constraints of neighbouring cells, structural and biochemical elements are closely related. This means, that whatever the precise point of initial injury or damaging stimuli may be, injury at one locus leads to a wide range of secondary effects. Tanno et al. (1992) showed that in rats, regions more remote from the site of impact showed transient breakdown of the BBB, and that the BBB at the site of impact remained permeable up to 72 hours after injury.

Morphologic changes of cellular injury become evident only after some critical biochemical systems within the cell have been deranged. As one would expect, the morphologic manifestations of irreversible damage take more time to develop than those of reversible damage (Laurer et al., 2000).

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Grady & Schmidt (1995) identified that the morphological damage or loss to the system occurred in the rat's ventro-basal forebrain cholinergic neurons. Another report demonstrated that, in fatally head-injured patients, cholinergic neurons in the nucleus basalis of Meynert (NBM) were damaged (Murdoch et al., 2002). Similarly, in a closed head injury in a rat model, the selective loss of forebrain cholinergic neurons caused significant disturbances in cognitive tasks associated with the neocortical and hippocampal cholinergic function (Schmidt et al., 1995).

Many studies Shaw et al. (2001); Smith et al. (1998); Raghupathi et al. (1995) have been undertaken to illustrate cellular change and lethal damage in TBI. Whilst changes such as cellular swelling, which may occur within a few minutes, are reversible, light microscopy may only show characteristics in keeping with cell death up to 12 hours after total ischaemia, as has been shown in myocardium. It has also been shown in the kidney, that when deprived of blood supply, tubular death occurs within 30 minutes, but that cells do not appear histologically dead until 12 hours later. In the brain, neurons suffer irreversible damage after 3 to 5 minutes when deprived of blood supply (Silver et al., 2005; Majno, 1998). Cell death by apoptosis has also been documented following experimental rat brain injury (Rink et al., 1995). More specifically, apoptosis by the activation of caspase proteases have been shown to occur after TBI (Namura et al., 1998). Cellular reaction is also dependent on cell type, state and adaptability of the cell, and the metabolic needs of that cell in its response to the injury.

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Historically, Virchow (1821-1902) established the concepts of 'cellular pathology'. His research was fundamental in recognizing that all disease processes originate in diseased cells. It was his pupil Julius Cohnheim (1839-1884) who provided one of the first, and proven to still be the best to date, microscopic descriptions of inflammation. These fundamental concepts and discoveries established the basis of describing the initial changes that are fundamental to inflammation (Kumar et al., 2005).

It can therefore be said that adaptation, reversible injury and cell death are states along a continuum of progressive encroachment on the cell's normal function and

structure, and that the inflammatory process either destroys, dilutes or contains the injurious agent and paves the way for repair.

The mechanisms underlying secondary or delayed cell death following TBI are poorly understood. Recent evidence from experimental models suggests that widespread neuronal loss is progressive and continues in selectively vulnerable brain regions for months to years after the initial insult (McIntosh et al., 1998). The mechanisms underlying delayed cell death are believed to result, in part, from the release or activation of endogenous "autodestructive" pathways induced by the traumatic injury. The development of sophisticated neurochemical, histopathological and molecular techniques to study animal models of TBI has enabled researchers to begin to explore the cellular and genomic pathways that mediate cell damage and death (McIntosh et al., 1998).

It has been shown, for instance, that interleukin 6 (IL-6) plays a dual role in response to head injury by releasing both pro- and anti-inflammatory mediators that promote tissue repair and also contribute to additional damage by releasing neurotoxic substances (Ott et al., 1994; Schindler et al., 1990). In transgenic mice whose microglia were fluorescently labelled, microglia became activated, accumulated and fused around the damaged and dead cells, forming a shield at the site of the laser-induced injury (Nimmerjahn et al., 2005). Activation of microglia causes phagocytosis of cellular debris, as well as the secretion of pro-inflammatory cytokines such as interleukin 1β (IL-1β) and tumour necrosis factor α (TNFα). IL-1β also promotes the proliferation of microglial cells and activates astrocytes, resulting in the elevated production of apolipoprotein E. Additionally, it induces the neuronal production of APP (Barger & Harmon, 1997; Buxbaum, et al., 1992). Increased synthesis of β-APP following FPI in rats was also evident after moderate and severe TBI. It was proposed that under certain circumstances of abnormal accumulation, β-APP may give rise to the pathological β- amyloid found in senile plaques in AD (Bramlett et al., 1997).

Ultra-structural evaluation of rat brains following TBI has revealed that the primary injury mechanism is the disruption of the BBB (Dietrich et al., 1994). One hour post-injury, rat gene expression for both interleukin-1 (IL-1) and tumour necrosis factor

(TNF) were up-regulated (Fan et al., 1996; Fan et al., 1995) and the BBB became permeable to blood-borne factors that consequently propagated secondary injury (Baldwin et al., 1996; Dietrich et al., 1994; Tanno et al., 1992). Reactive microglia, astrocytes and macrophages have also been found in the vicinity of the lesion in rat brains (Bellander et al., 2001; Bramlett et al., 1997; Dunn-Meynell & Levin, 1997). It was also shown that by increasing the severity level of FPI in rats, an increasing number of injured neurons were evident in the hippocampus (Hellmich et al., 2005).

Blais & Rivest (2003) reported that there is an innate immune system in the brain, which is transiently induced from the structure that is devoid of a BBB, and thereafter, within parenchymal microglia during systemic infection. Transcriptional activation of gene encoding proteins of the innate immunity also takes place in diseases of the central nervous system. This recent discovery raised the hypothesis that inflammation and innate immunity may be involved in the aetiology of neurodegenerative disorders. Nevertheless, this system is able to trigger the release of neurotrophic factors and to protect neuronal elements during brain infection and trauma. This innate immune response may therefore play a critical role in protecting neurons, and could be a possible cause of neuro-degeneration. The fate of this newly identified cascade of events is therefore likely to have a determinant impact on the central nervous system during infection and injury (Blais & Rivest, 2003).

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One of the most basic tenets of neurobiology is that glial cells function to provide metabolic, structural, and trophic support to neurons. Glia are essential supporting cells and without them nervous tissue would not be viable. Classic examples of glial cell support include myelination of axons by oligodendrocytes to facilitate nerve impulse conduction, and glutamate uptake by astrocytes to protect neurons from excitotoxicity. In the case of microglia, their normal physiological functions are not well understood. However, there is good reason to believe that microglia provide neuroprotection in a variety of ways (Silver et al., 2005). Research in recent years has helped consolidate the view of microglia as the brain's endogenous immune system and thus microglia should be seen as the first line of defence in acute "emergency" situations, such as physical/chemical or hypoxic injury or infectious diseases (Silver et al., 2005). There is now data showing that microglial cells are subject to age-

related structural deterioration and cellular senescence (Shafer et al., 2003; McCann et al., 1996).

In a study undertaken on archived material from the Department of Neuropathology, in the Institute of Neurological Sciences in Glasgow, to assess the long term response of microglia after TBI, Gentleman et al. (2004) report that both CD68 and CR3/43 immunoreactivity increased with ageing in an age matched cohort of controls, reflecting both hypertrophy and hyperplasia of microglia. In the head-injured cases there was however, a greater increase in CD68 immunoreactivity in both parasagittal and hippocampal white matter that was seen up to 4 years after the injury. This appeared not to be influenced by age. CR3/43 immunoreactivity had a greater load in both parasagittal and hippocampal white matter and was seen up to 16 years after the injury.

It also appears that microglial cellular senescence is exacerbated in the presence of Aβ, suggesting that Aβ may adversely affect physiological functions of microglia by hastening the cell's structural decline (Streit, 2002). Thus, as microglial cell function deteriorates with ageing, there may be an increasing disability of microglia to provide neuroprotection. This waning glial support could contribute to age-related neurodegenerative disease. At the same time, if microglial ability to clear away Aβ was also impaired by cell senescence, it would explain why Aβ accumulates extracellularly and forms amyloid plaques (Streit, 2002).

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However, when it comes to chronic neurological diseases, particularly neurodegenerative conditions such as AD, the role of microglia is not entirely clear. Streit et al. (2004) have recently discussed the relationship between microglia, Aβ, and neurodegeneration in some detail. Over the last fifteen years many researchers including Streit et al. (2004) have pursued the idea that neurodegeneration in AD is mediated in part by a chronic neuroinflammatory response of microglial cells, reactive astrocytes, acute phase proteins and complement factors within and around plaques in AD. This has led to clinical trials with non-steroidal anti-inflammatory drugs (NSAIDs).

By far the most compelling evidence supporting the critical role of microglia in neuroprotection comes from observing the cells' natural response to acute axonal injury in rodents. Axotomy of facial motor neurons in the periphery triggers a microglial response in the facial nucleus almost immediately after the injury has occurred. Within a few days, axotomized perikarya are surrounded by activated microglial cells, which tightly ensheath the injured neurons with their cytoplasmic processes. The number of activated microglia in the facial nucleus increases dramatically and suddenly through a proliferative burst that lasts from about day 2 to day 4 post-axotomy (Streit et al., 2004; Graeber et al., 1988). This mitotic response (hyperplasia) is unique to microglia, which are the only glial cells that divide. Astrocytes, which also respond to the axotomy lesion, do not divide but undergo hypertrophy. This is characterized by markedly increased production of the glial fibrillary acidic protein (GFAP) (Graeber & Kreutzberg, 1986).

The astrocytic reaction to motor neuron injury is a little slower to develop than the microglial response, a phenomenon that has been observed in other CNS injury models as well (Streit, 2002; McCann et al., 1996).

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2.9.2 Acute inflammation

With survival, there is a cellular response to traumatic injury. Almost all causes of cell injury provoke inflammation, mediated by released chemicals. Acute inflammation comprises the immediate and early response to an injurious agent. It is usually of short duration, lasting for a few minutes, several hours or one to two days. Simply put, the process of inflammation signals the marshalling of defensive elements in the immediate vicinity of the offending agent.

It is mainly characterized by three major overlapping components: firstly, the exudation of fluid and plasma proteins resulting in oedema (alterations in vascular calibre that lead to an increase in blood flow); secondly, structural changes in the microvasculature that permit plasma proteins to exude; and thirdly, the migration of predominantly neutrophils. It is normally a defensive reaction of the host. For example, neutrophil polymorphs increase in number by 24 hours after injury and migrate into the necrotic tissue (Silver et al., 2005). This is followed by activation of

microglia and the development of macrophages, which are usually prominent at the sites of contusion. However, it has been shown that activation of microglia is also present throughout regions demonstrating disruption of the BBB, including the hippocampus and the thalamus.

All three experimental models of TBI discussed typically produce focal contusion of the cortex, which appears histologically as haemorrhagic foci of necrosis that undergo changes characterized by absorption of the dead tissue, scarring and the development of a cavity (Silver et al., 2005).

A further feature of contusion is the disruption of the BBB. However, change can be seen well beyond the immediate vicinity of the contusion. This disruption facilitates the formation of vasogenic oedema, a decrease in regional cerebral blood flow (CBF) and an increase in glucose metabolism. Although blood flow adjacent to the contusion may not be at critical levels, it is apparent that oligaemia (when occurring with a hyper-metabolic response to trauma) creates an injury-induced vulnerability after traumatic injury. This may render the brain at risk to even minor changes in CBF, as well as increases in ICP (Silver et al., 2005).

The vascular response at a site of injury is fundamental to the acute inflammatory reaction. Without adequate blood supply, tissues cannot mount an inflammatory reaction. The vascular phenomena are characterized by increased blood flow to the injured area, resulting mainly from the opening up of capillary beds. Increased vascular permeability results in the collection of protein-rich extra-vascular fluid, which forms the exudates. Plasma proteins leave the vessels, either through widened inter-endothelial cell junctions of the venules, or by direct endothelial cell injury (Ray et al., 2002; Grammas & Ovase, 2001; Grammas, 2000). Leukocytes, predominantly neutrophils, also leave the microvasculature through the endothelial cell route and migrate to the site of injury under the influence of chemotactic agents (Ray et al., 2002; Grammas & Ovase, 2001; Grammas, 2000).

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2.9.3 Chronic inflammation

While some acute reactions disappear completely, some inflammatory stimuli go on for weeks, months or even years, as in some persistent infections and self perpetuating immunologic reactions. Persistent inflammatory stimuli lead to chronic inflammation (Kumar et al., 2005). Although the transition from acute to chronic is often difficult to pinpoint, chronic inflammatory responses have some features sufficiently unique to warrant a separate description.

Clinically, chronic inflammation can arise in a number of ways (Silver et al., 2005). It may follow acute inflammation, or the response may be chronic from the onset. Acute to chronic transition occurs when the acute inflammatory response cannot be resolved, due to either the persistence of the injurious agent or to some interference in the normal process of healing.

Histologically, the hallmarks of chronic inflammation are:

- Infiltration by mononuclear cells, principally macrophages, lymphocytes and plasma cells
- Proliferation of fibroblasts and often small blood vessels
- Increased connective tissue fibrosis

Infiltration by monocytes/macrophages is an important component of chronic inflammation, which will constitute the predominant cell type within 48 hours. When the monocyte reaches the extra-vascular tissue, it undergoes transformation into a much larger cell, the macrophage, which may become activated. In acute inflammation these macrophages eventually disappear (Kumar et al., 2005).

2.10 Cellular changes

Apoptosis is a pathway of cell death which is induced by a tightly regulated program. Cells that are destined to die activate enzymes that degrade the cells' own DNA and proteins. While the cell's plasma membrane remains intact, the apoptotic cell becomes a target for phagocytosis, and is cleared away before any cellular contents

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leak, thus preventing an inflammatory response from the host. This programmed cell death (PCD) differs from necrosis, which is characterised by loss of membrane integrity, enzymatic digestion of cells and frequently, a host response. However, apoptosis and necrosis can co-exist and may share some common features and mechanisms. Graham et al. (1995) and Graham et al. (1989) have shown a series of cellular events; PCD, which was first demonstrated by TUNEL histochemistry, gel electrophoresis and electron microscopy by Rink et al. (1995). This group showed that TUNEL positive cells could be detected for up to 72 hours after initial injury. More recent studies have confirmed that PCD and the nuclear changes of apoptosis can occur at 2 months after experimental TBI (Newcomb et al., 1999; Conti et al., 1998; Clark et al., 1997; Yakovlev et al., 1997; Colicos & Dash, 1996). The findings of these experimental models have also been replicated in clinical studies (Shaw et al., 2001; Smith et al., 2000; Clark et al., 1997). More recent work has identified TUNEL positive cells predominantly in white matter in patients surviving up to 12 months after TBI (Williams et al., 2001). Although the exact nature of the TUNEL positive cells in these studies was not established by either morphological or immunohistochemical criteria, they were considered to be predominantly macrophages occurring in association with Wallerian degeneration.

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2.11 Complement system

The complement system is an important mediator of inflammatory processes (McGeer et al., 1989; Rother & Till, 1988). It consists of more than 25 plasma proteins that are activated in a sequential process, as well as their cleavage products, which are found in greater concentration in plasma. It functions in the immune system by mediating a series of biological reactions, all of which serve in the defence against microbial agents. These reactions include increased vascular permeability, chemotaxis, opsonization prior to phagocytosis and lysis of target organisms. This system consists of activating and effector sequences.

Activation of the complement system can occur via either the classical pathway (the function of complement actually provides the major part of the antibody-mediated response), or by the alternate pathway, which is initiated by a variety of non-immunologic stimuli (McGeer et al., 1989; Rother et al., 1988).

The classical pathway is initiated by the binding of an antigen-antibody complex to C1, and cleaves C4 and C2. The resulting cleavage fragments form the complex C4b2a, also known as C3 convertase. C3 convertase is an important enzyme, since it splits C3 into two critical fragments, C3a and C3b. While C3a is released, C3b forms a trimolecular complex with *C4b2a* (C5 convertase). C5 convertase interacts with C5 to break off C5a; C5b combines with C6 and C7 into a complex C5b67. Further binding of *C5b67* with C8 and C9 produces C5b-9, the final lytic agent of complement (McGeer et al., 1989; Rother et al., 1988).

In the alternate complement pathway, C3 is activated directly by such stimuli as bacterial endotoxins, cobra venom and aggregated globulins, by bypassing C1, C4 and C2. The C3 convertase of this system is formed by interactions of factors B, D and C3b in the presence of magnesium (McGeer et al., 1989; Rother et al., 1988).

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The components of the complement system that have biologic activity in inflammation are as follows (Gallinaro et al., 1992):

1 C3a increases vascular permeability. In addition to being produced by the classic and alternate pathways, C3 can be directly cleaved (*in vitro*) by plasmin, bacterial proteases, and other C3-cleaving enzymes which may be found in various tissues.

2 C5a induces increased vascular permeability, is much more potent than C3a, and is highly chemotactic to both neutrophils and monocytes. It is released by complement activation or direct cleavage by trypsin, bacterial proteases and the enzymes found in neutrophil lysosomes and macrophages.

3 C3a and C5a, also known as anaphylatoxins, are split products of the corresponding complement components that stimulate histamine release from mast cells. Preformed histamine is widely distributed in tissue, the richest source being mast cells that are normally present in connective tissue adjacent to blood vessels. Physical injury such as trauma results in the release of histamine due to mast cell de-granulation. It is possible to suppress this activity by antihistamines or by depleting histamine containing mast cells. In humans, histamine is considered to be the principle mediator of the immediate transient phase of increased vascular permeability. It causes dilation of arterioles and increases the permeability of venules (McGeer et al., 1989;

Rother et al., 1988). However, this means that C3a and C5a can also induce vascular leakage through gaps in the endothelium.

4 C3b is an important opsonin, which recognizes receptors on neutrophils, macrophages and eosinophils.

5 C5b67 complex produced during activation of the complement system has no permeability effect but is also a chemotactic agent. Its role *in vivo* remains unclear.

6 The C5b-9 is the final lytic component of complement and is believed to be involved in injury to parenchymal cells (Gallinaro et al., 1992).

Several factors can initiate activation of the cascade, which usually functions on a cell surface, including immune complexes, proteases and polynucleotides (Gallinaro et al., 1992). The action of complement also attracts macrophages, which may scavenge the target cells of their products. As a result of activation, proteolytic cleavage products are produced and it is these products that have profound inflammatory effects.

Once the complement cascade is initiated, it progresses to the formation of a membrane attack complex (MAC). This complex inserts itself into cell membranes and causes cell lysis and eventually death (Fantone & Ward, 1995). The end result of complement activation is an inflammatory response that destroys local tissue and may expand to include immune cells, causing a cycle of continued tissue loss and further inflammation. Indeed, progressive loss of cortical and hippocampal tissue has been observed for as long as a year after TBI in rats (Pierce et al., 1998).

Interestingly, recent studies have also reported that Aß protein, a neurotoxic peptide and a hallmark of AD, which is capable of binding specifically to the C1q subunit of the first component of the complement system, can activate the complement pathway in a similar fashion to immunoglobulin (Halliday et al., 2000; Bergamaschini et al., 1999; Nybo et al., 1999; Daly & Kotwal, 1997). The deposition of complexes and the formation of immunomodulators by the cascade have been credited with activating the microglia/macrophages of the brain, which in turn cause a progression and the maintenance of inflammation (Daly & Kotwal, 1997).

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The amyloid deposition or fibril formation is capable of causing activation of both the classical and alternate complement pathways. This is accomplished by ionic interactions between the ß-pleated structure, comprising residues 14-26 of the collagen-like portion of the C1q subunit of the first component of the complement system, and the ß-pleated sheet structure of Aß (Jiang et al., 1994).

Key observations from experimental work in animals support a role of microglia as neuroprotective cells after acute neuronal injury. These studies point towards an important role of neuronal-microglial crosstalk in the facilitation of neuroprotection. Conceptually, injured neurons are thought to generate rescue signals that trigger microglial activation and, in turn, activated microglia produce trophic or other factors that help damaged neurons to recover from injury. Against this background, recent work from postmortem studies conducted in humans has revealed the occurrence of senescent, or dystrophic, microglial cells in the aged and AD brain. These findings suggest that microglial cells become increasingly dysfunctional with advancing age and that a loss of microglial cell function may involve a loss of neuroprotective properties that could contribute to the development of ageing-related neurodegeneration.

In addition to the presence of activated microglia, reactive astrocytes and acute phase proteins, complement factors within and around plaques in AD tissue are additional signs of an inflammatory response. This evidence points to an immune response as having an important role in the progression of AD (Daly & Kotwal, 1997; Cotman et al., 1996).

Additional support for the involvement of inflammatory and immune responses in the pathogenesis of AD has been shown by the central role that NF-kappaB, a major transcription factor, plays through an autoregulatory feedback system by I-kappaB (Yoshiyama et al., 2001). These observations indicate that the disruption of the autoregulatory mechanism of NF-kappaB in brain regions with neurofibrillary pathology may play a role in the pathogenesis of AD (Yoshiyama et al., 2001).

Despite immense advances in the management of clinical TBI, no treatment exists to date that can reverse the molecular and cellular mechanisms that lead to post

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traumatic death or the potential onset of AD. Currently, there is still no precise therapeutic strategy for the prevention of neuro-degeneration following TBI in humans. Therefore, understanding the pathobiology and histological changes that follow TBI is pivotal to halting and reversing the devastating effects of secondary brain injury.

In a study undertaken by Bellander et al. (2001), the results indicated an increased immunoreactivity for complement components C1q, C3b and C3d and the MAC, C5b-9, in the immediate vicinity of neurons in the penumbra area of the contusion after traumatic injury. These findings constitute histological evidence for activation of the complement cascade in the penumbra of cortical contusions in the human brain. Their findings also suggest that unknown compounds in the debris from injured neurons or myelin breakdown products trigger complement activation, including the formation of C5b-9. Activated complement components may stimulate the accumulation of inflammatory cells and the formation of brain oedema, as well as resulting in membrane destructive effects by the end product MAC, thereby being mediators in the development of secondary brain damage.

2.12 Therapeutic approaches to brain injury

Diffuse axonal injury occurs in nearly half of all severe cases of clinical TBI. Assessment of the extent of brain damage and prediction of outcome are relatively difficult in the early stages. Although it was once believed that DAI occurred as a direct result of the tensile forces of the initial trauma, it is now recognised to be a delayed process of progressive neurochemical changes that lead to disconnection (Park & Hyun, 2004).

The underlying neurological and cognitive deficits observed in patients are caused by both the primary and secondary injury following TBI. The primary insult initiates the secondary injury, which manifests long after the time of injury and includes the disruption of biochemical and physiological processes in the brain (DeKosky et al., 1998; Faden, 1993). Currently there are no neuroprotective agents available to counteract or repair secondary damage caused by TBI. Over the years, however, there have been efforts by Silver et al. (2005) to elucidate the pathophysiology of TBI as a

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means of establishing potential therapeutic targets. By unravelling the pathophysiology of the TBI, potential therapeutic targets could be identified.

Many therapeutic approaches are available for those who have experienced brain injury. It is however critical to conduct a thorough assessment of the patient before any intervention is initiated. There have been few controlled clinical trials to assess the effects of medication in patients with brain injury. Although these therapeutic approaches have shown promising results in experimental work, clinical trials have proved to be disappointing (Royo et al., 2003).

Despite this, Silver et al. (2005) report that in the case of treatment for psychiatric disorders such as depression, panic disorder and obsessive-compulsive disorder, a combination of therapeutic interventions administered simultaneously often provides more effective treatment than using a single modality. They also report that due to the frequent changes in the clinical status of patients after TBI, continuous re-assessment is necessary to determine whether each prescribed medication is still required. This requires vigilance on the part of the clinician due to drug to drug interactions. It is also important, where possible, to ensure that the selections of medications are guided by an understanding of the relationship between the neurochemistry most likely related to the symptom as well as the injury location.

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The number of human studies using alternative treatments in TBI is limited (Silver et al., 2005). Review of the literature takes advantage of the overlaps in the pathophysiology of TBI with AD, age-associated memory impairment, stroke and animal models of trauma and ischaemia. The probable mechanisms by which alternative agents improve brain function can be placed within a pathophysiological framework using four constructs: neurotransmitter hypotheses, biochemical and metabolic derangements, neuroanatomy, and brain wave patterns (Silver et al., 2005).

Others (Durmaz et al., 2003), have shown that administering Lazaroid (U-83836E) prophylactically after cerebral trauma in rats appears to reduce oedema and neuronal necrosis, possibly by inhibiting increases in lipid peroxidation and by stabilizing ATPase. It has also been shown that post traumatic administration of Cyclosporin

(CY-A) in sheep has a downregulatory effect on increased APP expression caused by TBI (Van den Heuwel et al., 2004).

Additional animal studies have demonstrated involvement of glutamate and DA systems in recovery after TBI. Schmidt & Grady (1995) report cholinergic deficits in rats after TBI as well as in TBI patients' postmortems (Murdoch et al., 2002). Dixon et al. (1991) report that after TBI in rats, there is an increase in neurotrophic factors such as nerve growth factor, which attenuates cholinergic deficits.

Magnesium has been shown to be neuroprotective in rats that sustained TBI, as it was possible to alter N-Methyl-D-Aspartate (NMDA) receptor permeability to calcium and sodium (Bareyre et al., 2000). Other inhibitors, such as non-selective and selective inhibitors of nitric oxide synthase, free radical scavengers and caspase inhibitors, have also shown success as therapeutic targets in animal models (Royo et al., 2003).

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2.13 Vaccinia Virus Complement Control Protein

Pox viruses are complex double-stranded DNA viruses that are capable of causing disease in a wide variety of animals and humans. Much of the success of this family of viruses is due to their ability to encode several proteins that can subvert the host's immune response. Pox viruses produce two major families of proteins which are involved in the evasion of host defence: cell associated cytokine response-modifying proteins and secreted proteins called virokines (Upton et al., 1992). These secreted proteins are made up of viroreceptors, a neurovirulence factor and a complement control protein. The VCP is one of these cytokine-chemokine-binding proteins. Over an evolutionary period of time VCP has developed mechanisms to evade the consequences of immune surveillance (Kotwal, 1996).

Vaccinia virus complement control protein is a 35kDa major virokine, experimentally found to be expressed early in the course of vaccinia infection. Containing four short consensus repeats (SCR) (Kotwal & Moss, 1988), VCP is structurally and functionally similar to the family of human complement regulatory proteins. These regulatory proteins are: decay accelerating factor (DAF), factor H, complement

receptor 1 (CR1), membrane co-factor protein (MCP) and complement 4b binding protein (C4b-BP) (Kotwal & Moss, 1988).

Unlike the large and complex human complement control proteins, VCP is much smaller and yet it retains the functionality of the human proteins. Functionally VCP, whose crystal structure was only determined a short while ago (Jha & Kotwal, 2003), is the only known structure of an intact and complete complement control protein which resembles CR1, and which is able to bind to both the third and the fourth components of the complement system (Kotwal et al., 1990). This inhibits the formation of chemotactic factors C3a, C4a and C5a, which mediate inflammatory responses, thus preventing tissue destruction (Kotwal et al., 1990).

To a lesser extent, VCP has also been shown to inhibit the alternate pathway by using the same mechanism, resulting in the cleavage of C3b into inactive C3b (iC3b). This prevents the alternate pathway C3 convertase. By blocking complement activation at multiple sites, there is a large reduction of C3a, C4a and C5a pro-inflammatory chemotactic factors, resulting in reduced cellular influx and inflammation (Kotwal et al., 1990).

One of the more recently identified properties of this multifunctional protein is its ability to bind heparin. This ability inhibits chemotaxis of monocytes across endothelial cells, antibody binding to endothelial cells and inhibition of naïve neutrophils and natural killer (NK) cells from killing endothelial cells (Kotwal, 2000). It has also been experimentally proven to inhibit complement in a dose-dependent manner (Al-Mohanna et al., 2001).

The recent *in vivo* study conducted by Anderson et al. (2003) has shown that VCP contributes significantly to the reduction of cardiac tissue damage in a guinea pig-to-rat cardiac xenograft by blocking hyperacute rejection and therefore prolonging graft survival. Kahn et al. (2003) have also shown the ability of VCP to inhibit human anti-Gal alpha 1-3 Gal antibody attachment to cultured porcine endothelial cells. This attachment subsequently reduced human neutrophil and NK killing of pig aortic endothelial cells through its ability to bind heparin sulphate.

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Human complement regulatory proteins function to prevent inadvertent injury to adjacent cells and tissue during complement activation (Kotwal, 2000). Thus, VCP can effectively block the complement pathway at an early stage following complement activation, and thereby potentially block the downstream events leading to complement induced inflammation and cell death.

Smith et al. (2000) have shown that VCP remains fully active after adverse exposure to high temperatures, freeze-thawing cycles and lyophilisation. They have also shown VCP to have a shelf life of 30 days at room temperature (the longest time point tested). These findings demonstrate advantages for shipping and storage conditions. Furthermore, VCP has also been shown to tolerate extreme acidic conditions (Smith et al., 2000). Considering the gastric environment, this suggests the possibility for a route of oral administration.

These studies suggest that VCP retains functionality following adverse conditions and that it is therefore robust and stable. Such evidence thus supports the multi-functionality and stability of this immunomodulatory protein, making the use of VCP promising in the treatment of complement-mediated diseases such as spinal cord injury (Reynolds et al., 2004; Reynolds et al., 2003), xenograft transplant rejections (Anderson et al., 2003), peritonitis (Scott et al., 2003) head injury (Keeling et al., 2000), and AD (Daly & Kotwal, 1998).

It has now become possible to exploit such strategies used by micro-organisms to prevent the harmful effects of the immune system. To this end, VCP, a viral inhibitor of both the classical and alternate complement pathways is proposed as a novel attenuator of inflammation and cellular injury in TBI with the potential to support the development of a clinically viable neuro-protective/regenerative agent for human TBI.

2.14 Conclusion

Chapter 2 has provided a detailed discussion on TBI as well as discussing the complex mechanisms of cellular injury and repair.

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Chapter 3 follows with a detailed discussion of the study design and conditions, as well as looking at the parameters that were reviewed for analysis of the data obtained.

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CHAPTER 3: METHODOLOGY

3.1 Ethics

This study formed part of a larger project for which ethics approval had already been granted (Ethics application Ref: REC REF 03/013) by the University of Cape Town Animal Research Ethics Committee.

3.2 Rats

A total of 48 male Wistar Kyoto (WKY) rats, with an average mass of 250-300 grams at time of surgery, were used for the purpose of the study. The rats were sourced from the University of Cape Town Animal Unit. Each rat, which was housed in a separate cage and allowed free access to food and water, was marked appropriately for the treatment group and was identified on the tail with indelible ink. All animals were housed with a 12-hour light/dark cycle.

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3.3 Experimental design

The 48 rats were divided into 2 groups, n=24 (3 month group) and n=24 (2 week group.) Each of these two groups was further divided into 4 subgroups (each subgroup n=6). See Table 3.1 for tabular representation.

The animals were subjected to the following procedures.

Following anaesthesia, the animals underwent surgery for craniotomy construction. Randomly selected animals underwent TBI by means of FPI. Animals were subjected to either VCP or saline administration at point of craniotomy. After surgical recovery, the rats were euthanized at two time intervals, (2 weeks and 3 months), by using transcardial perfusion. Whole brains were removed for histological investigation and evaluation.

Subgroups 1-4 were euthanized after 2 weeks. Subgroup 1 were not injured, but were injected with saline at the site of craniotomy. Subgroup 2 were treated similarly, but

were injected with VCP at the site of craniotomy. The animals in subgroups 3 and 4 were injured by means of FPI. Subgroup 3 had saline administered at the site of craniotomy and the animals in subgroup 4 received VCP at the site of craniotomy.
The animals in subgroups 5-8 were euthanized after 3 months. Subgroup 5 were not injured, but were injected with saline at the site of craniotomy. Subgroup 6 were treated similarly, but were injected with VCP at the site of craniotomy. The animals in subgroups 7 and 8 were injured by means of FPI. Subgroup 7 had saline administered at the site of craniotomy and the animals in subgroup 8 received VCP at the site of craniotomy. The 2 week group received an injury of 2.7-3.0 ATM. and the 3 month group received an injury of 0.1-1.1 ATM. The severity of FPI was determined by survival rate of the rats as discussed in 3.4.2.

Table 3.1. Tabled Treatment groups

Subgroup	**Procedure**	**Harvest interval** (post procedure)	**N**
1	Sham & saline	2 weeks	6
2	Sham & VCP	2 weeks	6
3	FPI & saline	2 weeks	6
4	FPI & VCP	2 weeks	6
5	Sham & saline	3 months	6
6	Sham & VCP	3 months	6
7	FPI & saline	3 months	6
8	FPI & VCP	3 months	6

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3.4 Procedures

3.4.1 Anaesthesia

Isoflurane has the desirable effect of rapid induction and recovery. As an added benefit, depth of anaesthesia is easy and rapid to alter, as it has the tendency to cause respiratory depression. It has very little effect on liver microsomal enzymes. It is non-explosive and non-flammable (Forman, 1998).

Rats have a relatively small total muscle mass and are prone to develop muscular atrophy or nerve damage following intramuscular (IM) injections (Short, 1987). Thus subsequent anaesthesia was administered by means of intraperitoneal injection (IP) of Equithesin at a dose of 1ml/300g (Thurmon et al., 1996). See Appendix B for the composition of the Equithesin and the physiological saline.

Respiration was carefully monitored as surgical anaesthesia is attained in most species only when doses close to those which cause respiratory failure have been given. Anaesthesia was monitored in the following manner (Forman, 1998):

1. Response to pain: Pedal withdraw reflex: using the fingernails to pinch the web of skin between the animal's toes. If the limb was withdrawn, muscles twitched or the animal cried, the depth of anaesthesia was considered too low.
2. Ear pinch: Upon pinching the ear, if the animal shook its head or vocalized, anaesthesia was considered too light.
3. Alteration in eye reflexes: Palpebral reflex: This was difficult to assess as it was noted that this reflex may only be lost when anaesthesia levels are dangerously deep.
4. Alterations in respiratory function: Rate, depth and pattern were monitored by observation of the chest wall.

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3.4.2 Fluid percussion injury

Optimising the Fluid percussion device

As it was the first time that the FPD was being used in this laboratory with the intention of measuring the effects of VCP, it was important to evaluate the percussion shock administered in relation to the survival of the animal. It was decided to commence with a high-grade or severe percussion and therefore set the pendulum to the upper limits of tolerance.

As a means of optimising the FPD, Pillay et al. (2005) used ten rats in a preliminary study to determine the survival rate of the rats. Four rats were first subjected to a most severe percussion at the pendulum angle of 40° (which corresponded to 4 ATM)

before the strike-angle was reduced to 33°, 30° and 26° angles, respectively Four of the rats that were subjected to percussion greater than 3.0 ATM died immediately after impact of fluid on the brain. McIntosh et al. (1989) reported similar results of rat mortality at this level of injury. All the other six rats that were subjected to FPI equal to or less than 3 ATM survived. It was therefore evident that the most severe injury that could be caused by the FPD without fatality was at 3.0 ATM. As a means of maintaining survival of the rats over the long term, it was decided that the 3 month group would be subjected to an injury of 0.1-1.1 ATM. During the FPI a single pressure pulse (21-23 ms) was delivered into the closed cranial cavity.

The FPI device (Hicks et al., 1996; Schmidt & Grady, 1995) reproduces clinically relevant features associated with human head injuries by increasing ICP by controlled measurable liquid impact to the brain with resultant displacement and deformation of neural tissue into the closed cranial cavity.

Hicks et al. (2002) reported that a moderate head injury is induced at a level of 4.5 ATM. However, according to classification criteria as presented by McIntosh et al. (1989), this level of injury would be classified as a severe injury. As this classification has been adopted (as described in Chapter 2), the current value of the FPI (2.7-3.0 ATM) is within the severe range of injuries and has been used for the purposes of this study. This study also investigates the efficacy of VCP at a lower dose (1.7μg/μl) than previously reported (2.25μg/μl) (Hicks et al., 2002).

Induction of injury

Rats were humanely anaesthetized by 4% isoflurane inhalation and then with Equithesin (1ml/300gIP) (Forman, 1998). After anaesthesia the rat was placed in a stereotaxic frame to immobilize the head.

Under aseptic conditions and after reflection of the scalp and temporal muscles, a 5mm diameter and 2.7mm depth craniotomy over the left parietal area centred between bregma and lambda and 3mm lateral to the sagittal suture was constructed leaving the dura intact (Paxinos & Watson, 1998). A Luer-loc hub was carefully and

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securely fixed to the skull with dental acrylic and the cement allowed to dry, such that a fluid percussion would be directed horizontally onto the cortex.

To induce injury to the brain, the hydraulic system was attached to the rat via hydraulic tubing and a Luer-loc which had been fitted to the exposed skull. A high pressure stainless steel T-manifold coupler permitted the percussion shockwave to be transmitted to both the animal and to the pressure transducer simultaneously, enabling a direct recording of the actual pressure at the coupling site to the animal.

The hub was then attached to the FPI device (Scientific Instruments, Univ. Washington), which was calibrated to produce an injury of mild severity (0.1-1.1 ATM) and severe injury (2.7-3.0 ATM). The 3 month group received an injury of 0.1-1.1 ATM and the 2 week group received an injury of 2.7-3.0 ATM.

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3.4.3 Experimental conditions

As discussed in Chapter 2, the model selected to induce TBI in animals needs to include procedures to control for surgical technique, anaesthesia, and brain damage due to the placement of intracranial probes or head restraints.

For the purpose of this study, sham animals were subjected to the same surgical procedures and craniotomy construction as the injured animals. These uninjured animals were coupled to the FPI device but were not subjected to the single pulsed injury as were the injured animals.

After injury, and removal of the Luer-loc, animals were injected at the site of craniotomy, centered 3mm lateral to the sagittal suture and 4.5mm posterior to bregma in the A-P plane, by means of a Hamilton syringe, which was attached to a stereotaxic injection frame, with either 10 µl of VCP at a concentration of 1.7µg/µl, or with sterile isotonic saline. This was delivered to a depth of 6.8mm as determined on the stereotaxic injection frame scale, into the rat cortex at the site of injury. Following brain trauma, the tubing was removed, the scalp was sutured together and the rats were allowed to recover under heating lamps for at least 2 hours before being returned to the animal holding room.

The VCP that was administered to the animals was synthesised at the Division of Medical of Virology, Faculty of Health Sciences, University of Cape Town (Pillay et al., 2005; Kotwal et al., 1990; Kotwal & Moss, 1988).

3.4.4 Euthanasia

Deep sleep was induced by inhalation of anaesthetic. Subsequent euthanasia was performed by transcardial perfusion initially using physiological saline and then 10% formalin pH 7.0 at room temperature. See Appendix B for the composition of the formalin and physiological saline.

Transcardial perfusion was performed in the following manner to ensure adequate fixation of the brain and to minimise agonal and post-mortem changes:

1. Ensuring that the head was down, the rat was anchored at the tail with adhesive tape.
2. A midline incision was made above the thorax and the ziphoid cartilage was located. Transverse cuts were made through the diaphragm.
3. Following midline incision through the sternum, the heart was exposed by clamping open the thorax.
4. The saline primed canula with an 18 gauge needle was inserted into the left ventricle by grasping the base of the heart with the thumb and index finger.
5. The saline tap was immediately opened to ensure that the heart swelled. A tiny nick was then made in the right atrium. Perfusion was rapid with a high hydrostatic pressure for 2.5 – 3 minutes of approximately 250 ml of saline.
6. After closing of the saline tap and opening of the tap for fixative, perfusion was rapid with 200ml of 10% formalin and then reduced to 1-2 drops per second, totalling 400ml of fixative.

Perfusion was considered successful if the following were noted:

1. Rapid fasciculation of the limbs for the first minute of fixative perfusion.
2. Rigid forelimbs and flaccid hind-limbs as well as clear, blood-free fluid running from the nostrils.

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Following transcardial perfusion, the head was shaved and the skull carefully opened. The head was decapitated between the atlas vertebrae and the base of the skull, ensuring that the ear canals remained intact (Forman, 1998). Brains were fixed in-situ for 24 hours.

Retrieval of the brains was undertaken in the following manner:

1. The skull was placed in the stereotaxic frame with the ear bars located in the external auditory meatus.
2. The brain was cut with a stereotaxically placed scalpel blade between the cerebral and cerebellar cortices, ensuring that the cut completely transected the brain at the posterior level. The cerebellum was not included.
3. The process was anteriorly repeated, ensuring that the canula placement was within the boundaries of the two cuts.
4. Whole brains were removed and post-fixed overnight in 10% formalin.
5. The brains were then coronally sliced (Figure 3.1), and placed in appropriately marked tissue cassettes for processing and embedding in paraffin wax.

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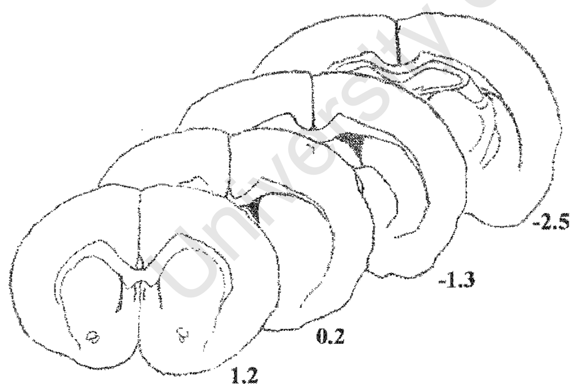

Figure 3.1: Coronal sections correspond to the levels 1.2; 0.2; -1.3; -2.5mm from bregma in the sterotaxic atlas of Paxinos and Watson (1998).

As a baseline for all neurohistology, two animals underwent no treatments, but were euthanized in the same manner. This was done as a means of controlling the procedures of fixation and tissue processing as well as staining techniques and identifying cell types. The size and shape of the normal nucleus helps in the light microscopic distinction of one glial cell type from another. Astrocytes typically have

round to oval nuclei with evenly dispersed, pale chromatin, oligodendrocytes have a denser, more homogeneous chromatin in a rounder and smaller nucleus, and microglia can be recognised by their elongated, irregular shaped nucleus with clumped chromatin. On Haematoxylin and Eosin (H+E) staining, ependymal cells are columnar epithelial-like cells with a ciliated/microvillous border facing the ventricular surface. These cells have pale, vesiculated nuclei, which are seen at the abluminal end of the cell.

3.5 Neurohistology

3.5.1 Processing

Buffered formalin is a widely used fixative for routine histopathology. However, no single fixative is ideal for all investigations. When subsequent techniques or tests following fixation require precise morphology and special investigations, the choice of fixation is always a compromise between allowing maximum biological activity to be demonstrated and the best morphological preservation possible. Transcardial perfusion with 10% formalin was deemed the most suitable fixative and method to render the brains suitable for investigation (Bancroft & Gamble, 2002).

The aim of tissue processing is to embed the tissue in a solid medium firm enough to support the tissue and give it sufficient rigidity to enable thin sections to be cut, but yet soft enough not to damage the knife or tissue (Bancroft & Gamble, 2002).

Manual methods of processing were selected as being the method of choice due to the delicate nature of the tissue and the most suitable means of minimising processing artefact. Automated methods which employ heat and vacuum impregnation techniques were deemed unsuitable for this study (Adams, 1984).

The most satisfactory embedding material for routine histology is paraffin wax. Most fixatives are aqueous-based and are not miscible with paraffin wax. To enable impregnation with this medium, the tissue was processed through the following stages:

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- Dehydration: to remove fixative and water from the tissue and replace it with dehydrating fluid. This was done by using a graded series of ethanol, commencing with a 50% solution through to absolute ethanol to ensure that the brains were not rendered hard and brittle.
- Clearing: this involves removing the dehydrating fluid with a fluid that is totally miscible with both the dehydrating fluid and the embedding medium. Xylol was used as the clearing agent.
- Impregnation: this is the method by which the clearing reagent was replaced by the embedding medium, namely, paraffin wax.
- Embedding was performed by blocking the tissue in metal moulds with paraffin wax.

3.5.2 Staining

After sectioning the wax blocks, the sections were floated out on a flotation water bath with a temperature maintained at 52° C. The sections were mounted on positively charged glass slides and incubated in a Memmert incubator maintained at 60° C for 1 hour. Routine H+E stains were performed for microscopic assessment.

H+E stained sections were screened using light microscopy. Two histologists (the researcher and one trained pathologist, Dr L Lange Veterinary Pathologist; Drs Dietrich, Voigt and Mia) independently scored morphological changes using the criteria as set out in Section 3.5.3. After the scoring was audited, any discrepancy was noted and jointly re-screened and a consensus score allocated.

3.5.3 Indicators for analysis

Neurons and glia show limited and fairly stereotyped patterns of response to injury. Although the glia may survive influences that destroy the neurons, any damage to neurons is almost invariably accompanied by some glial response. The inflammatory response consists of two main components, viz. a vascular reaction and a cellular reaction (Kumar et al., 2005).

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Prepared slides were microscopically reviewed for the presence or evidence of the following indicators of inflammation and injury:

1) Inflammation

a) Neutrophils

b) Lymphocytes

c) Plasma cells

Acute inflammation is rapid in onset with the main characteristics of exudation of fluid and plasma proteins and the emigration of leucocytes from the microcirculation, predominantly neutrophils, to the site of injury. Chronic inflammation is of longer duration and is associated histologically with the presence of lymphocytes and macrophages.

d) Microglial/macrophages: The microglia is part of the reticuloendothelial system – the histiocytes of the CNS. In response to injury or destruction, microglial cells become activated and assume the shape of a macrophage (Gitter cells, compound granular corpuscles), or they proliferate and adopt a rod-like form. They remain alone in a focus of injury to remove the remnants of other dead cells. This process is termed neuronaphagia and begins about 48 hours after the onset of a destructive process. The H+E sections would also be screened for any evidence of haemosiderin within the macrophages. Morphologically, iron pigment appears as a coarse, golden brown pigment within the cytoplasm. When the basic cause is the localised breakdown of red blood cells, the pigment is first demonstrable in the phagocytes in the immediate area.

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2) Gliosis

a) Gliosis: The proliferation of fibrillary astrocytes with the formation of many glial fibres. Gliosis is the most important histopathologic indicator of CNS injury, regardless of aetiology. Glial cells can be identified as small cells with round centrally located nuclei and scant, eosinophilic, homogenous cytoplasm.

b) Astrocytosis: Astrocytes participate in this process by undergoing hypertrophy and hyperplasia. Astrocytes may proliferate and form a glial scar to replace dead neurons (gliosis). Some astrocytes are further described as gemistocytic because of their 'stuffed' appearance with abundant eosinophilic homogeneous cytoplasm. The

cytoplasm expands irregularly. This surrounds an eccentric nucleus, from which stout processes emerge. (Scored as follows: 0 = absent; 1 = present).

3) Neuronal changes

a) Early neuronal change is indicated by a diffuse eosinophilia of the cytoplasm and, somewhat less pronounced, decreased basophilia also of the nucleus. This change resembles that induced by anoxia. Another type of early neuronal change that could occur can be see where the cells are dark and elongated and often have wavy apical dendrites. These are to be interpreted with caution, as similar dark neurons may occur as an artefact in non-perfused tissue, possibly caused by mechanical pressure on the brain. The number and location of such cells, if observed, need to be interpreted within context and sequence from dark neurons in early stages to similarly shaped eosinophilic shrunken cells with wavy dendrites. This indicates that dark neurons may, at least in part, be an early sign of neuronal death by trauma.

b) Red degeneration: Ischaemia causes neuronal shrinkage and angulation, the cytoplasm becomes hyper-eosinophilic, and the nucleus pyknotic and triangular. Once these changes occur, they are irreversible and indicate cell death. The pigmented Nissl substance in the cytoplasm disappears (chromatolysis) and the cytoplasm assumes a heterogeneous, brightly eosinophilic appearance.

Red neurons are evident with H+E stains at approximately 12-24 hours after an irreversible ischaemic injury. (Scored as follows: 0 = early neuronal change (eosinophilia); 1 = dark, elongated neurons; 2 = wavy dendrites of neurons; 3= red degeneration).

c) Satellitosis: The most vulnerable of the glial cells is the oligodendroglia. These are normally found streaming between the axons in the white matter and clustered about the neurons in the grey matter. In grey matter, the oligodendrocytes swell in response to nearly any type of injury to the neuron. They may also increase in number around a damaged neuron.

4) Oedema: Denotes an excess of fluid in the tissue. The mechanisms of inflammatory oedema are largely related to local increases in vascular permeability (vasogenic oedema), allowing fluid to escape from the intravascular compartment into the intercellular spaces of the brain. Oedema may also be generalised depending on the extent of the injury. Cytotoxic oedema implies an increase in intracellular fluid

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secondary to neuronal, glial or endothelial cell membrane injury. Conditions associated with generalised oedema often have elements of both vasogenic and cytotoxic oedema. (Scored as follows: 0 = < 5% of microscopic field; 1 = > 5 – 30% of microscopic field; 2 = 30 – 60% of microscopic field in more than 1 field; 3 = > 60% of microscopic field in more than 1 field at x 4 magnification.).

5) Vascular changes

a) Neovascularisation: Denotes new vessel formation. (Scored as follows: 0 = < 5% of microscopic field; 1 = > 5 – 30% of microscopic field; 2 = 30 – 60% of microscopic field; 3 = > 60% of microscopic field at x 10 magnification). Endothelial swellings, as well as thrombosis of small intracortical vessels are additional features screened for.

b) Endarteritis: Denotes inflammation of the tunica intima of an artery.

6) Meningeal changes

a) Haemorrhage. (Scored as follows: 0 = confined to the meninges; 1 = petechial haemorrhage in the cortex; 2 = multifocal, < 0,5μm; 3 = diffuse, multifocal > 0,5μm).

b) Calcification

c) Vascular: Repair usually begins early in inflammation. If resolution has not occurred, fibroblasts and vascular endothelial cells begin to proliferate to form a specialised type of tissue, which is the hallmark of healing. This is known as granulation tissue. These new vessels are leaky, which allows for the passage of proteins and red blood cells.

7) Apoptosis: Death by apoptosis is a normal phenomenon that serves to eliminate cells that are no longer needed. Apoptosis is important in the physiologic situation such as in neutrophils in the acute inflammatory process and lymphocytes at the end of an immune response. In these situations cells undergo apoptosis because they have been deprived of the necessary survival signals, such as growth factors. Evidence of apoptosis (PCD) is best seen with the electron microscope. However, on histologic examination of H+E sections, apoptosis involves single cells or very small clusters of cells. The apoptotic cell appears as a round or oval mass of intensely eosinophilic cytoplasm with dense nuclear chromatin fragments. Because cell shrinkage and formation of the apoptotic bodies occurs rapidly, the fragments are quickly

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phagocytosed. Considerable apoptosis may occur before it becomes apparent in histologic sections. Apoptosis, in contrast to necrosis, does not elicit inflammation, making it more difficult to detect by histologic means. Evidence of pyknosis characterised by nuclear shrinkage and increased basophilia are seen in the apoptotic cell on H+E staining. Evidence of increased apoptosis would also be taken into consideration as a possible response to VCP administration.

8) Other: Overt cavitation, inclusion bodies, neurofibrillary tangles (a particular type of neuronal degeneration that occurs in a number of diseases), and plaque formation would also be screened by light microscopy supported by special investigations.

3.5.4 Additional investigations

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Histochemical and immunocytochemical as well as tinctorial staining procedures were undertaken on those sections of brain that showed evidence of the criteria described above. This was done in an attempt to evaluate and categorise any cellular changes that may have occurred across the subgroups. These investigations include techniques such as the Perl's Prussian blue histochemical reaction for the determination of haemosiderin pigment. See Appendix C for method.

Tinctorial staining

Phosphotungstic Acid Haematoxylin (PTAH) (Appendix D) and Cresyl violet staining (Appendix E) were also performed. The PTAH stain was used as a means of demonstrating reactive astrocytes and cellular pattern. The Cresyl violet stain was used to demonstrate neurons as well as Nissl substance (an indicator of gliosis). These stains were performed on those sections specifically selected from the H+E sections.

Immunocytochemistry

Immunocytochemistry, a technique used for identifying cellular or tissue components (antigens) by means of antigen-antibody interactions, the site of antibody binding being identified either by direct labelling of the antibody, or by use of a secondary

labelling method, was performed on selected sections which demonstrated evidence of the above criteria.

Glial fibrillary acidic protein (GFAP) to identify glial cells and the role of astrocytes in this process, as well as CD 68 immunocytochemistry staining to identify microglia were performed. See Appendix F for method.

Leong et al. (2003) reported a cautionary note of false negative detection of amyloid by conventional tinctorial staining methods. This can be avoided by increasing the level of detection by using both immunocytochemical and Congo red staining methods. Beta-amyloid (Dakocytomation) immunocytochemistry staining (Appendix F) was also performed in conjunction with Congo red staining (Appendix G) to identify amyloid plaques.

Glial fibrillary acidic protein, a 50kD intermediate filament, is relatively resilient to fixation. Most antibodies are immuno-reactive in routinely fixed and processed tissue sections. The monoclonal antibody GFAP (Dakocytomation) is fixative sensitive. Leong et al. (2003) reported that GFAP immunoreactivity is mildly enhanced by heat induced epitope retrieval (HIER).

Leong et al. (2003) also reported that the best macrophage reagents produced to date are those recognising the CD68 antigen. They further reported that anti-macrophage reagents recognising formalin-resistant epitopes require microwave or enzyme pre-treatment prior to immunocytochemistry staining as a means of reducing background staining. For the purposes of this study, these recommendations were found to be unsuitable for the accurate demonstration of CD68 (clone KP1; Dakocytomation). This was attributed to the extended fixation time of the brains in 10% formalin which may have caused an excess of cross-linking aldehyde bonds with the antigenic sites to be demonstrated. The KP1 clone labels monocytes and macrophages in a wide range of tissues and is conserved in the rat species. Optimal demonstration of this 110kD macrophage marker was best demonstrated once the sections had been treated with HIER by means of pressure cooking with Tris EDTA at pH 9.0.

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Immunocytochemical staining for Aβ was best demonstrated by pre-treating the dewaxed sections with formic acid for 3 mins as a means of antigen retrieval.

For the purposes of this study, it was necessary to adapt traditionally used protocols to unmask antigenic sites formed by formalin cross-linking during the fixation process of the brains. This was optimized by using a range of retrieval solutions and types of equipment.

The equipment included the use of a conventional Panasonic microwave oven, a 6 litre capacity stainless steel Presto pressure-cooker and a water bath. The solutions that were used for antigenic retrieval ranged from citrate buffer at pH 6.0 to EDTA at pH 8.0 and Tris EDTA at pH 9.0 and pH 10.0 as well as concentrated formic acid (Appendix F).

During procedures to remove the whole brain, one rat presented with a cyst that was found on the surface of the skull contralateral to the craniotomy site.

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3.6 Data analysis

3.6.1 Histological description

Histological screening was done as described in paragraph 3.5.2 and the findings were described according to the criteria as described in paragraph 3.5.3. The descriptions were supported by photomicrographs.

3.6.2 Statistical analysis

Over time, as concepts of axonal change and diffuse brain injury have developed, the overriding hypothesis was that of a clinical spectrum, all due to a common axonal pathology, but differing in amount, severity and location of change (Gennarelli, 1996).

The injury status of the animals was scored using a modified scaling system (Gennarelli, 1996) to determine amount, severity and location of general injury.

Based on this, the brains were assigned a ranking of damage based upon the criteria as described above. They were assigned a DAI score on a continuous scale of increasing damage. Three randomised power fields were analysed and graduated on a scale of 0-3. Animals were scored as follows:

- 0 (detectable changes), evidence of inflammation
- 1 (mild damage), minimal alterations (< 30% of the microscopic field)
- 2 (moderate damage), moderate alterations (30-60% of the microscopic field)
- 3 (severe damage), (> 60% of microscopic field)

As all the animals in the study underwent surgery, there was no need to make provision for scoring the absence of injury.

In addition to the DAI score, morphological changes were scored as follows (see 3.5.3):

- Detectable (0): evidence of inflammation eg neutrophil influx
- Mild damage (1): as with 'detectable changes', including early neuronal changes such as eosinophilia, macrophages and oedema.
- Moderate damage (2): as with 'mild damage', including evidence of neuronal death (dark wavy, apical dendrites), haemorrhage, gliosis, oedema and vascular changes.
- Severe damage (3): as with 'moderate damage', including red degeneration, chromatolysis, necrosis, satellitosis and calcification. Distribution of injury was also accounted for.

The result of the above scoring was subjected to statistical analyses. For each measure, a comparison between the four experimental groups was analysed using inferential statistics, namely one-way ANOVAs. Where appropriate, significant differences between groups were ascertained using Bonferroni's test for post hoc comparisons.

Specific indicators were assigned a similar score using this modified scaling system (Gennarelli, 1996). Comparisons of specific morphologic findings of the injury groups (VCP vs saline) were examined using t-tests for independent samples, which

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were confirmed using non-parametric analysis, in this case, T-tests for independent samples.

3.7 Conclusion

Chapter 3 provided a detailed analysis of study design, specimen harvesting and criteria for defining and describing of histological changes.

Chapter 4 follows with a detailed description and analysis of the findings using the methods described above.

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CHAPTER 4: RESULTS

4.1 Diffuse axonal injury

Diffuse axonal injury was used as an indicator of generalized diffuse injury. Injury was considered as diffuse (multifocal), which describes a number of pathologies, some of which are a consequence of acceleration/deceleration forces applied to white matter, whereas others are vascular in nature, and some may be secondary to hypoxia. The individual injury scores are presented in Appendix H. These scores were then subjected to one-way ANOVA, with Bonferroni's post hoc test.

4.1.1 Acute group (2 weeks)

When the four subgroups were compared, there were significant differences in their DAI scores [$F(3, 20) = 73.24$; $p<0.0001$]. Rats subjected to FPI + VCP showed less injury than rats with FPI + saline ($p<0.0001$). Rats that were injured by FPI and had received VCP or saline, showed more diffuse injury than rats with sham surgery (VCP and saline) ($p<0.001$ for all). The mean and standard deviation of the four subgroups are presented in Table 4.1.

Table 4.1. Mean and standard deviation of the DAI score of the two week group

Subgroup	Procedure	Mean	N	SD
1	Sham & saline	0.00	6	0.00
2	Sham & VCP	0.50	6	0.55
3	FPI & saline	3.00	6	0.00
4	FPI & VCP	1.33	6	0.52

This demonstrates that a dose of 10 µl of VCP at a concentration of 1.7µg/µl, administered immediately after injury, has significant beneficial effects on injured rats at 2 weeks post-injury.

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It further demonstrates that the FPI did in fact induce significant injury, apart from the effects of surgery, and that this device can be used to produce reproducible brain injury in the rat. This model may be used to evaluate the neurochemical correlates of brain trauma and/or the response to pharmacologic interventions.

4.1.2 Chronic group (3 months)

When the 4 subgroups were compared, there were significant differences in their DAI scores [$F(3, 20) = 6.26$; $p<0.01$]. Rats with FPI + VCP showed less injury than rats with FPI + saline ($p<0.05$). Further, rats with FPI + saline showed more injury than rats with sham surgery and saline ($p<0.01$). The mean and standard deviation of the four subgroups are presented in Table 4.2.

Table 4.2. Mean and standard deviation of the DAI score of the three month group

Subgroup	Procedure	Mean	N	SD
5	Sham & saline	0.00	6	0.00
6	Sham & VCP	0.50	6	0.55
7	FPI & saline	1.33	6	0.87
8	FPI & VCP	0.33	6	0.52

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This demonstrates that a dose of 10 µl of VCP at a concentration of 1.7µg/µl, administered immediately after injury, has significant beneficial effects on injured rats at 3 months post injury.

4.1.3 Other comments

The data suggests that these effects may be more clearly seen in the more acute phases of cellular injury than in the chronic phase. However, the severity of the injury may have played an important role here, and the above conclusion cannot be drawn without further research.

4.2 Specific morphological findings of the two week severe grade injury group

In this study the effect of VCP administration following severe (2.7-3.0 ATM) fluid percussion injury was evaluated. After a period of 2 weeks specific indicators were scored and the injury groups compared using t-tests for independent groups. Means and standard deviation of both groups for specific indicators is presented in Table 4.3 and visually presented in Figure 4.1.

Evidence of injured neurons was based on alterations of morphology and affinity for acid fuchsin, which was originally used to identify neurons with irreversible damage following hypoglycaemia. Duchen (1992) observed that Nissl-stained neurons were not an indicator for irreversible damage, but that acid fuchsin stained neurons were moribund.

Changes included evidence of neuronal injury, evident by red degeneration, vascular changes, gliosis, oedema, haemorrhage, necrosis and calcification (Plate 1A). The most striking early neuronal change was evident by the demonstration of eosinophilia of the cytoplasm and, a less pronounced, decrease in basophilia of the cell nucleus. Cell and nuclear membranes of the eosinophilic neurons became distinct. Another neuronal change noted was that of dark, elongated apical dendrites. These occurred consistently within the damaged tissue and not in the adjacent normal cortex (Plate 1B). This suggests that these changes were caused directly by the FPI. Glial cells appeared to be swollen with round, centrally located nuclei.

Following FPI trauma, the injured brain demonstrated significant histological changes, which were predominantly unilateral (Plate 1C). There was no histological evidence to support a 'contrecoup' concussive injury in the short term acutely injured group or the mildly injured long term group

H+E stained sections revealed that in the severely injured subgroup that received saline, injury and haemorrhage was diffuse (multifocal) and widespread. Evidence of trauma could be seen from the left subcortical hemisphere and the external capsule, and extended approximately 3.5mm-4.0mm, beginning slightly caudal to the level of the optic decussation (Plate 1D).

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In the severe acutely injured group, the haemorrhage was extensive in its distribution and was visible in all of the animals. The haemorrhage was seen in the corpus callosum, the subcortical white matter and pons and in the ipsilateral cortex and hippocampus. Haemorrhage was prominent at the level of the optic decussation, where it was primarily associated with the fimbria and external capsule and often associated with interruption of blood into the third ventricle. The haemorrhage extended to the level of the pons in regions including the substantia nigra, external capsule, dentate gyrus and in an area just medial to the brachium of the inferior colliculus. Left hippocampal (CA3 region) and cortical haemorrhage (frontoparietal, striate and entorhinal cortex) was common.

These haemorrhages varied in severity between groups, ranging from tiny foci of scattered peri-vascular petechiae to larger areas of haemorrhage (Plate 1E). Tiny foci of haemorrhage were observed in the majority of animals that were injured and had received VCP. The pattern of haemorrhage changed to large, more extensive haematomas in the injured subgroup that had received saline. Based on the scoring system, the rats that received VCP showed significantly less haemorrhage than the rats that received saline ($p<0.01$). Thrombosis of small intracortical vessels was present to a lesser degree in the animals that had received VCP.

Comparisons of the severe acutely injured group, FPI + saline (subgroup 3) indicate that haemorrhage was more extensive than that of the low injury chronic, FPI + saline (subgroup 7). The low injury chronic group had evidence of haemorrhage largely confined to the meningeal surfaces with evidence of scar formation.

There was significantly less oedema ($p<0.01$) seen in the sections of the rats that had received VCP. Previous studies undertaken by Durmaz et al. (2003), who administered Lazaroid (U-83836E) prophylactically after cerebral trauma in rats also showed evidence in the reduction of oedema and neuronal necrosis.

Neuronal loss was evident and more severe in both the hilar regions of the dentate gyrus and the CA2/3 region of the hippocampus of the FPI + saline subgroup when compared with the injured animals that had received VCP. Some neurons in the FPI + saline subgroup showed marked differences in morphology, with shrunken and

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triangulated cell bodies with darker staining on H+E damage (Plate 1F). In the neocortex, neuronal damage post injury was most severe in the ipsilateral parietal, occipital and temporal cortices

In the ipsilateral dorsal and mid-hippocampus, injured neurons were principally observed in pyramidal cell layers CA2 and CA3 (Plate 2A; 2B). The severity and distribution of these changes was markedly and consistently reduced in all the animals of the acute injury group that had received VCP, whose scores were significantly less than that of the saline group ($p<0.01$) (Plate 2C; 2D; 2E).

Hippocampal astrocytes and oligodendrocyte populations appeared to be unaffected by the injury.

Large areas of necrosis were exhibited in the acute FPI + saline subgroup. Necrotic cells showed increased eosinophilia with a glassy homogenous appearance. The eosinophilia is in part attributed to the loss of the normal basophilia imparted by ribonucleic acid (RNA) in the cytoplasm and by the increased binding of eosin to denatured intracytoplasmic proteins. The cytoplasm was expanded and irregular and stained bright pink on H+E (Plate 3A). CD68 immunocytochemical demonstration showed macrophage infiltration, oedema formation and haemorrhage (Plate 3B; 3C). Injury scores of the VCP group were significantly lower than those of the saline group ($p<0.01$).

Neovascularisation was prominent and marked in the acute FPI + VCP subgroup, but not in the FPI + saline subgroups (Plate 3D). The differences in the scores of the two subgroups reached significance ($p<0.05$).

No overt cavitation was noted in any of the subgroups, as has been noted and reported by McIntosh et al. (1989). Three animals in the FPI + saline subgroup showed foci of calcification (Plate 4A).

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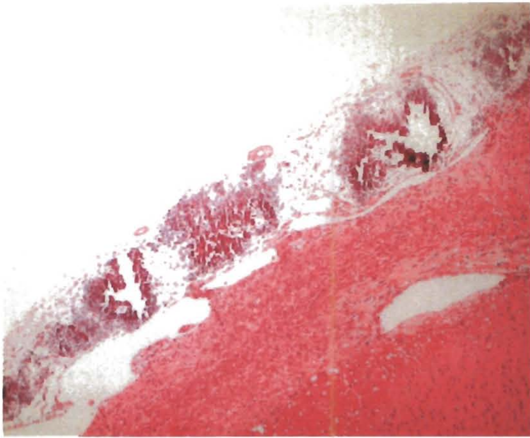
PLATE 1



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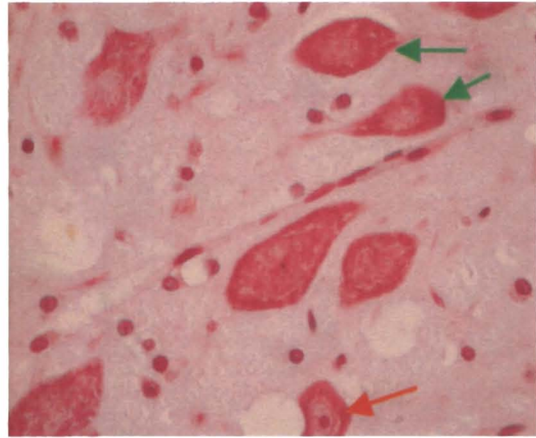

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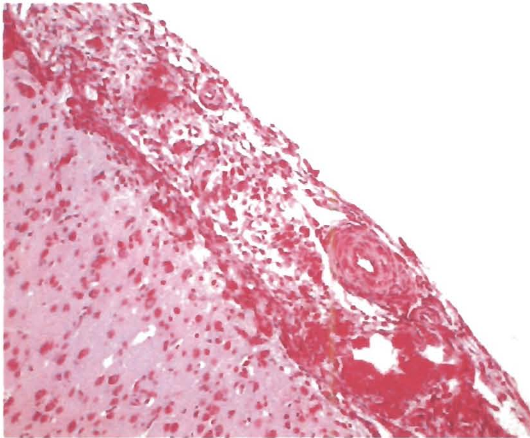

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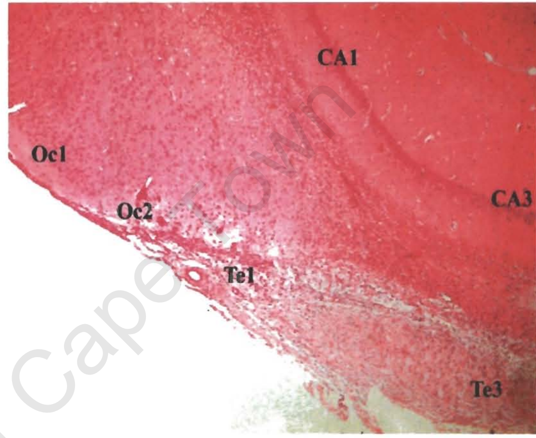

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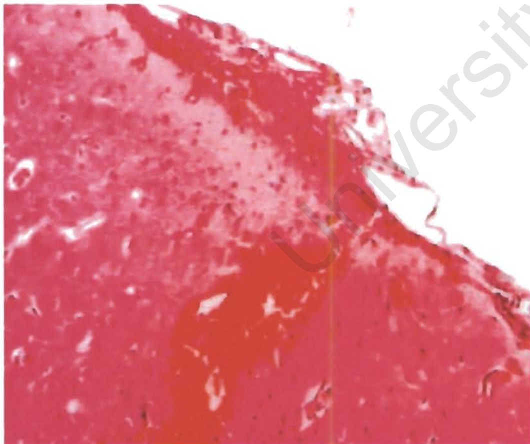

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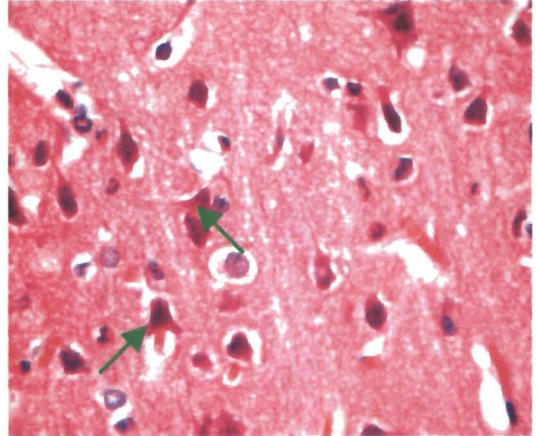

F.

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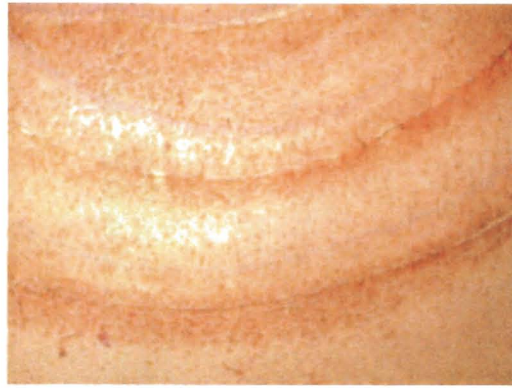
Subgroup 3: (A) H+E demonstrates that the injury induced was significant enough to produce cellular damage which includes oedema, increased vascularisation and calcification (x 4).
Subgroup 4: (B) Cresyl Violet stain showing necrotic neurons indicated by green arrows. Red arrow indicates a normal neuron (x 40).
Subgroup 3: (C) Cresyl Violet stain showing neuronal damage in the cerebral cortex (x 4).
Subgroup 3: (D) H+E showing neuronal damage, oedema, haemorrhages and scar formation in the following subdivisions of the cortex: Oc1, Oc2, Te1, Te3 and cell layers CA1 and CA3 (x 4).
Subgroup 3: (E) H+E stain showing large area of haemorrhage (x 10).
Subgroup 3: (F) H+E stain. Green arrows indicate shrunken, triangulated cell bodies showing necrotic neurons (x 100).

PLATE 2

A.



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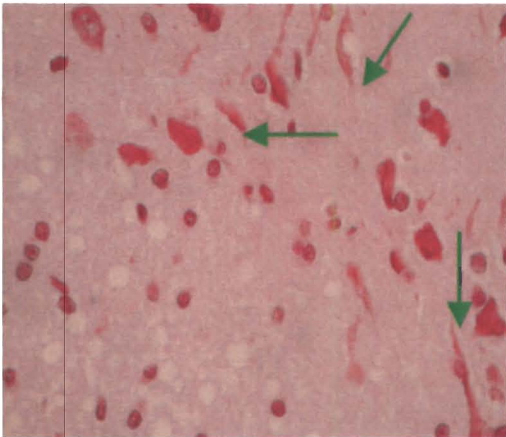

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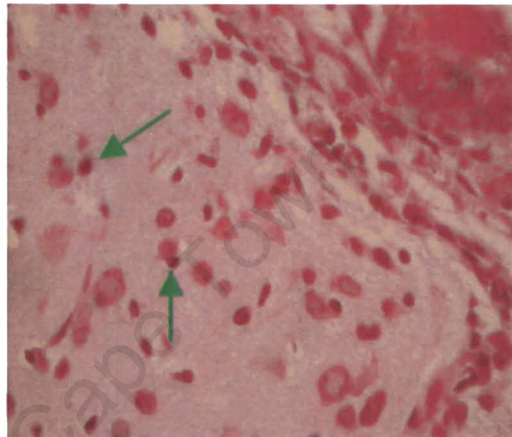

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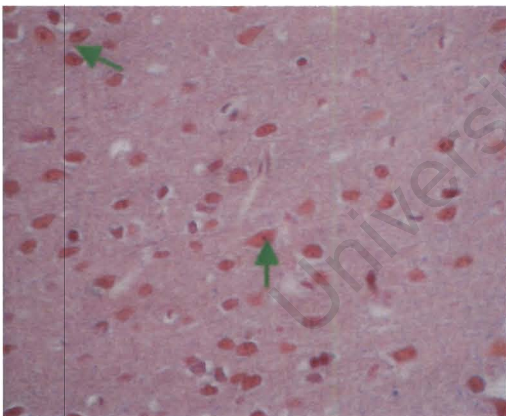

E.

Subgroup 3: (A) GFAP staining of dentate gyrus and hippocampus of injured brain (x 4).
Subgroup 3: (B) Contralateral staining of GFAP demonstrated in (A) (x 4).
Subgroup 4: (C) Cresyl Violet stain. Green arrows indicate necrotic glia (x 10).
Subgroup 4: (D) Cresyl Violet stain. Green arrows indicate shrunken neurons with wavy processes indicating early neuronal death (x 10).
Subgroup 4: (E) PTAH stain. Green arrows showing reactive neurons (x 4).

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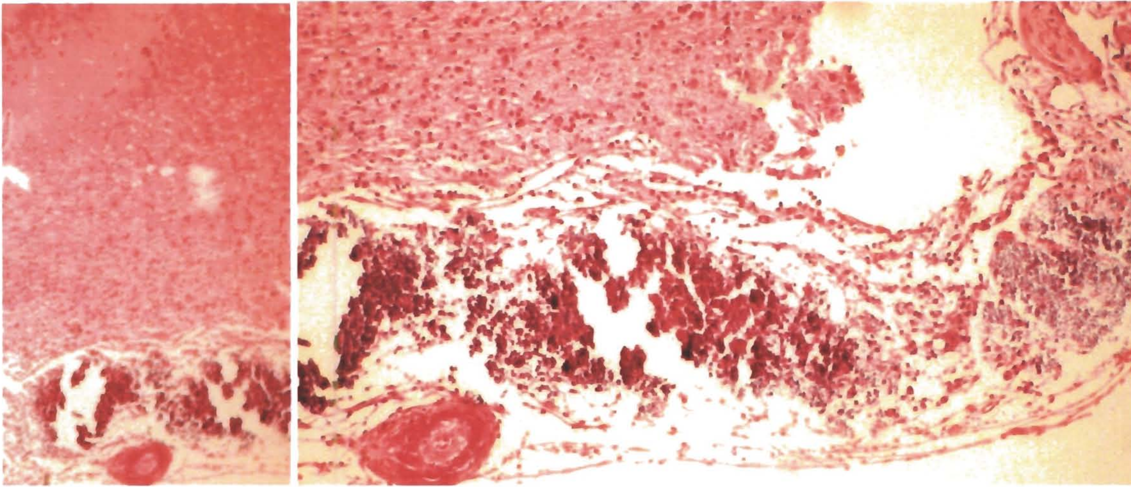
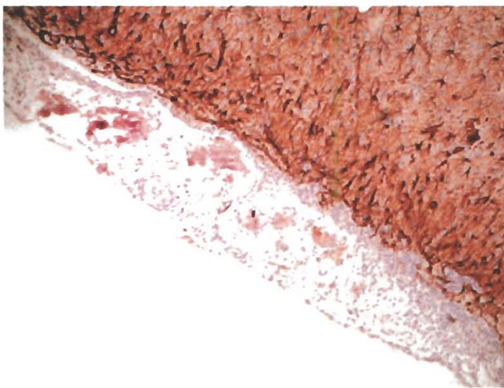
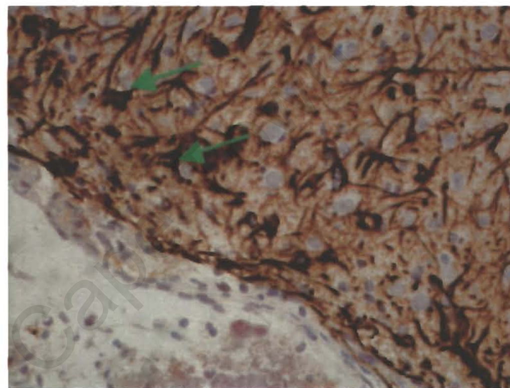
PLATE 3



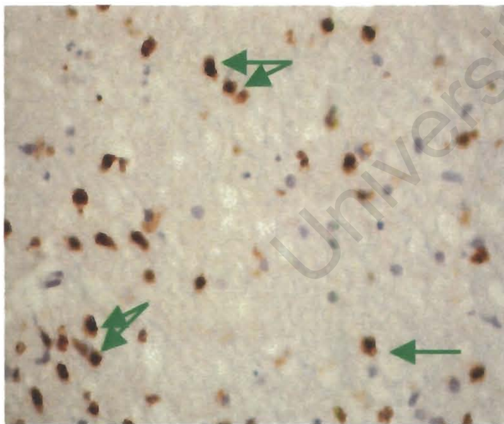
A. (a) A. (b)



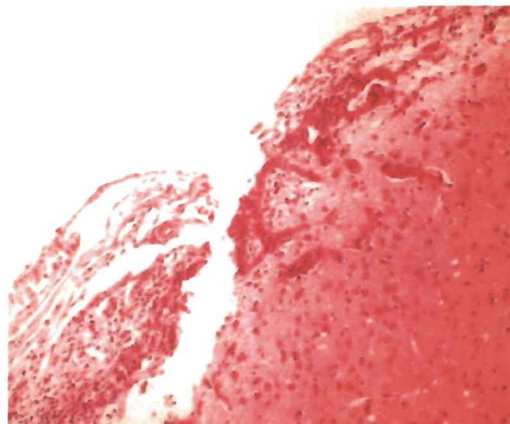
B. (a)



B. (b)



C.



D.

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Subgroup 3: (A. a) H+E stain which shows necrosis, oedema formation, haemorrhage and areas of calcification (x 4).
Subgroup 3: (A. b) Higher magnification of (A. a) (x 10).
Subgroup 3: (B. a) GFAP staining of extensive injury indicating reactive astrocytes in response to tissue injury (x 4).
Subgroup 3: (B. b) Higher magnification of (B. a). Green arrows indicate GFAP positive astrocytic perinuclear cytoplasm and well developed processes (x 10).
Subgroup 4: (C) CD 68 positive macrophages (x 10).
Subgroup 4: (D) H+E stain showing oedema and neovascularisation. Injury was confined to Oc1, Oc2, Te1 and Te3, but not extending to cell layers CA1 and CA3 (x 4).

4.3 Specific morphological findings of the three month low grade injury group

In this study, the effect of VCP administration following mild (1.0 -1.1 ATM) FPI over a 3 month period was evaluated. Specific indicators were scored and the injury groups compared using t-tests for independent groups. Means and standard deviation of both groups for specific indicators is presented in Table 4.3 and visually presented in Figure 4.1.

Within the FPI + VCP subgroup, a specific and consistent pattern of neuronal injury was observed, with only minor variations noted between the animals. Graded scores on neuronal changes, haemorrhage and oedema were uniform throughout. In contrast, the same scores of the group that received saline showed a slightly wider range.

The primary site of cortical damage appeared to be confined to the area directly under the impact site which coincides with Par1, the primary somatosensory cortex (Plate 4B).

Histology showed meningeal changes. Neuronal changes were evident by features consistent with shrinkage of the cell body, pyknosis of the nucleus and disappearance of the nucleolus, accompanied by intense eosinophilia of the cytoplasm. Meningeal thickening was seen in the sections of the FPI + saline but not in the FPI + VCP subgroup (Plate 4C). Sections of subgroup 7, (FPI + saline) that were screened for morphology showed a neutrophil influx, and evidence of inflammation was supported by the presence of macrophages demonstrated by immunocytochemical detection of CD68 positivity (Plate 4D; 4E). Gliosis was also present and evident by GFAP positivity with fine GFAP-containing processes which formed a mat-like arrangement in which stellate cell bodies were evident.

Neovascularisation was prominent, but confined to the meninges. Neovascularisation was more pronounced in the FPI + VCP than the FPI + saline subgroup but did not reach statistical significance ($p=0.07$). Based on the scoring system, there was significantly less haemorrhage noted in the VCP recipients, when compared with those of the saline group ($p<0.05$).

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The FPI + saline subgroup exhibited more oedema than that of the FPI + VCP subgroup ($p<0.05$) (Plate 5A; 5B). All cellular changes exhibited in the FPI + VCP subgroup were less severe than those seen in the FPI + saline subgroup of both time intervals. Histology showed focal neuronal change with some red degeneration exhibited in some sections (Plate 5C). Small foci of peri-ventricular, subependymal haemorrhage were also seen. In the FPI + VCP subgroup, there were no neurons showing red degeneration in the hippocampus. The FPI + saline subgroup showed more neuronal change than the FPI + VCP subgroup ($p<0.05$) (Plate 5D).

Haemosiderin was present in one case of the FPI + saline long term study. On H+E staining this is seen as a coarse golden brown pigment. Histochemical demonstration of haemosiderin by means of the Perl's Prussian blue technique was performed on this section (Plates 6A; 6B). Minimal evidence of red degeneration was noted in the long term groups.

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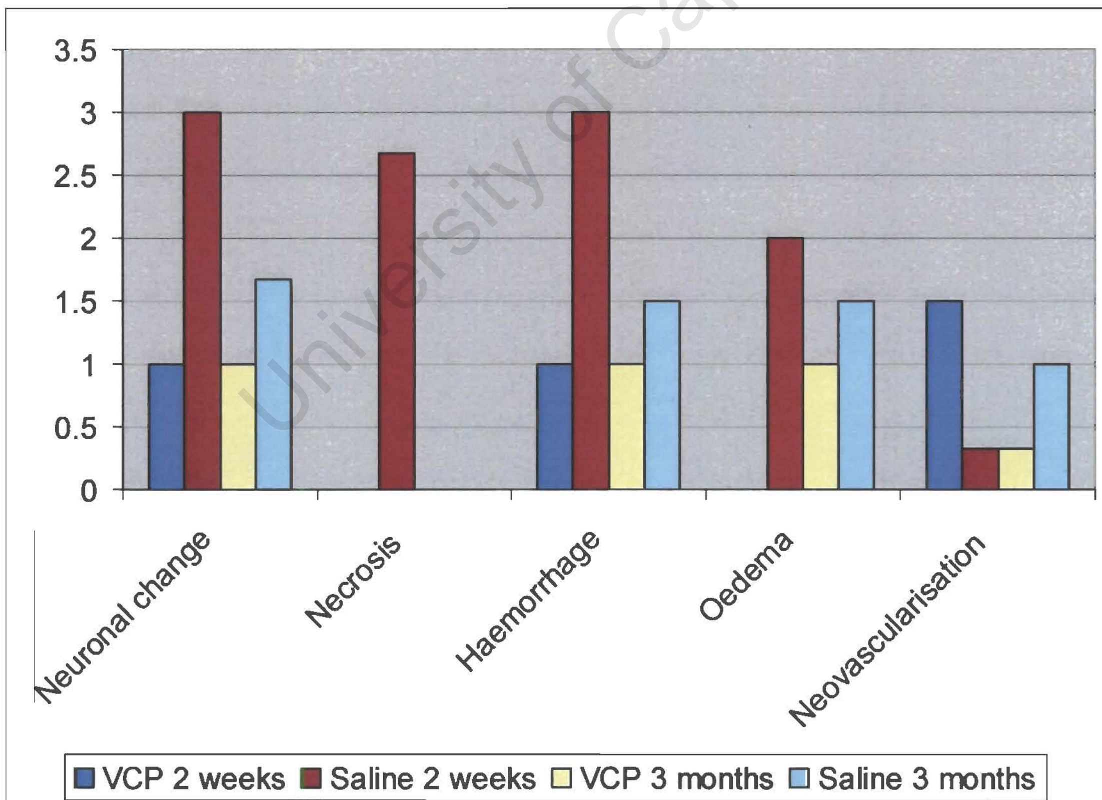
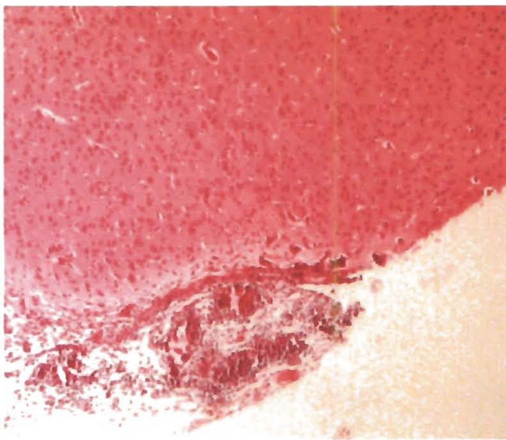


Figure 4.1 Comparative scores for VCP and saline of the 4 subgroups

PLATE 4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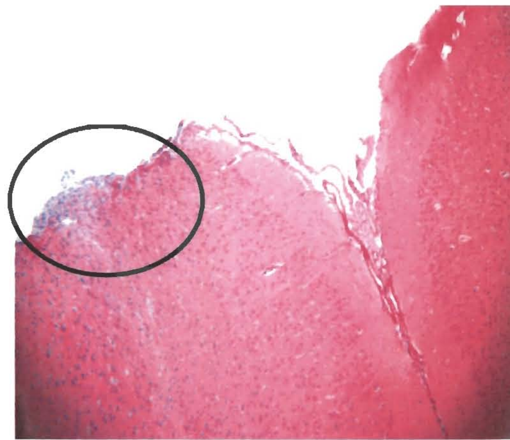
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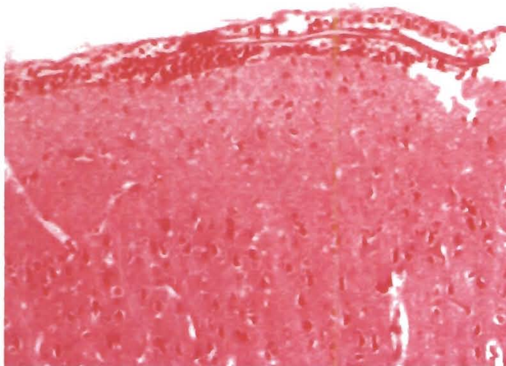
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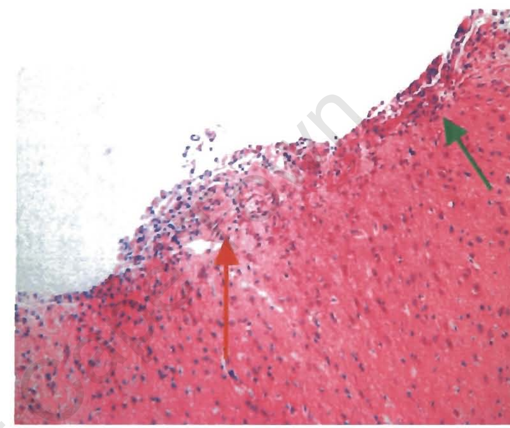
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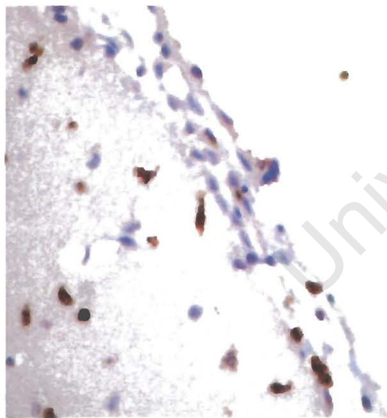
E.

Subgroup 3: (A) H+E stain with evidence of calcification (x 4).
Subgroup 7: (B) H+E stain. Injury in the retrosplenial region and bordering on the primary visual cortex at site of FPI (x 4).
Subgroup 7: (C) H+E showing meningeal thickening (x 10).
Subgroup 7: (D) H+E stain. Macrophages and mild oedema in the retrosplenial region (red arrow). Blood vessel formation is indicated by the green arrow (x 10).
Subgroup 7: (E) CD 68 positive macrophages (x 10).

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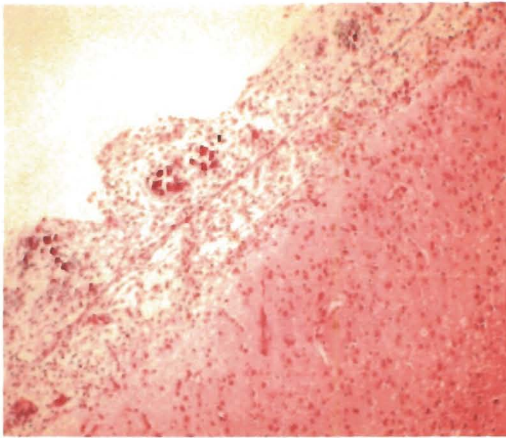
PLATE 5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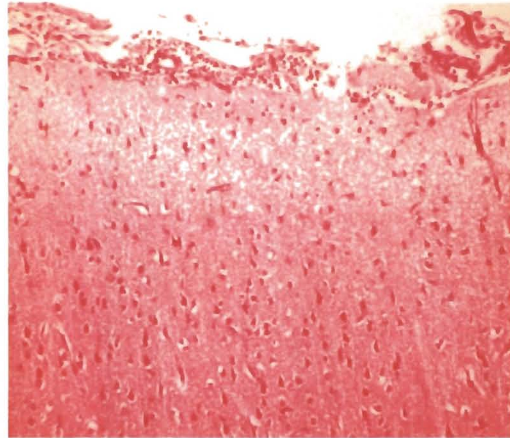
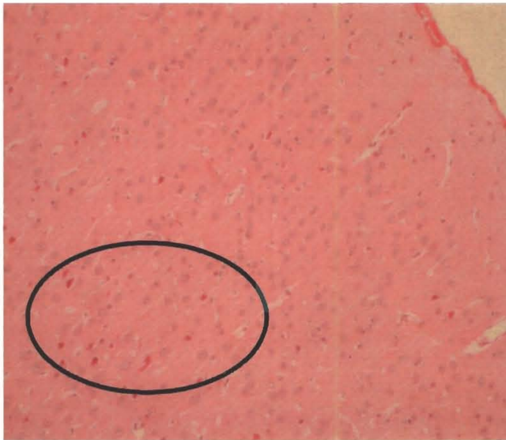
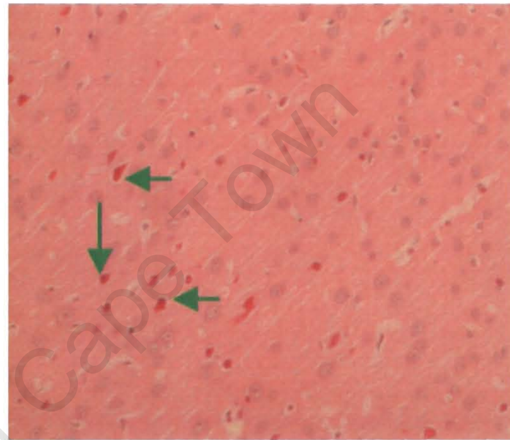
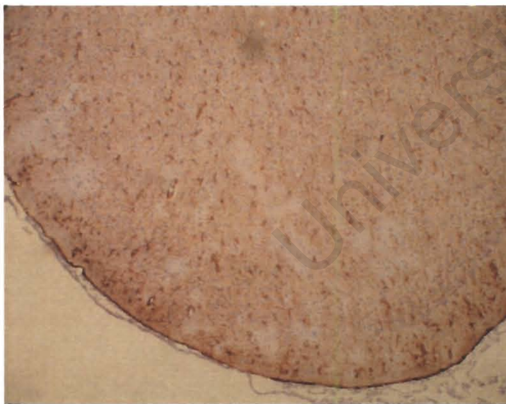
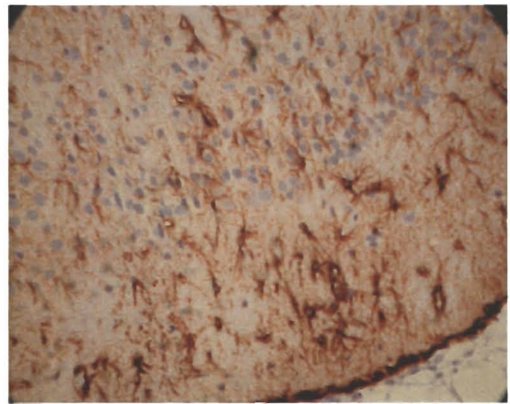

B.


C. (a)


C. (b)


D. (a)


D. (b)

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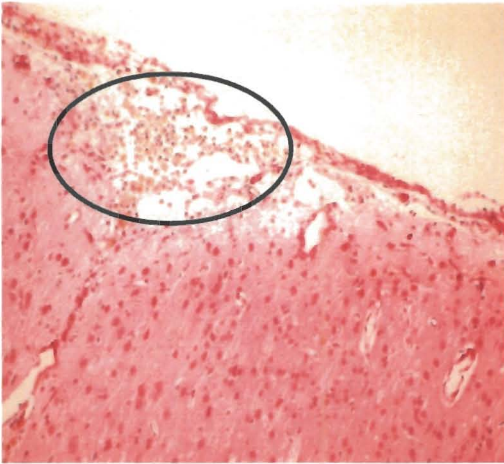
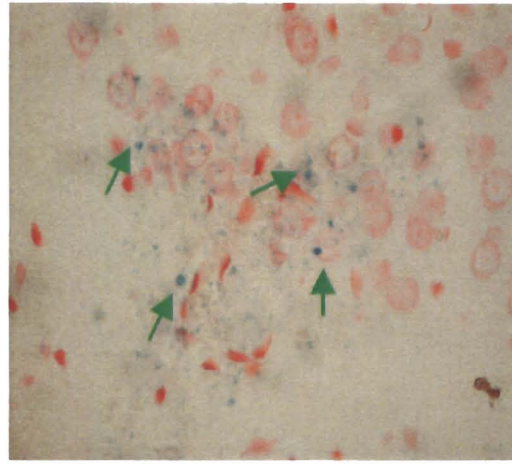
Subgroup 7: (A) H+E stain showing evidence of oedema (x 10).
Subgroup 8: (B) H+E stain showing evidence of oedema (x 10).
Subgroup 7: (C. a) H+E stain. Acute neuronal injury with red degeneration (x 4).
Subgroup 7: (C. b) Red degeneration indicated by green arrows (x 10).
Subgroup 8: (D. a) GFAP stain for reactive astrocytes in the injured brain (x 4).
Subgroup 8: (D. b) Higher magnification (D. a) of fine GFAP-containing processes which form a mat-like arrangement in which stellate cell bodies are evident (x 10).

PLATE 6


A.


B.

Subgroup 7: Haemosiderin as coarse golden brown pigment on H+E stain (x 10).
Subgroup 7: Haemosiderin pigment indicated by green arrows with the Perl's Prussian blue technique (x 40).

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Table 4.3. Means and standard deviations of the 4 subgroups

	VCP 2 weeks		Saline 2 weeks		VCP 3 months		Saline 3 months	
Indicators	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**
Neuronal change	1	0	3	0	1	0	1.67	0.52
Necrosis	0	0	2.67	0.52	0	0	0	0
Haemorrhage	1	0	3	0	1	0	1.5	0.55
Oedema	0	0	2	0	1	0	1.5	0.55
Neovascularisation	1.5	0.84	0.33	0.52	0.33	0.52	1	0

4.4 Negative findings

In both the acutely and mildly injured groups of animals, no evidence of neurofibrillary tangles or neuritic plaque formation was noted. There was no evidence on assessment of the H+E sections to support basophilic fibrillary structures and no positive Congo red stained sections to support evidence of amyloid deposition. Sham animals showed no significant changes.

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4.5 Other observations

Three animals in the mildly injured group showed bilateral changes on histological examination. The changes were mild, and were confined close to the meningeal surface. It is unclear if this was due to surgery or changes within the craniotomy when FPI was induced.

Histological examination of H+E sections of all the groups, including the sham animals, showed no morphologic evidence that VCP administration contributed to the cellular changes.

Histological examination of H+E sections of all the groups, including the sham animals, showed no morphologic evidence that VCP administration contributed to the cellular changes.

4.6 Conclusion

Chapter 4 provided a detailed analysis of the data and the specific parameters, which were identified as being significantly influenced by the administration of VCP. These results will be discussed in Chapter 5.

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CHAPTER 5: DISCUSSION

5.1 Discussion of findings

5.1.1 Location and distribution of changes

Morphologic assessment of the H+E sections and analysis of the data indicated that the injury sustained was severe enough to produce significant damage to the underlying hippocampal structures and that the effects of surgery and the administration of the VCP or saline did not influence the results. Additionally, it did not obscure the ability to apply the established cell recognition criteria to delineate cell type and effects of trauma. From the cellular changes and pattern of injury described, it would appear that the cortical regions of the brain display susceptibility to TBI, and that the extent of damage seen beyond this point, both necrotic and diffuse damage, is dependent on the force of the physical impact.

As discussed in Chapter 2, the resultant neuronal damage of the central nervous system caused by TBI occurs as a result of at least two mechanisms. There is a direct, mechanical lesion, which is surrounded by an area in which the indirect damage occurs.

The goal of the repair process is to restore tissue to its original state. The inflammatory process, which was set in motion by the FPI, attempts to contain damage, eliminate the damaging stimulus and elicit the recruitment of cell-matrix interactions and extra-cellular matrix (ECM) components in the area of injury. The different patterns of cell loss between the fluid percussion brain injury time periods of this study may reflect characteristic alterations in underlying cellular pathology.

Shifts in the location of the craniotomy, either small or large, result in different patterns of neuropathology and behavioural deficits (McIntosh et al., 1989). Neuronal loss in the hippocampus after experimental TBI has been suggested as a mechanistic link to cognitive impairments that arise from this type of injury (Hicks et al., 1996), as well as behavioural deficits (Petullo et al., 1998).

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In this study, the severity of FPI compares to that which was first described by McIntosh et al. (1989), who classified low-grade injury as being 1.0 ATM, moderate as 1.5-2.0 ATM and severe injury as 2.5-3.6 ATM.

In the sections examined from the severely injured animals, evidence of trauma could be seen from the left subcortical hemisphere and the external capsule, which extended approximately 3.5mm-4.0mm, beginning slightly caudal to the level of the optic decussation. These findings are consistent with those reported by Cortez et al. (1989). In the ipsilateral dorsal and mid-hippocampus, injured neurons were principally observed in pyramidal cell layers CA1 and CA3. There was no significant damage noted in CA1 and CA3 or the dentate gyrus as was seen in the 2 week group. This could be attributed to the severity of the injury induced.

While the mechanism(s) remain unclear, these regions have previously been shown to be selectively vulnerable in the FPI brain injury model (Hicks et al., 1996). The severity and distribution of these changes were markedly and consistently reduced in all the animals of the acute injury group that had received VCP. Hippocampal astrocytes and oligodendrocyte populations appeared to be unaffected by the injury, which further supports the selective vulnerability of neurons to FPI.

The microglial population of cells increased in number in the hilus of the dentate gyrus as well as in the ipsilateral areas CA2 and CA3 after injury. This is believed to be due to the breakdown of the BBB, which may allow passage of macrophages and lymphocytes into the brain, potentially initiating inflammatory and autodestructive processes. The presence of microglia reflects the ongoing pathology associated with the significant neuronal injury reported throughout the hippocampus and the possible inability of microglia to provide neuro-protection. Unfortunately, due to the histological techniques used, discriminating the difference between activated and resting microglia was not possible.

See Appendix I for a diagrammatic representation of location of injury.

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5.1.2 The implications of time and TBI

In a study undertaken by Keeling et al. (2000), the activation of the complement system during the acute periods following traumatic brain injury has been described over a period of 1 hour to a period of 7 days. The 2 week survival period was selected as previous studies have established that APP, although not Aß, is present 2 weeks following moderate FPI (Bramlett et al., 1997; Pierce et al., 1996) and therefore, this time point would serve as a positive control for the potential formation of amyloid protein in the brain.

The 3 month survival period was selected because inflammation has been reported at this time after TBI in rats (Holmin & Mathiesen, 1999). This has been expanded to investigate the role of complement activation on the long-term effects of traumatic brain injury.

The possibility exists that the acceleration/deceleration impulses selectively damage small blood vessels in the brain and that the initial injury (2 week group) was severe enough to induce rapid morphological and histological change.

The infiltration of macrophages is an important component of chronic inflammation, and within 48 hours they will usually constitute the predominant cell type. Macrophages were identified in the chronic low injury group of animals that received VCP. This suggests that the process of inflammation may have been delayed. As discussed in Chapter 2, it has been reported that macrophages usually disappear in short-duration acute inflammation; that such tissue would be devoid of inflammatory infiltrate, and that oedema and increased vascularity would have largely disappeared. This can be attributed to the ability of macrophages and activated endothelium to elaborate a variety of growth factors and cytokines. However, this may set the stage for the persistence of the inflammatory response.

5.1.3 The effects of VCP

It should also be considered that the improvements following the administration of therapeutic intervention, namely VCP, occurs as a result of the attenuation of other

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forms of neuropathology, namely haemorrhage, oedema and neuronal injury. Gennarelli (1996) reported the presence of multiple petechial haemorrhages in the cortex, and that after non-missile head injuries in humans, petechial haemorrhage had been noted in both the frontal and temporal lobes often coinciding with death or a poor prognosis.

The relationship between the micro-vascular response and clinical outcome is unclear. As discussed in Chapter 2, the vascular response at a site of injury is fundamental to the acute inflammatory reaction. Healing is modified by a number of known influences and some unknown ones, frequently impairing the quality and adequacy of both inflammation and repair. These influences include both systemic and local host factors. Systemic influences include the circulatory status of the tissue, which in turn can modulate wound healing. Without adequate blood supply, tissues cannot mount an inflammatory reaction. The vascular phenomena are characterized by increased blood flow to the injured area, resulting mainly from the opening up of capillary beds. Increased vascular permeability results in the collection of protein-rich extra-vascular fluid, which forms the exudates. Plasma proteins leave the vessels, either through widened inter-endothelial cell junctions of the venules, or by direct endothelial cell injury (Ray et al., 2002; Grammas & Ovase, 2001; Grammas, 2000).

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Majno (1998) reported that after day 5 of injury, neovascularisation is maximal in ischaemic organs and cutaneous wounds. Majno (1998) has also reported that it has been well known for many years that necrotic cells elicit inflammatory reactions that serve to eliminate these cells. The molecular basis for this reaction is largely unknown.

New vessels are leaky, allowing the passage of proteins and red blood cells into extra-vascular space, often causing oedema. The significance of neovascularisation in the animals that received VCP suggests that the host is attempting to mount an inflammatory response. While this mechanism remains unclear, it is possible that the administration of VCP inhibits the stimulation of histamine, decreases endothelial cell damage and subsequently reduces vascular permeability and the production of oxygen free radicals. The capacity for VCP to bind heparin and thereby inhibiting chemotaxis of monocytes across endothelial cells and preventing NK cells from killing

endothelial cells, may explain the presence and significance of neovascularisation. Despite the diversity of factors that may participate in the various steps in angiogenesis, cytokines and growth factors released during the injury process would have produced conditions favourable for the stimulation of vascular endothelial growth factor (VEGF).

It further suggests that the administration of VCP, irrespective of injury severity or time period, had a therapeutic benefit on parameters such as oedema and subsequent necrosis. There was a significant reduction in neuronal red degeneration and oedema in the rats who had received VCP prophylactically after cerebral trauma. As previously discussed, activated complement components may stimulate the accumulation of inflammatory cells and the formation of brain oedema. Increased vascular permeability is caused by, among other substances, the anaphylatoxins C3a and C5a, which are split products of the corresponding complement components that stimulate histamine release from mast cells. As a cell associated cytokine response modifier, VCP may bind the third and fourth complement components, subsequently decreasing the pro-inflammatory chemotactic effects of C3a, C4a and C5a.

Significant results on these two parameters have been exhibited by the prophylactic administration of Lazaroid in TBI rats. Durmaz et al. (2003) hypothesized that this could be due to Lazaroid inhibiting increases in lipid peroxidation and stabilizing ATPase. The results of the current study further indicate that the incidence and severity of haemorrhage was directly related to the acute injury of the FPI + saline group.

Less severe haemorrhagic changes were exhibited in both the long term low injury group as well as in the severely injured acute group that received VCP. Petechial haemorrhage in the cortex was common.

As already discussed in Chapter 2, IL-1β has been implicated in an array of pathological and non-pathological processes, including apoptotic cell death, leukocyte-endothelial adhesion, BBB disruption, oedema, astrogliosis and neovascularisation. IL-1β also promotes the proliferation of microglial cells and activates astrocytes resulting in the elevated production of apolipoprotein E. VCP

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may act as an anti-inflammatory agent that inhibits a variety of macrophage responses as well as acting as a suppressor of cytokine response by blocking the expression of TNF and IL-1β. This may enhance synthesis and secretion of their endogenous antagonists. It may further act on endothelial interactions that promote pro-coagulation and inhibit the extravasations of blood cells.

5.1.4 Clinical implications

It is of interest that in this model of FPI, there was no histological evidence to support a contrecoup contusive injury. The evidence of haemorrhage within both cortical and subcortical structures was restricted to the ipsilateral hemisphere receiving the impact. With this model, the unilateral distribution of pathological changes of the brain reflect, in part, both the intensity of the injury and the vectors of force which, in combination, do not appear to cause a contrecoup injury.

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The substantia nigra has a high number of NMDA receptors. The neuronal injury in the substantia nigra is of potential interest clinically because of the Parkinson-like syndrome that is associated with dementia following repeated blows to the head (Semchuk et al., 1993). Since cell loss in the substantia nigra has also been characterized in rodent models of ischaemia, and in aged animals, excitotoxic or other mechanisms may confer selective vulnerability on this region (Shuaib et al., 1992).

Furthermore, there is no histological evidence to show that the administration of VCP worsened brain injury and that, in fact, data supports that the cell loss and damage is significantly reduced, in both time periods in the animals that received VCP.

Although the methods of assessing damage might have missed very subtle changes, it is unlikely that major differences in cell loss and evidence of trauma would have remained unnoticed.

Previous studies by Pillay et al. (2005) and Hicks et al. (2002) showed cognitive improvements as measured by the use of a Morris water maze (MWM) when VCP was used as a therapeutic agent for head trauma in mild and moderate injury. Pillay et al. (2005) also demonstrated that the administration of VCP had a favourable

influence on the motor system of treated WKY rats. Cumulative data shows that early VCP administration has a positive influence on neuronal outcome and on the subsequent sequelae of TBI.

5.2 Discussion of methodology

5.2.1 Methods

An interesting observation was made with regard to the differences in surgical procedures. Some experimental models of TBI indicate that the animals had surgery and underwent recovery on a heating pad as a means of maintaining body temperature between 36.5 and 37.5° C to prevent brain hypothermia. It has been reported that brain hypothermia may act in a neuro-protective manner in animal models (Van der Staay et al., 1996).

The animals in this study were not monitored in this manner. The data obtained indicates that the induced TBI was indeed significant and that the absence of a heating pad throughout surgery and recovery had no bearing on neuro-protection in any of the subgroups. This may be species related.

5.2.2 Limitations

This study formed part of a larger study in which all the animals underwent swim training and regular neurological evaluations on an incline board. Due to the different degrees of severity of induced injury, comparison of data between the acute severely injured 2 week group and that of the mild injury 3 month group was not possible on a time period continuum. Possibly, animals exposed to a mild injury of 0.1-1.1 ATM for a two week period would have served to determine whether the acute response to this injury is similar to that observed in the rats that had been exposed to the severe injury of 2.7-3.0 ATM. Furthermore, Diamond et al. (1985) have reported that studies with rats have shown that the most significant loss of neurons and glial cells occurs early in life and not after the attainment of adulthood. They also reported that there is a thickening of the cortex with training and enrichment, which is to some degree reflected in the structure of the brain.

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Because other studies made use of different strains of rats, generalisation of results should be done with caution.

5.2.3 Future directions

Female rats were deliberately excluded from the study due to the neuroprotection conferred by the influences of oestrogen. Male rats were specifically selected for this study, as research has shown that female sex hormones are acutely neuroprotective in experimental models of traumatic brain injury (Behl et al., 1995). In contrast to the clinical literature, experimental studies have delineated several potential mechanisms for the acute neuroprotective effects of female sex hormones. It remains unknown whether endogenous female sex hormones confer lasting neuroprotection or affect behavioural or histological markers of recovery. It also remains unclear whether female sex hormones have any impact on response and efficacy of treatment paradigms (Roof & Hall, 2000).

The neuroprotective effects of oestrogen have been well documented (Behl et al., 1995) and include maintenance of an auto regulatory function (Pelligrino et al., 1998), as well as a reduction of Aß production (Shi et al., 1998). Oestrogen has also been cited for its effective antioxidant effects (Behl et al., 1995) and reduced excitoxicity with glutamate exposure (Mendelowitsch et al., 2001). The neuroprotective effects of oestrogen have also been implicated with an increased expression of the anti-apoptotic factor bcl2 (Garcia-Segura et al., 1998), and the activation of mitogen-activated protein kinase pathways. (Mize et al., 2003).

Subsequent research has also shown that oestrogen assists in the maintenance of adequate levels of cerebral blood flow after experimental TBI. This may in part be responsible for minimizing other aspects of secondary injury such as oedema production (Roof & Hall, 2000).

In contrast to the clinical literature, recent studies suggest that females may be at risk for poorer long term outcomes of TBI (Farace & Alves, 2000). It may be of value to compare, under similar conditions, the above data, using female rats as a means of

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delineating the role of sex hormones on mechanisms of recovery and response to intervention after TBI.

Future research should examine the best method of delivering functional VCP to the brain. Studies using more time intervals would serve to evaluate the histological progression of changes in response to VCP over time. Lastly, in light of the reported histo-morphological changes, the possibility of directly measuring complement activation and interleukins release may serve to discriminate the relative roles of these agents.

This study administered a lower dose of VCP than other studies. Further studies comparing the histological effects of different dosages of VCP would assist in determining the optimal dose.

Lastly, comparing the effects of VCP on different degrees of severity of injury may be of importance.

5.3 Implications of the study

Analysis of the data shows a significant positive outcome for the FPI + VCP animals on the parameters investigated. This includes a marked reduction in oedema, haemorrhage and necrosis, with a significant increase in vascularisation. The occurrence of oedema in the 3 month group of animals compared to the 2 week group may be related to the half life of the VCP.

Presented data indicates that the administration of VCP offers some neuroprotective and/or neuroregenerative benefits in TBI and could be exploited as a novel therapy in attenuating the cascade of devastating events following brain injury, and that it may be considered as an alternative approach to manipulating the inflammatory response.

5.4 Conclusion

Traumatic brain injury is a major public health problem, yet receives little support or attention from the media or health care policy makers. The lack of attention to the

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consequences of TBI may be due to the lack of scientific, understandable information on the neuropsychological sequelae.

It would be ideal if the cognitive impairments, psychosis, depression, aggression and agitation after TBI could be controlled without medications. Diseases of the brain present a challenge for therapeutic interventions not only because the brain is an anatomically and functionally complex structure, but also because few molecules are able to cross the blood-brain barrier. While viral vectors used in gene therapy have become alternative and adjuvant options to pharmacological intervention in neurological diseases, these therapies are not without obstacles of their own.

As discussed in Chapter 2, due to the similarities in cranial circulatory anatomy, this study used an animal model, viz. that of the rat, that closely resembles the human condition of mild and severe head trauma to test a strategy targeted at attenuating the complement system by the use of VCP.

The presented data suggests that VCP may indeed offer some attenuation of non-specific neurological impairments, thus positively influencing spatial learning, memory and behavioural deficits that are exhibited in individuals who are victims of TBI. It may assist in conceptualising the therapeutic and neuro-protective or neuro-regenerative effects of VCP as a viable means of aiding comprehensive rehabilitation.

Previous studies undertaken by Durmaz et al. (2003), who administered Lazaroid (U-83836E) prophylactically after cerebral trauma in rats also showed evidence in the reduction of oedema and neuronal necrosis.

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APPENDICES

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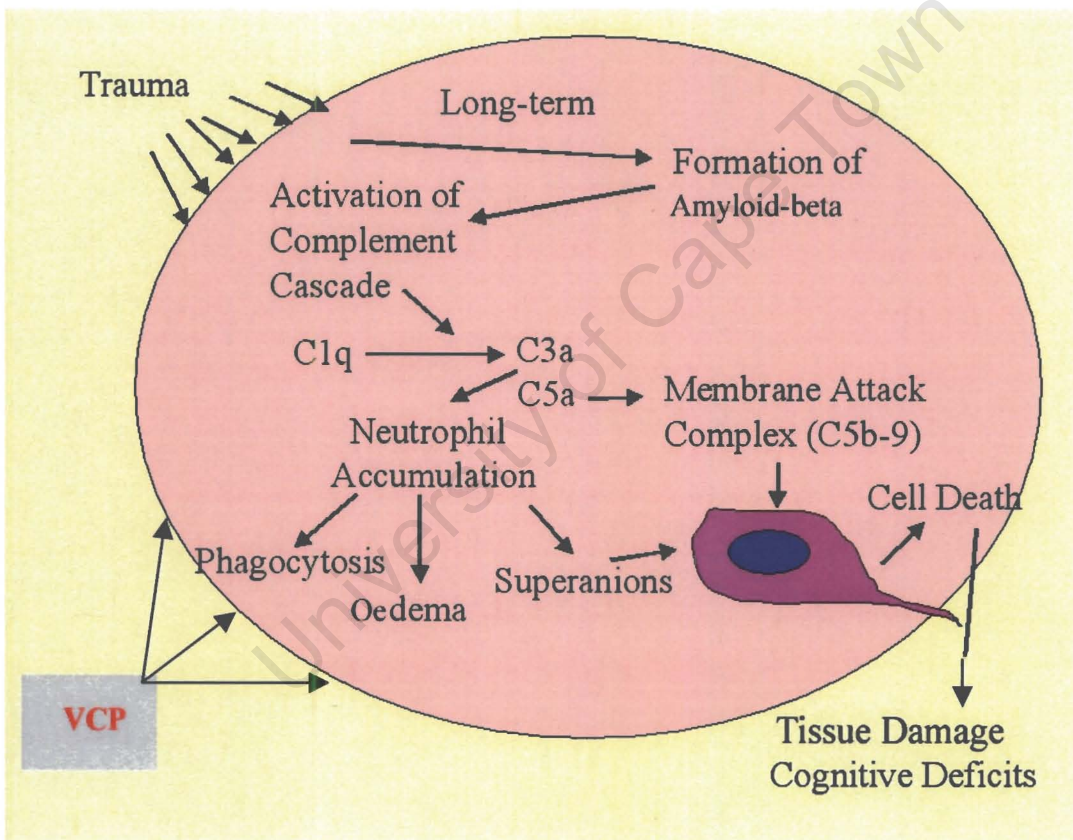
Appendix A: **Diagrammatic representation of the events following brain trauma, leading to the activation of the complement cascade and possible formation of Aβ, which in turn leads to further activation of complement. Ultimately, activation of the complement system binding to the first complement subunit (C1q) or the third subunit (C3b) may contribute to cell death and cognitive dysfunction. VCP is a potent inhibitor of the complement cascade and may attenuate the long term consequences of TBI.**



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Appendix B: Reagents and chemicals used in study design

Equithesin

Equithesin is no longer commercially available, and was prepared in the following manner:

Method

- Dissolve 8.5 mg of chloral hydrate in 20ml 95-100% ethanol.
- Add 1.96g pentobarbital (Nembutal=50mg/ml) and 4.25g $MgSO_4$.
- Once the above ingredients are in solution, add 60ml propylene glycol.
- Bring to a total volume of 200ml with distilled water.

Dose for rats is 1ml/300g IP.

10% Buffered Formalin

The 10% buffered formalin consisted of:

Formaldehyde	200ml
$Na_2HPO_4.H_2O$	15g
$NaH_2PO_4.2H_2O$	8g
Distilled water	2000ml

Physiological Saline

NaCl	9g
Distilled water	1000ml

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Appendix C Perl's Prussian blue reaction for ferric iron

Treatment with an acid ferrocynanide solution will result in the unmasking of ferric iron in the form of the hydroxide, Fe $(OH)_3$, by dilute hydrochloric acid. The ferric iron then reacts with a dilute potassium ferrocyanide solution to produce an insoluble blue compound, ferric ferrocyanide.

Fixation

Avoid the use of acid fixatives. Chromates also interfere with the preservation of iron.

Sections

Works well on all types of sections, including resin sections.

Ferrocyanide solution:

- 1% aqueous potassium ferrocyanide 20ml
- 2% aqueous hydrochloric acid 20ml
- Prepare the solution fresh just before use.

Method

1. Take sections to water.
2. Treat sections with freshly prepared acid ferrocyanide solution for 10-30 min. (Depending on the amount of ferric iron present, it may be necessary to vary the staining times).
3. Wash well in distilled water.
4. Lightly stain the nuclei with 0.5% aqueous neutral red solution.
5. Wash rapidly in distilled water.
6. Dehydrate, clear and mount.

Results

Ferric iron	blue
Nuclei	red

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Appendix D: PTAH stain for Astrocytes

Fixation

Formalin

Sections

Paraffin, 5-10μm

Solutions

PTAH (naturally ripened)

Haematoxylin	1g
Phosphotungstic acid	20g
Distilled water	1000ml

Permanganate

Potassium permanganate	1g
Distilled water	100ml

Oxalic acid

Oxalic acid	5g
Distilled water	100ml

Method

1. Take sections to water.
2. Mordant sections in Zenkers' fixative for 60 mins at 50° C.
3. Wash in running tap water, 15 mins.
4. Place in Lugol's iodine, 15 mins.
5. Decolourise in 95% alcohol for 60 mins.
6. Wash well in distilled water.
7. Oxidise in permanganate solution, 5 mins.
8. Decolourise in oxalic acid solution, 5 mins.

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9. Stain in PTAH solution 12-24 hours at room temperature.
10. Rinse rapidly in three changes of absolute alcohol.
11. Clear in xylene and mount.

Results

Astrocyte fibrils	blue
Nuclei	blue
Myelin	blue
Neurons	pink

Note: The mordanting in a mercuric fixative, such as Zenker's, enhances staining of glial fibrils.

Zenker's fluid

Distilled water	950ml
Potassium dichromate	25g
Mercuric chloride	50g
Glacial acetic acid	50g

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Appendix E: Cresyl fast violet (Nissl) stain for paraffin sections

Fixation

Alcohol, Carnoy's fluid or formal saline.

Sections

Paraffin 7-10μm

Preparation of stain

Cresyl fast violet	0, 5g
Distilled water	100ml

Differentiation solution

Glacial acetic acid	250μl
Alcohol	100ml

Method

1. Dewax sections and bring to water.
2. Cover with filtered cresyl fast violet; stain for 10 min.
3. Rinse in distilled water.
4. Differentiate in 0.25% acetic alcohol until most of the stain has been removed.
5. Briefly pass through absolute alcohol into xylene and check microscopically.
6. Repeat steps 5 and 6 if necessary.
7. Rinse well in xylene and mount.

Results

Nissl substance	purple-dark blue
Neurons	pale purple-blue
Cell nuclei	purple blue

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Appendix F: Immunocytochemical detection of GFAP, CD68 and β-amyloid by means of an indirect two-step visualisation system.

Immunocytochemical Protocols

Antibody	Antibody Supplier	Recommended retrieval	Optimised Retrieval	Retrieval Time & Method	Dilution of 1° Antibody
GFAP	Dakocytomation	None / HIER pH 6.0	HIER TRIS EDTA pH9.0	2 mins. Pressure cooker	1:1000 30 mins RT
CD 68	Dakocytomation	Enzyme	HIER TRIS EDTA pH9.0	2 mins. Pressure cooker	1:50 30 mins RT
Beta 4-Amyloid	Dakocytomation	None	Conc. Formic Acid	3 mins at R.T	1:50 30 mins RT

Summary and Explanation of visualisation system

Bond Polymer Refine Detection is a biotin-free, polymeric horseradish peroxidase (HRP)-linker antibody conjugate system for the detection of tissue-bound mouse and rabbit IgG and some mouse IgM primary antibodies. It is intended for staining sections of formalin-fixed, paraffin-embedded tissue on the Bond automated system.

Immunocytochemical techniques can be used to demonstrate the presence of antigens in tissues and cells. Bond Polymer Refine Detection utilises a novel controlled polymerisation technology to prepare polymeric HRP-linker antibody conjugates. The detection system avoids the use of streptavidin and biotin, and therefore eliminates non-specific staining as a result of endogenous biotin.

Bond Polymer Refine Detection works as follows:

- The specimen is incubated with hydrogen peroxide to quench endogenous peroxidase.
- The user-supplied specific primary antibody is applied.

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- A post primary antibody solution enhances penetration of the subsequent polymer reagent.
- A poly-HRP anti-mouse/rabbit IgG reagent localises the primary antibody.
- The substrate chromogen, 3, 3' diaminobenzidie (DAB), visualises the complex via a brown precipitate.
- Haematoxylin (blue) counterstaining allows the visualisation of cell nuclei.

Reagents

- Peroxide block (3.0%).
- Post primary Polymer penetration enhancer containing 10% (v/v) animal serum in Tris-buffered saline and 0.09% ProClin™ 950.
- Polymer Poly-HRP anti-mouse/rabbit IgG containing 10% (v/v) animal serum in Tris-buffered saline and 0.09% ProClin™ 950.
- DAB Part 1, 66mM 3, 3'-diaminobenzidine tetrahydrochloride, in a stabiliser solution.
- DAB Part 2, 0.05% (v/v) Hydrogen peroxide in a stabiliser solution. (Applied twice).
- Haematoxylin 0.02%.

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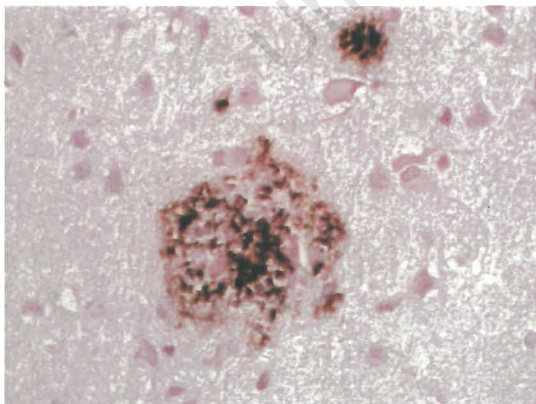

High power micrograph of a senile plaque showing a dense core of amyloidal deposition.
Human positive control: Beta-4 amyloid plaques by immunocytochemical detection.
Courtesy of archived control blocks: Drs Dietrich, Voigt and Mia. Pathcare Laboratories.

Appendix G: Alkaline Congo red technique

This method obviates the need for a differentiation step by the inclusion of a high concentration of sodium chloride; this reduces background electrochemical staining whilst enhancing hydrogen bonding of Congo red to amyloid, resulting in a progressive and highly selective technique. The solutions must be freshly made prior to use.

Fixation

Not critical

Stock solution A

Saturated sodium chloride in 80% ethanol.

Stock solution B

Saturated Congo red in 80% ethanol saturated with sodium chloride.

Stock solution C

1% aqueous sodium hydroxide.

Working solutions

To 100ml of stock A add 1ml of 1% aqueous sodium hydroxide and filter.

To 100ml of stock B solution add 1ml of 1% aqueous sodium hydroxide and filter.

Method

1. Take sections to water.
2. Stain nuclei in alum haematoxylin, differentiate and blue.
3. Immerse in alkaline sodium chloride solution for 20 min.
4. Transfer directly to the alkaline Congo red solution for 20 min.
5. Rinse briefly in alcohol, clear and mount.

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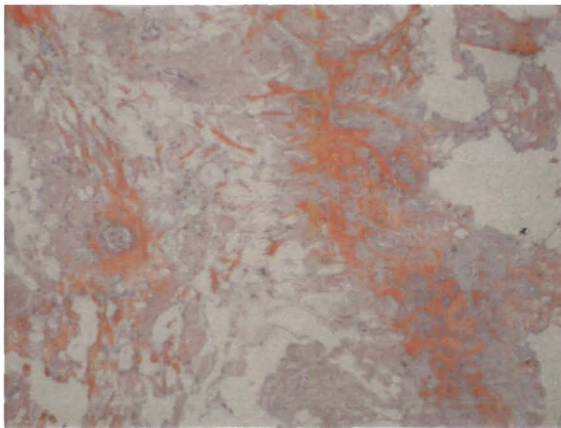
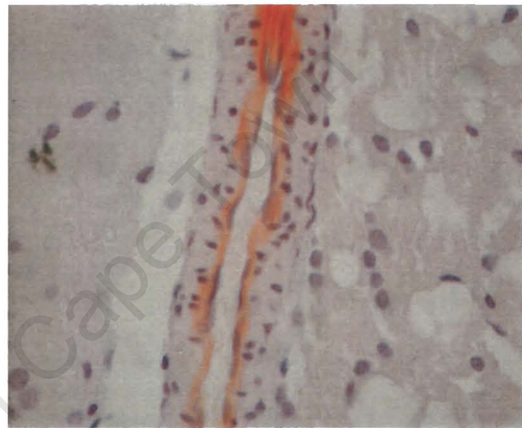
Results

Amyloid, elastic fibres, eosinophil granules	red
Nuclei	blue

Note: It is essential to make use of a positive control with all test sections.



A.



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(A) Human positive control for Congo red stain. Courtesy of archived control blocks: Drs Dietrich, Voigt and Mia. Pathcare Laboratories.

(B) Normal internal control of elastic fibres for Congo red stain seen in the rat brain.

Appendix H: Spreadsheet of raw data

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Rat no	Group	Time	Condition	DAI	Neuronal change	Necrosis	Haemorrhage	Oedema	Neovascu-larisation
1	2W-I-V	2 weeks	FPI + VCP	2	1	0	1	0	1
2	2W-I-V	2 weeks	FPI + VCP	1	1	0	1	0	1
3	2W-I-V	2 weeks	FPI + VCP	1	1	0	1	0	1
4	2W-I-V	2 weeks	FPI + VCP	1	1	0	1	0	1
5	2W-I-V	2 weeks	FPI + VCP	1	1	0	1	0	3
6	2W-I-V	2 weeks	FPI + VCP	2	1	0	1	0	2
7	2W-I-S	2 weeks	FPI + saline	3	3	3	3	2	1
8	2W-I-S	2 weeks	FPI + saline	3	3	3	3	2	0
9	2W-I-S	2 weeks	FPI + saline	3	3	3	3	2	0
10	2W-I-S	2 weeks	FPI + saline	3	3	2	3	2	1
11	2W-I-S	2 weeks	FPI + saline	3	3	2	3	2	0
12	2W-I-S	2 weeks	FPI + saline	3	3	3	3	2	0
13	2W-S-V	2 weeks	Sham + VCP	0					
14	2W-S-V	2 weeks	Sham + VCP	0					
15	2W-S-V	2 weeks	Sham + VCP	1					
16	2W-S-V	2 weeks	Sham + VCP	1					
17	2W-S-V	2 weeks	Sham + VCP	0					
18	2W-S-V	2 weeks	Sham + VCP	1					
19	2W-S-S	2 weeks	Sham + saline	0					
20	2W-S-S	2 weeks	Sham + saline	0					
21	2W-S-S	2 weeks	Sham + saline	0					
22	2W-S-S	2 weeks	Sham + saline	0					
23	2W-S-S	2 weeks	Sham + saline	0					
24	2W-S-S	2 weeks	Sham + saline	0					

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1	3M-I-V	3 months	FPI + VCP	0	1	0	1	1	1
2	3M-I-V	3 months	FPI + VCP	0	1	0	1	1	0
3	3M-I-V	3 months	FPI + VCP	0	1	0	1	1	1
4	3M-I-V	3 months	FPI + VCP	1	1	0	1	1	0
5	3M-I-V	3 months	FPI + VCP	1	1	0	1	1	0
6	3M-I-V	3 months	FPI + VCP	0	1	0	1	1	0
7	3M-I-S	3 months	FPI + saline	2	2	0	2	2	1
8	3M-I-S	3 months	FPI + saline	2	2	0	1	2	1
9	3M-I-S	3 months	FPI + saline	1	1	0	1	1	0
10	3M-I-S	3 months	FPI + saline	1	2	0	2	1	1
11	3M-I-S	3 months	FPI + saline	0	1	0	1	1	1
12	3M-I-S	3 months	FPI + saline	2	2	0	2	2	2
13	3M-S-V	3 months	Sham + VCP	0					
14	3M-S-V	3 months	Sham + VCP	0					
15	3M-S-V	3 months	Sham + VCP	1					
16	3M-S-V	3 months	Sham + VCP	1					
17	3M-S-V	3 months	Sham + VCP	1					
18	3M-S-V	3 months	Sham + VCP	0					
19	3M-S-S	3 months	Sham + saline	0					
20	3M-S-S	3 months	Sham + saline	0					
21	3M-S-S	3 months	Sham + saline	0					
22	3M-S-S	3 months	Sham + saline	0					
23	3M-S-S	3 months	Sham + saline	0					
24	3M-S-S	3 months	Sham + saline	0					

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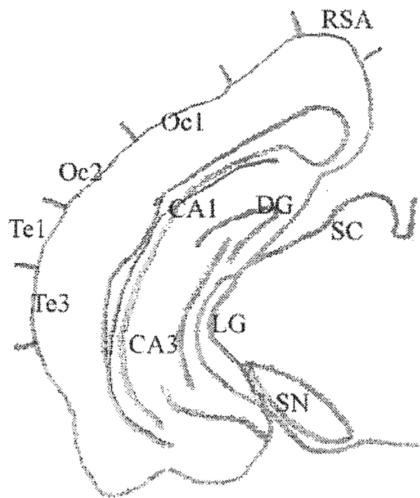
Appendix I: Diagrammatic representation of location of TBI.



I.1. Diagrammatic representation of the layers of the brain

RSA: Retrosplenial agranular cortex; Oc1: primary visual cortex; Oc2: secondary visual cortex; Te1: primary auditory cortex; Te3: association auditory cortex; CA1 and CA3: pyramidal cell layer; DG: dentate gyrus; LG: Lateral geniculate body; SC:superior colliculus; SN: substantia nigra.

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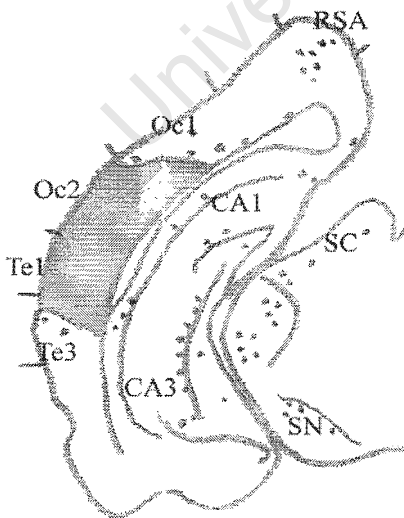
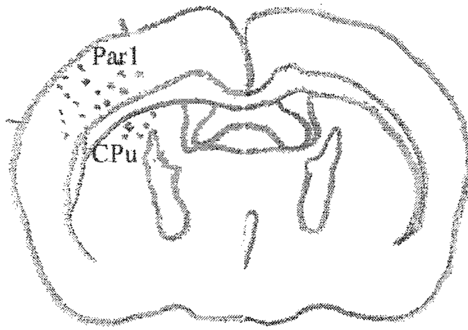


I.2. Diagrammatic representation of the two week acute injury group. Shaded area represents the site of the majority of the injury



I.3. Diagrammatic representation of the three month chronic injury group. Par1 indicates the site of the majority of the injury

Par1: secondary parietal cortex; CPu: caudate putamen.

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