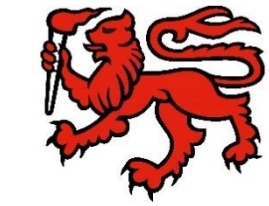

Investigating the Therapeutic Efficacy of Non-anticoagulant Fractions (Dp2 and Dp4) of Enoxaparin following Experimental Traumatic Brain Injury

by

Mimieveshiofuo E. Aiyede

B.Sc, MRes

School of Pharmacy and Pharmacology

University of Tasmania

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Doctor of Philosophy

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So then it is not of him that willeth, nor of him that runneth,

but of God that sheweth mercy.

- **Romans 9:16 (KJV)**

Declaration of Originality

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Statement of Ethical Conduct

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University.

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List of Abbreviations

AFXa	Anti-factor Xa
AG-1	Arginase-1
ASM	Airway smooth muscle
ATP	Adenosine triphosphate
AQP4	aquaporin-4
BAX	Bcl-2 associated X protein
BBB	Blood brain barrier
Bcl-2	B-cell lymphoma-2
C3	Complement component 3
CA1	Cornu ammonis-1
CA3	Cornu ammonis-3
Ca^{2+}	Calcium
CBF	Cerebral blood flow
CCI	Controlled cortical impact
CCL11	C-C motif chemokine ligand-11/eotaxin-1 (eosinophil chemotactic protein)
CCL2	chemokine ligand-2
CCL5	chemokine ligand-5
CD3	T-cell marker

CDC	Centre for Disease Control
CDK5	Cyclin-dependent kinase-5
CE	Capillary electrophoresis
CHI	Closed head injuries
CK	creatinine kinase
CNS	Central nervous system
COX-2	Cyclooxygenase-2
CRP	C-reactive protein
CSF	Cerebrospinal fluid
CT	Computed tomography
DAI	Diffuse axonal injury
Dlk1	Delta-like homologue-1
DNA	Deoxyribonucleic acid
Dp2	Degree of polymerization - 2
Dp4	Degree of polymerization - 4
DSS	Dextran sulfate sodium
DVT	Deep venous thrombosis
ED	Emergency department
EPO	Erythropoietin

EPOR	Erythropoietin receptor
FIZZ1	Found in inflammatory zone-1
FPI	Fluid percussion injury
GCS	Glasgow Coma Scale
G-CSF	Granulocyte colony-stimulating factor
GDNF	Glial cell line derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GAT4	GABA transporters-4
$GABA_A$	Gamma (γ)-amino-n-butyric acid
$GABA_AR$	Gamma (γ)-amino-n-butyric acid receptor
HIF-1α	Hypoxia-inducible factor-1 alpha
HMG	3-hydroxy-3-methylglutaryl
HMG CoA	3-hydroxy-3-methylglutaryl reductase coenzyme A
HMGB1	High mobility group box 1
HP-SEC	High-performance size exclusion chromatography
HSP	Heat shock protein
IBA-1	Ionized calcium binding adaptor molecule-1
IC	Ion-exchange chromatography

ICAM-1	Intercellular adhesion molecule-1
ICP	Intracranial pressure
ICU	Intensive care unit
IFN-γ	Interferon gamma
IL-1β	Interleukin-1 beta
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-13	Interleukin-13
IL-15	interleukin-15
iNOS	inducible nitric oxide synthase
IP	Intraperitoneal
K^+	Potassium ion
kDa	Kilodaltons
KC	Keratinocyte chemoattractant
LDL	Low-density lipoprotein
LFPI	Lateral fluid percussion injury
LMWHs	Low molecular weight heparins

LOC	Loss of consciousness
MAP2	Microtubule-associated protein-2
mbTBI	Mild blast-induced traumatic brain injury
MCP-1	Monocyte chemoattractant protein
MIP-2	Macrophage inflammatory protein-2
Mg^{2+}	Magnesium
MHC	Major histocompatibility complex
MMP-9	Matrix metalloproteinase-9
MRI	Magnetic resonance imaging
mTBI	Mild traumatic brain injury
mTOR	Mechanistic target of rapamycin
MWM	Morris water maze
Na^{+}	Sodium ion
NeuN	Mature neuron marker
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
NICD	Notch intracellular domain
NIMP-R14	Marker for neutrophils
NMDA	*N*-methyl-d-aspartate
NO	*Nitric oxide*

NPCs	Neural progenitor cells
NRs	Nuclear hormone receptors
NR2B	*N*-methyl-d-aspartate receptor subunit
Nrf2	Nuclear factor erythroid-2-related factor-2
NSS	Neurological Severity Score
OCT	Optimal cutting temperature
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PFA	Paraformaldehyde
p-JAK2	Phosphorylated Janus kinase-2
p-STAT3	Phosphorylated signal transducer and activator of transcription-3
PMN	Polymorphonuclear neutrophil
PPAR	Peroxisome proliferator-activated receptor
PPAR-α	Peroxisome proliferator-activated receptor alpha
PPAR-δ	Peroxisome proliferator-activated receptor delta
PPAR-γ	Peroxisome proliferator-activated receptor gamma
PR	Progesterone receptor
PTA	Post-traumatic amnesia
rhEPO	Recombinant human erythropoietin

RNS	Reactive nitrogen species
RPIP-HPLC	Reversed-phase ion pair high-performance liquid chromatography
ROS	Reactive oxygen species
RTC	Road traffic collision
SAH	subarachnoid haemorrhage
sAPPα	Soluble forms of amyloid precursor protein-alpha
SCID	Severe combined immunodeficient
SC	Subcutaneous
SMC	Smooth muscle cell
SNAP5114	GABA transport inhibitor
SNpc	Substantia nigra pars compacta
SOD-1	Superoxide dismutase-1
SOD-2	Superoxide dismutase-2
TBARS	Thiobarbituric acid-reactive substances
TBI	Traumatic brain injury
TGF-β	Transforming growth factor beta
TLR-9	Toll-like receptor-*9*
TNF-α	Tumour necrosis factor alpha
UFH	Unfractionated heparin

UK	United Kingdom
US	United states
WDI	Weight drop injury
WHO	World Health Organisation
Xa	Activated factor X

Conference abstracts

- **Aiyede M.**, Lim XY., Guven N., Patel R., Bye N. (2019) Dp4, non-anticoagulant fraction of Enoxaparin: A potential anti-inflammatory treatment for Traumatic Brain Injury (TBI). 10th Australian Neurotrauma Workshop, Alfred Centre, Melbourne, VIC, Oct 2019; Oral presentation.

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- Sumargo N., Wang L., Chen H., **Aiyede M.**, Guven N., Bye N. (2018) Neuroprotective potential of the antioxidant idebenone following focal traumatic brain injury in mice. Australian Neuroscience Society 38th Scientific Meeting, Brisbane Convention and Exhibition Centre, QLD, Dec 2018; poster presentation.

- **Aiyede M.**, Lim XY., Guven N., Patel R., Bye N. (2018) Investigating the therapeutic efficacy of Dp4 following experimental Traumatic Brain Injury (TBI). University of Tasmania Annual 12th Graduate Research Conference, Hobart, TAS, Sept, 2018; Poster presentation.

Abstract

Traumatic brain injury (TBI) is a debilitating neurological disorder that is fast becoming a global public health problem. It is prevalent among the active population (<45 years of age) and results from external forces encountered due to falls, explosions, bomb blasts, accidents, or sports-related collision, leading to brain dysfunction and neurodegeneration in most cases. With the increasing global incidence of TBI, it has been estimated that 69 million people worldwide will suffer from TBI yearly.

TBI triggers a cascade of events that are both primary and secondary injury mechanisms. While primary injury comprises the direct tissue damage from the impact, secondary injury occurs minutes, hours, or days after the initial impact. Neuroinflammation, a crucial component of the secondary injury mechanisms, contributes to ongoing neuronal degeneration and poor outcome following TBI. Trauma elicits neuroinflammatory responses including disruption of blood brain barrier (BBB), increased activation of microglia and astrocytes, infiltration of peripheral immune cells, and elevated levels of inflammatory cytokines/chemokines, as well as other neurotoxic molecules like reactive oxygen species. While all these responses on one hand are necessary for host defence and repair following injury, on the other hand, over time, they can become detrimental leading to further brain damage. Thus, it is now well established that neuroinflammation displays dual roles in the pathophysiology of TBI.

Despite extensive preclinical and clinical research into identifying and developing treatment for TBI, to date, there is no effective neuroprotective treatment available, as promising candidates have failed to replicate beneficial results in the clinic. With the suppression of deleterious neuroinflammatory processes linked to better outcomes in experimental TBI, drugs targeting the inflammatory cascade are being investigated as potential new therapies. Heparin and low molecular weight heparins (LMWHs), such as enoxaparin, have exhibited potential neuroprotective properties following administration in both clinical and experimental TBI, with studies showing reduced inflammation, decreased brain oedema, attenuated motor function impairment, and also ameliorated cognitive and neurological dysfunction. Although the exact mechanism underlying this remains unclear, there is evidence that the beneficial effects demonstrated by both heparin and enoxaparin are associated with their anti-inflammatory properties, which are separate from their well-known anticoagulant effects. However, despite these potential beneficial effects, treatment with heparinoids is often delayed or withheld from TBI patients in the clinic, due to an increased risk of cerebral

haemorrhage. Importantly, the recently isolated and characterised di-saccharide and tetra-saccharide fragments of enoxaparin, Dp2 and Dp4, respectively, have robust anti-inflammatory effects but no anticoagulant activity. In *in-vitro* and *ex-vivo* studies, Dp2 and Dp4 inhibited the release of pro-inflammatory cytokines from human pulmonary epithelial cells and peripheral blood mononuclear cells, respectively. However, unlike enoxaparin, Dp2 and Dp4 do not have anticoagulant activity and would therefore pose no risk of bleeding to TBI patients. Therefore, in this thesis, I evaluated the effect of enoxaparin, Dp2 and Dp4 on acute neuroinflammation and long-term functional outcome following experimental TBI, with the hypothesis that Dp2 and Dp4 will significantly decrease the levels of neuroinflammation and improve recovery following TBI.

Firstly, I investigated the effect of enoxaparin and Dp4 on gliosis in the injured brain. I hypothesised that treatment would decrease the accumulation of activated astrocytes and microglia in the pericontusional cortex. To address this, 10–14-week-old mice were subjected to a moderate focal TBI induced by the controlled cortical impact injury model, while sham animals underwent surgery only. Enoxaparin, low and high doses of Dp4, or saline (vehicle) were administered either through continuous subcutaneous (SC) infusion or multiple intraperitoneal (IP) injections over 3 days post-TBI. Mice were killed at 3 days post-TBI and their brains were collected for immunohistochemistry using antibodies to detect GFAP, IBA-1 and CD68, to quantify astrocytes and microglia/macrophages in the pericontusional cortex. Our results demonstrated that irrespective of dose and mode of administration, enoxaparin and Dp4 did not attenuate astrocyte and microglial gliosis following experimental TBI.

To further explore the effect of these drugs on neuroinflammation, I measured the levels of pro- and anti-inflammatory cytokines and chemokines in the injured cortex. Additional cohorts of mice of the same experimental groups described above were killed at 6 hours post-TBI and protein levels of 23 cytokines and chemokines were quantified in the pericontusional cortex using BIO-RAD Bio-Plex Pro assay. As expected, I found that inflammatory mediators including IL-6, IL-1, MCP-1, G-CSF, KC, MIP-1α and RANTES, were increased after trauma; however, their levels were not reduced by enoxaparin or Dp4, regardless of mode of drug delivery. Interestingly, the levels of T-cell related cytokines, MIP-1β and IL-9, were significantly reduced by IP injection, but not SC infusion, of enoxaparin. These results suggest that while some components of neuroinflammation are not affected, treatment with enoxaparin may decrease recruitment of T-cells into the contused brain. Since

infiltrated T-cells can contribute to poor outcomes post-TBI, by blocking them, enoxaparin could potentially lead to neuroprotection.

Consequently, I undertook another study to investigate the long-term effects of treatment on functional outcomes after trauma. Here, I introduced Dp2 and the combination of Dp2 and Dp4 (Dp2+Dp4) as treatment options, to explore if both drugs together could have an additive effect, since earlier *in vitro* studies showed they suppressed the production of different pro-inflammatory cytokines in peripheral immune cells. Following CCI, mice received repeated SC injections of enoxaparin, Dp2, Dp4, Dp2+Dp4 or vehicle (saline) and behaviour was assessed on the ledged beam, hanging wire and open field at multiple timepoints across four weeks post-TBI. Our results revealed that none of the treatments ameliorated the motor function deficits that were observed for up to 7 days after trauma on the ledged beam. Also, there were no differences between the performances of the injured animals and sham animals on both the hanging wire task and the open field arena, so no effect of treatment on muscle strength or anxiety could be assessed.

Together, the results from these experimental studies were surprising, since it has been reported that heparin and enoxaparin have numerous beneficial effects after trauma. Therefore, to gain a more detailed insight into the efficacy of these drugs in the treatment of TBI, and to better understand our findings within this context, we performed a systematic review and meta-analysis on the available literature evaluating the potential neuroprotective effects of unfractionated heparin and LMWHs following experimental TBI. Given the relatively small number of publications, all studies reporting the effect of unfractionated heparin, desulfated heparin and LMWHs (enoxaparin and dalteparin) in experimental TBI were included irrespective of outcomes measured. Our results identified 11 studies published between 2000 and 2020, from which 23 outcomes were extracted. A meta-analysis was conducted on only two outcomes: brain oedema and neurologic function, because they had the highest number of comparisons and were more likely to best answer our research questions. SYRCLE's risk of bias tool and a modified checklist from CAMARADES assessed study quality. Both tools revealed that while most of the included studies described baseline characteristics and documented group randomization, blinded outcome assessment or detection bias were not reported. Meta-analysis indicated that heparin and enoxaparin decreased brain oedema and improved neurologic function. While subgroup analysis found that there were no differences between the drug types, drug dose could have a small influence on decreased brain oedema, as overall heterogeneity was slightly reduced following subgroup analysis. However, due to

insufficient data, subgroup analysis was not performed for neurologic function outcome. Furthermore, trim and fill analysis suggest that study heterogeneity, and not necessarily publication bias, accounted for funnel plot asymmetry. Overall, this review demonstrated that heparin and enoxaparin decreased brain oedema and improved neurologic function albeit with high levels of heterogeneity between studies. Individual studies also reported the beneficial effects of both drugs in other outcomes including reactive gliosis, apoptosis, and brain infarct volume. However, the findings from this review should be interpreted with caution as it was based on a very few numbers of studies whose study quality were mostly fair.

In conclusion, the studies in this thesis contribute to the body of research that is investigating the potential therapeutic effects of enoxaparin after trauma and suggest that its beneficial actions may not include attenuating glial activation. Furthermore, for the first time, the therapeutic effect of non-anticoagulant fractions of enoxaparin, Dp2 and Dp4, were evaluated in the brain, and no beneficial effects of these drugs were detected. However, based on the range of therapeutic actions attributed to heparin and enoxaparin following experimental TBI in the literature, further studies exploring different doses and pharmacodynamics of these drugs are warranted to more fully determine their potential as a pharmacotherapy for TBI.

CHAPTER 1

INTRODUCTION

1.1 Traumatic Brain Injury

Traumatic Brain Injury (TBI) has become a serious global health and socio-economic problem. It is sometimes referred to as a "silent epidemic" due to the common underestimation of its severities (1). TBI is one of the leading causes of death and disability among the young population of less than 45 years and it has been estimated that 69 million people worldwide will suffer from TBI yearly due to its increasing global incidence (2).

TBI is caused from an external physical force or blow to the head due to falls, motor vehicle accidents, industrial accidents, bomb explosions, or sport-related activities/injuries, that result in the damage and dysfunction of the brain (3, 4). Injuries to the head resulting in TBI can be broadly characterized into closed head injuries and open or penetrating head injuries. The closed head injuries are injuries where the dura is undamaged, while penetrating injuries include a compromised skull and dura (1).

1.2 Causes of TBI

The causes of TBI are diverse and vary greatly by country as a result of a number of factors, which include modernization as well as the economic status of the country (5). In 2007, Hyder et al. reported that the three leading causes of TBI in the United States (US) were road traffic accidents, violence and falls which accounted for 62%, 24% and 8% respectively (5). However, in 2010, the Centre for Disease Control (CDC) reported that falls were the leading cause of TBI and motor vehicle traffic injury was the primary cause of TBI-related deaths in the US (6, 7). In 2004-2005, TBI were diagnosed as either the principal (62.5%) or as an additional diagnosis (26.1%) and as a principal diagnosis, falls were also reported to be the most common cause of TBI in Australia, followed by transportation and assault (8). However, motor collisions were identified as the main cause of TBI in Australia and New Zealand following a 6 months prospective study of major trauma centres (9). Other causes of TBI include struck by/against, assaults, and combat injuries commonly found among military personnel (10, 11).

1.2.1 Falls

Falls are one of the most common causes of TBI, accounting for approximately 47.2% of all TBIs annually within the general population especially among young children and the elderly in the US (11). The CDC reported that in 2013, the older population of ≥75 years with

an estimate of 1,859 per 100,000 had the highest rate of TBI-related falls followed by children aged 0-4 years with an approximate rate of 1,119.3 per 100,000 in 2016 and 2017 respectively (11, 12). The Australian Institute of Health and Welfare also reported that in 2004-2005, falls accounted for 42% of injury cases with TBI as a principal diagnosis in Australia (8). In the same report, the rate of TBI-related falls was recorded to have increased by over 50% from the 60-64 years age group to the over 85 years age group. This increase can probably be attributed to the increased average human life expectancy in most developed countries (13).

1.2.2 Road Traffic Collisions

Road traffic collisions include collisions involving cars, motorbikes/bicycles and pedestrians, and were the main cause of TBI related deaths especially among young adults aged 20-24 years in the US (6). Meanwhile, following falls, over 29% of TBI as principal diagnosis was due to transport-related injuries in Australia (8). Over the years, declining rates of road traffic collisions in most industrialized countries, are most likely due to increased road safety awareness programs. These includes regulations for the use of helmets and seatbelts, campaigns against driving under the influence of alcohol, improved vehicle safety measures and innovations like airbags (14-16). Also, electronic stability control systems in cars (17) as well as the implementation of legislative policies and schemes directed at drivers are aimed at reducing the TBI related death rate (11, 18). Nevertheless, road traffic collisions remain the leading cause of TBI-related deaths and disabilities in low and middle income countries due to the increased use of cars, bad or poor road quality and networks, poor implementation of road safety regulations as well as unavailability of adequate healthcare facilities and services (2, 19-21).

1.2.3 Struck by/against events

Struck by/against events are defined by the CDC as injuries sustained from hitting a moving or stationery object such as in sports activities or workplace related injuries (14, 22). It is the second most leading cause of TBI in the US particularly in young adults, and has gained a lot of interest lately due to the increased media awareness of sports related concussions (11). The rate of sport-related TBI/concussions is increasing and they are often linked to a number of specific sports activities such as football, wrestling, cycling, equestrian, soccer, basketball (23).

1.2.4 Self-inflicted/Suicide

Self-inflicted/Suicide has recently become the leading cause of TBI-related deaths in the US with a sum total of 18,321 deaths recorded in 2013 (22). In 2008, 2,191 deaths were reported in Australia from suicide and 78% of cases were males with the highest suicide death rate of 26.4 per 100,000 in the 40-44-years age group (24).

1.2.5 Assaults

Assaults encompass violence including domestic violence as well as abusive head traumas (25). It is the third cause of TBI in Australia accounting for 14% of TBI as principal diagnosis between 2004-2005 (8). A CDC report in 2015 listed a total of 232,842 TBI cases in 2010 including emergency department (ED) visits, hospitalizations and deaths result from assaults (22), which is a significant increase from the previous estimate in 2002-2006 of 169,625 per year which accounted for approximately 10% of all TBIs (6). It is worth noting that this comparison is between studies that are 4-8 years apart.

1.2.6 Others/Unknown causes

TBI can also result from unknown/unspecified causes across all age-groups (11) and this cause had previously accounted for over 20% of all TBI cases (26).

1.3 Epidemiology of TBI

The reported epidemiological statistics for TBI probably do not reflect the real extent of TBI worldwide because many cases of TBI go unreported, are missed or undiagnosed by health professionals and the number of people who seek treatment in other ways, such as in military facilities or as an outpatient in a hospital, are not included (27, 28). There seem to be gross paucity of data on the incidence of TBI in Australia as there are no available recent TBI statistics for the entire country.

The AIHW reported that the overall TBI hospitalisation rate in Australia between 2004-2005 was 107 per 100,000 population (29). Gardner and Zafonte, stated in their 2016 review (30) that 1.9% of the Australian population is estimated to suffer from TBI related disability with an approximate rate of 46.7 per 100,000 trauma related deaths (31).

In the US, there are more regular TBI surveillance reports by the Centre for Disease Control (CDC) compared to Australian TBI reports. The table (Table 1.1) below shows the data for TBI-related emergency department visits, hospitalizations and death (TBI-EDHDs) across several years in the US. A steady increase in the overall number of cases was seen over time with an estimated 2.8 million cases in 2013 (11). While the overall number of emergency department visits in 2016 and 2017 were not reported, there was an increase in the number of deaths for both years as against the previous years (32). Although, it is important to note that from 2015, CDC implemented new changes by modifying the international classification of diseases to a new code which changed the inclusion criteria for surveillance reports as well as the methodology of how TBI-related deaths are calculated (32).

Table 1.1: Annual rate of TBI-related emergency department visits, hospitalizations and deaths in the U.S.

Year	Emergency department visits	Hospitalizations	Death	Approximate Total	References
2002-2006	1,365,000	275,000	52,000	1,692,000	(6)
2010	2,213,826	283,630	52,844	2,550,300	(33)
2013	2,500,000	282,000	56,000	2,838,000	(11)
2016	NR	227,000	59,000	286,000	(34)
2017	NR	224,000	61,000	285,000	(34)

Meanwhile, the National Institute for Health and Clinical Excellence in the UK reports that approximately 1.4 million people with new TBI cases will visit the emergency departments per year (35) and an overall rate of all TBI severities was 453 per 100 000 (30).

A recent study systematically reviewed the incidence of TBI in Europe for the year 2015 and the TBI rate was reported to widely range between 47.3 to 849 per 100,000 population per year (36). This was definitely higher than the previous overall incidence rate of TBI in Europe between 1990 and 2014 which was 262 per 100,000 (37).

The incidence of TBI is higher in developing countries due to modernization. For instance, in Asia, TBI rate is estimated to be 344 per 100,000 and 160 per 100,000 in India (26).

Overall, males are more likely to experience TBI than females (26), while falls in the elderly and road traffic collisions in the young remain the most common mechanism of injury across Europe, the US and in Australia (8, 32, 36). Although, this is dependent on time and other factors like age, gender and race and ethnicity (25).

1.3.1 Economic cost

In addition to the health burden, TBI also presents a huge cost to the economy of a country. These costs could be direct or indirect costs. Direct costs are the costs of medical treatments to an individual mostly right after the injury and follow-up treatments/rehabilitation afterwards, while indirect costs are the costs from loss of benefits and wages due to disability from the injury or not being able to return to work (26).

In 2004-2005, the direct costs of hospital care excluding costs for rehabilitation care, palliative care and other non-acute care as reported by the Australian Institute of Health and Welfare was estimated to be over AU$184 million in Australia (8). Furthermore, in 2008, the total cost of TBI in Australia was estimated to reach AU$8.6 billion, with AU$3.7 billion for moderate and AU$4.8 billion for severe cases, respectively (38). Meanwhile, the annual total costs of TBI was recorded to be €33 billion (US$39.2 billion) in Europe, US$76.5 billion in the US (39) and US$146.5 million in New Zealand (40). These figures further portray the burden of TBI and the need for its prevention and treatment.

1.4 Types of TBI

TBI is classified as two different types: focal and diffuse TBI. While each type of TBI can be found to present independently in an individual, in most cases they co-exist (41).

1.4.1 Focal TBI

Focal TBI occurs as a result of contact forces (struck by an object or against) acting directly and/or indirectly to cause damage to precise area(s) or a certain portion of the brain (42) to produce injuries such as skull fractures, contusions and mass lesions like intraparenchymal haemorrhage, epidural and subdural haematomas that eventually contributes

to ischemia and neuronal cell death (4, 43). They are a common feature of most moderate and severe TBIs (44) and the approximate mortality rate for severe focal injuries is recorded to be about 40% (4).

The pathophysiological mechanisms of focal brain injury are dependent on the severity and location of impact to the skull and include a variety of ongoing processes, such as excitotoxicity, mitochondrial dysfunction, necrosis and apoptosis (41, 45), which will be described further in Section 1.6, below.

1.4.2 Diffuse TBI

Diffuse TBI is caused by rapid linear or rotational acceleration and deceleration forces of the head, which are often produced by motor vehicle accidents, assaults and falls (46). Diffuse TBI creates extensive damage throughout the brain resulting in axonal injury, diffuse oedema, hypoxic-ischemic injury and diffuse vascular injury (4, 41). Diffuse TBI occurs in about 70% of TBI patients with an approximate mortality rate of 25% in severe diffuse injuries (4, 47).

Diffuse axonal injury (DAI) is the most significant pathology of diffuse TBI and it is often characterised by disconnected and swollen axons that can mostly be identified by microscopic examination, so this pathology is frequently missed with standard non-invasive techniques such as magnetic resonance imaging (MRI) or computed tomography (CT) scans (48, 49). DAI is considered a disease of disconnection, which could include tissue tears in the white matter with intra-parenchymal haemorrhage in severe cases (49, 50). Furthermore, it involves breakdown of the axonal cytoskeleton, impaired axonal transport, altered axolemma permeability, secondary axotomy or axonal degeneration, and mitochondrial swelling (46, 51).

1.5 Classification of TBI severity

There is currently no internationally accepted method to determine TBI severity as each country has its own health regulations and pattern. However, TBI severity is mostly classified using the following parameters:

- clinical severity using the Glasgow Coma Scale (GCS)
- mechanism of injury which could be penetrating, blast, crash or closed.
- extent of structural damage using imaging technologies like CT scan (52).

The GCS is most widely used to classify TBI severity. It is often used in emergency departments (ED) but can also be employed at the scene of injury to assist the transport of the patient to the most suitable facility. The GCS assesses three components: eye opening, motor and verbal responses (Table 1.2) of an individual ranging from 3-15 to classify TBI severity into mild, moderate and severe forms (53). A GCS score of 13-15 categorizes the injury as mild TBI (mTBI), scores of 9-12 as a moderate TBI and scores 3-8 as a severe TBI (53).

Table 1.2: Glasgow coma scale score (4)

Eye opening	Best verbal response	Best motor response
4: spontaneous	5: oriented	6: obeys commands
3: to speech	4: confused	5: localizes
2: to pain	3: inappropriate words	4: withdraws
1: none		3: abnormal flexion
	2: incomprehensible sounds	2: extension
		1: none
	1: none	
	TOTAL GCS SCORE: 3-15	

Note: For a clearer image, I replicated this table from (4)

Other measures of assessing TBI severity include the duration of loss of consciousness (LOC) and post-traumatic amnesia (PTA). LOC records the duration of unconsciousness in an individual while PTA measures the time of amnesia in an individual post-injury (26). TBI is classified (Table 1.3) as mild when there is a LOC of less than 1 hour and a PTA of less than 24 hours, moderate if LOC is between 1 and 24 hours or PTA of 1-7 days and severe if LOC is more than 24 hours or PTA is more than 1 week (4). The challenge in using these measures of TBI assessment is that not all TBI patients experience LOC and/or PTA and they do not have to be present for TBI to be diagnosed, although when present, they could serve as a good measure for the extent of functional and cognitive deficits in an individual (30).

Table 1.3: Classification of TBI severity

GRADING/CRITERIA	**TBI SEVERITY**		
	MILD	**MODERATE**	**SEVERE**
Glasgow Coma Scale	13-15	9-12	3-8
Loss of Consciousness (LOC)	< 1 hour	1-24 hours	> 24 hours
Post Traumatic Amnesia (PTA)	< 24 hours	1-7 days	> 7days

1.5.1 Mild TBI

Mild TBI (mTBI) and concussion are often used interchangeably. Of the various TBI severities, mTBI are the most common with about 80% of all TBI cases, which sometimes go undiagnosed and unreported (28, 54). It occurs from various activities particularly sports related ones like rugby, football, baseball, cheerleading, wrestling and hockey (23). The symptoms of mTBI are mostly acute with about 10-15% of patients experiencing chronic symptoms (55). Acute symptoms can be behavioural (hyperactivity, emotional susceptibility, and irritability), physical (headache, vomiting, dizziness, and nausea) and cognitive (poor concentration, attention problems, and confusion) (55, 56). Persistent symptoms lead to post-concussive syndrome (PCS), which affects 10-15% of patients (55). Mild TBI patients recover full neurological function within 7-10 days in most cases while patients with PCS show a slower recovery from 1-12 weeks (54, 56).

1.5.2 Moderate TBI

Moderate TBI is more serious than mTBI and accounts for about 10% of all TBIs (26). Over 80% of patients with moderate TBI are treated in the intensive care unit (ICU) during the acute phase due to extra-cranial and intra-cranial injuries with anaemia, hyperglycaemia and hyperthermia seen in patients staying for longer than 3 days in ICU (57). Generally, moderate TBI is associated with an estimate of a 15% mortality rate and those who survive often show neurocognitive and psychological deficiencies (58).

1.5.3 Severe TBI

The most severe form of TBI also accounts for 10% of all TBI cases and is associated with a devastating outcome that includes severe neurological disability, vegetative state and

death (4). Patients are often comatose, mostly require surgery to relieve intracranial pressure and present with hypoxemia, brain swelling, hypotension, and seizures (4, 52).

1.6 Pathophysiology of TBI

TBI triggers a series of both primary and secondary injury mechanisms. The acute primary injury resulting from the initial mechanical impact at the time of trauma is irreversible and involves direct damage to neurons, blood vessels, axons and glia as well as the breakdown of the blood-brain barrier (BBB) (59, 60). All these effects together initiate the secondary injury mechanisms. The extent of primary injury is dependent on the severity of the trauma, and can consist of focal or diffuse brain injury patterns or a combination of both (61). These primary injuries can only be reduced using preventive measures such as the use of helmets or other protective equipment. Conversely, secondary injury mechanisms occur within minutes to days and even months after the initial mechanical damage. Secondary injury results from the activation of a range of metabolic, cellular and molecular processes, which significantly alters numerous physiological pathways in the brain (62).

As illustrated in Figure 1.1, secondary injury processes following TBI involve biochemical changes including ion imbalance, reduced production of adenosine triphosphate (ATP), mitochondria dysfunction, increased production of free radicals like reactive oxygen species (ROS) and nitric oxide species (NOS) and eventually cell death (50, 63).

The ion imbalance following TBI causes a rapid influx of sodium (Na^+) and calcium (Ca^{2+}) with an efflux of potassium (K^+) resulting in excessive intracellular Ca^{2+} accumulation and high levels of extracellular K^+ thereby disrupting the normal synaptic membrane potential and neurotransmitter activities (64, 65). In a bid to maintain ionic equilibrium, there is rapid use of glucose leading to depletion of energy reserves, which may be due to reduced cerebral blood flow (65, 66). Also, increased Ca^{2+} influx into the mitochondria alters its integrity due to the compromised membrane potential of the mitochondria resulting from the positively charged Ca^{2+} (61, 67). This leads to decreased ATP production and the release of ROS and NOS, which together with elevated glutamate stimulation and activation of N-methyl-D-aspartate (NMDA) receptors could also contribute to mitochondrial impairment and ultimately oxidative stress (68-71). Additionally, elevated levels of intracellular protein can further induce the activation of caspases which are involved in apoptosis (43, 45, 72, 73). Cerebral ischaemia results in a

shift from aerobic to anaerobic metabolic processes, in which astrocytes induce the increased production of lactate as an alternative source of energy for neurons (74).

These biochemical and metabolic processes described above are associated with neuronal apoptosis, cerebral ischemia, brain oedema, neuroinflammation and axonal damage, which eventually leads to neurodegeneration, cell death and functional impairment (75-78). (For detailed reviews of TBI pathophysiology, see (61, 64, 79, 80)).

Therefore, to identify and develop effective therapeutic interventions for the treatment of TBI, it is essential that the different molecular pathologies associated with TBI are well understood. The delayed and progressive nature of the secondary injury has been suggested as a window for therapeutic intervention to prevent tissue damage and improve functional recovery after injury (61, 81). The summary of TBI pathophysiologies described above shows the interconnection between various TBI secondary injury mechanisms. A brief overview of more specific pathology-related role is provided below for some of the main TBI pathologies that contribute to poor outcomes, with emphasis on neuroinflammation, as the novel drugs that are being investigated in this thesis have potential anti-inflammatory effects.

1.6.1 Excitotoxicity

Excitotoxicity is an important secondary injury mechanism. Injury to the brain stimulates excessive release of excitatory amino acids like glutamate (82) which over-activates the NMDA receptor leading to an ionic imbalance and resulting in continuous depolarization (79, 83). This ionic imbalance involves the influx of sodium and calcium ions and an efflux of potassium ions (84). Together, this impairs the voltage-dependent magnesium blockade of the NMDA receptor (85, 86) thereby, resulting in further depolarization leading to neuronal cell death.

1.6.2 Mitochondrial dysfunction

Mitochondria are organelles that play a prominent role in the modulation of cellular homeostasis as well as producing energy in the form of ATP within the cells (87). Therefore, under pathological condition, its dysfunction could result in very serious outcomes.

After brain injury, mitochondria dysfunction occurs from the disruption in the mitochondrial membrane potential due to excessive accumulation of calcium. This results in the increased

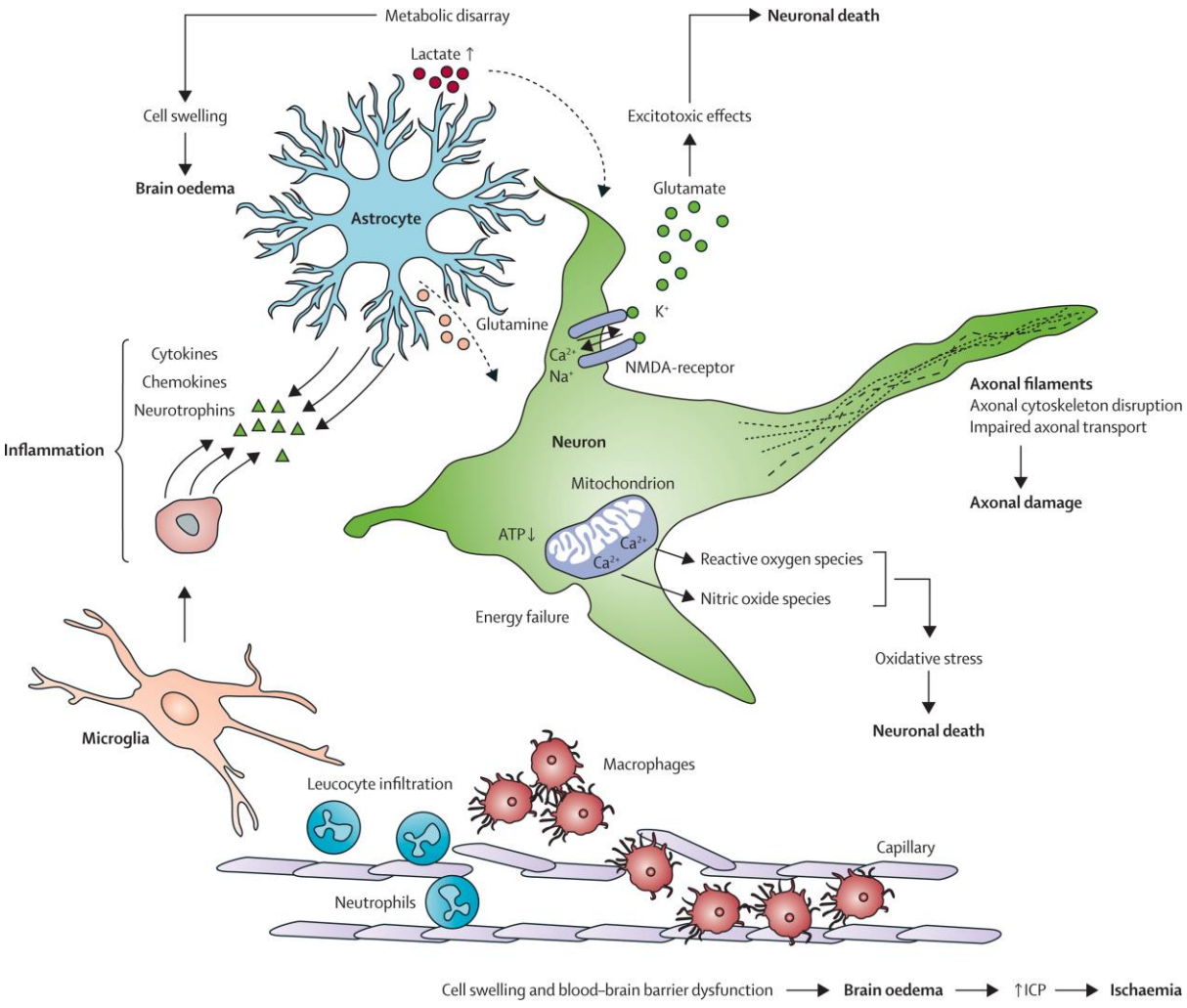


Figure 1.1: The pathophysiology of Traumatic Brain Injury (63)

The biochemical and metabolic changes associated with TBI are intricate and dynamic, involving a multitude of cellular pathways. The ion imbalance resulting from TBI triggers this process, leading to abnormal intracellular Ca^{2+} accumulation, which results in mitochondria impairment causing the release of free radicals like ROS, decreased ATP production and cell death. Together, these changes drive neuroinflammation, brain oedema, axonal damage, ischaemia and neuronal cell death. (Figure from Rosenfeld et al., 2012 (63))

formation of ROS and NOS, reduced production of cellular energy (88) and the release of cytochrome c (89), which induces apoptotic cell death (72, 73, 90). Together, all these lead to neuronal damage by impairing the functions of the mitochondria. Moreover, the degree of mitochondria damage varies depending on the region of the brain that has been affected. For instance, mitochondrial permeability and the generation of ROS were observed to be

significantly higher in mitochondria from the cornu ammonis (CA)-1 than mitochondria from the CA3 region (91) of the hippocampus.

1.6.3 Oxidative stress

Oxidative stress is an imbalance between pro-oxidant and anti-oxidant molecules and can be the result of increased production of free radicals (92). Oxidants are by-products of aerobic metabolism and under physiological conditions their levels are maintained and regulated by endogenous antioxidant mechanisms. Oxidants such as ROS and RNS are involved in many biological processes as well as in the pathophysiology of a broad range of diseases including neurodegenerative disorders (93).

Following TBI, elevated levels of ROS and RNS have been reported to react with proteins, deoxyribonucleic acid (DNA) and lipids to cause oxidative damage (93-95). Factors that contribute in making the brain more susceptible to oxidative damage include increased rate of oxidative metabolic activity in the brain coupled with the non-replicating nature of its neuronal cells (96). Also, increased production of reactive oxygen metabolites, reduced antioxidant capacity, low repair mechanism activity and the high ratio of cell membrane surface to cytoplasm can enhance oxidative damage (96). Oxidative damage plays a role in glutamate-induced excitotoxity, apoptosis, mitochondrial dysfunction and ion haemostasis (71, 97-99).

1.6.4 Cerebral oedema

Cerebral oedema is a significant feature of secondary injury after trauma. It is the abnormal accumulation of water in the brain and it is broadly classified into vasogenic and cytotoxic oedema. Vasogenic oedema represent extracellular accumulation of water in the brain tissue due to the disruption of the BBB (and extravasation of serum proteins). In contrast, cytotoxic oedema are the intracellular accumulation of water characterized by swelling of glial, neurons and dendrites with no increase in BBB permeability (100). There is also a rare form of brain oedema known as osmotic brain oedema, which results from osmotic imbalances between tissue and blood (101).

Following TBI, the mechanisms underlying cerebral oedema are complex and involve both vasogenic and cytotoxic oedema processes that in most cases depend on injury severity (102). Injury to the brain triggers the activation of numerous ion channels including aquaporin 4 and $Na^+/K^+/Cl^-$ channels leading to an ionic imbalance which allows the movement of water into different brain cells (103). Other processes such as BBB breakdown, increased release of

arginine vasopressin, inflammatory mediators like matrix metalloproteinase and the release of neurotransmitters like glutamate can contribute to a worsening of cerebral oedema (102, 104). For instance, upregulated glutamate levels lead to the intracellular influx of Na^+ and Ca^{2+} through its NMDA-receptor and contribute to cytotoxic oedema in astrocytes (69, 105).

Oedema leads to increased intracranial pressure (ICP) following TBI (106) and it is associated in the regulation of cerebral blood flow (107) (Figure 1.1). In fact, ICP monitoring is very important as increased ICP leading to ischaemia is one of the factors that could predict outcome following TBI (108).

1.6.5 Neuroinflammation

Since I explored the effects of novel potential anti-inflammatory drugs within this project, the neuroinflammatory cascade and the roles of inflammatory mediators are discussed with greater detail here than the other secondary injury mechanisms described above.

Neuroinflammation is a fundamental host-defence mechanism that repairs and protects the brain against an injury, infection or neurological diseases (109). It is one of the major secondary injury mechanisms contributing to ongoing neuronal degeneration and poor outcomes following TBI (109, 110). Likened to a "double edged sword", neuroinflammation plays dual roles that can be both beneficial and detrimental (111). Neuroinflammation involves a cascade of complex events (Figure 1.2) including the activation of resident immune cells, microglia and astrocytes, the release of inflammatory mediators within the brain, as well as the recruitment of immune cells from the peripheral system (112).

Neuroinflammation is a characteristic feature that is associated with several neurological diseases (109, 113) and can persist for a prolonged period of time after the acute injury (114). Although, some aspects of the neuroinflammatory events can differ based on the form of disease or brain insult (109, 115). Understanding the neuroinflammatory responses following an insult to the brain is increasingly garnering interest as potential new therapies targeting the inflammatory cascade are being investigated for the treatment TBI (116, 117). Below is a detailed description of some inflammatory mediators known to be important following TBI, and their various roles in the neuroinflammatory cascade.

1.6.5.1 Astrocytes

Astrocytes are divided into two types based on their anatomical location and cellular morphology. The protoplasmic astrocytes are located all through the grey matter and are characterised by numerous fine branching processes originating from various stem branches,

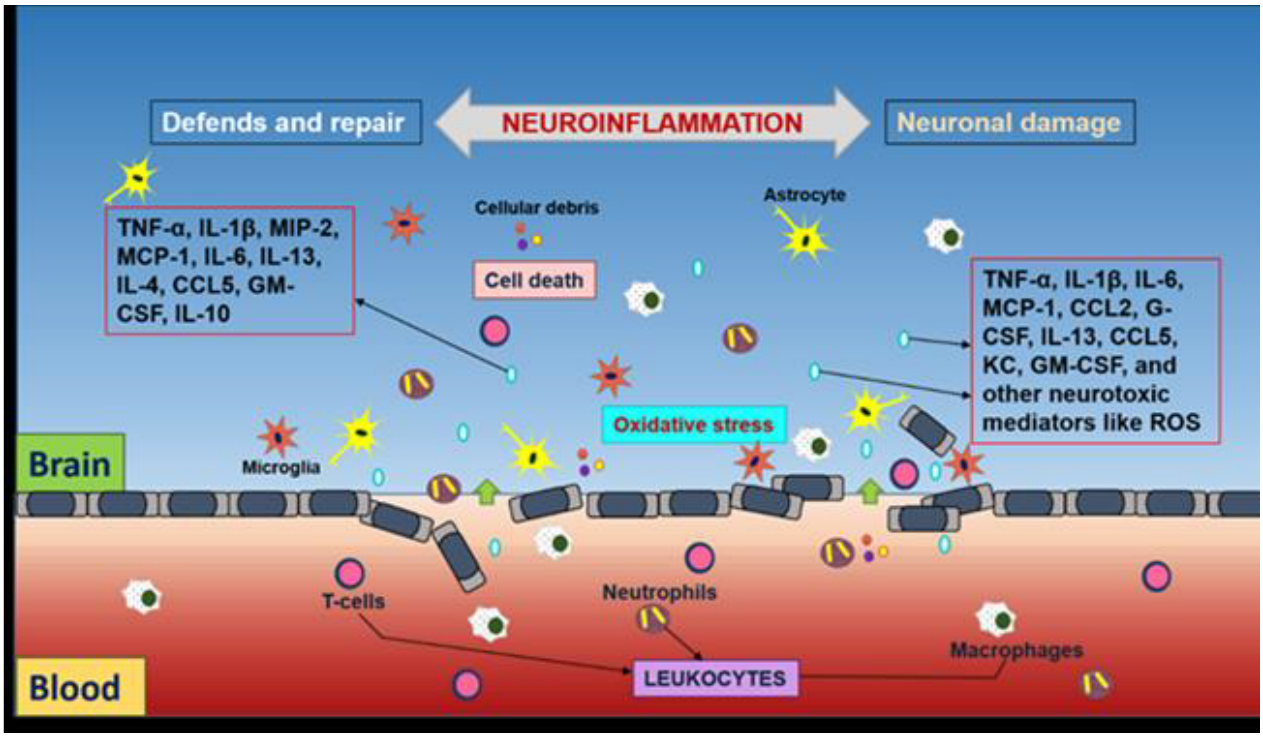


Figure 1.2: Neuroinflammation after Traumatic Brain Injury.

Neuroinflammation can be beneficial or detrimental. Injury to the brain activates the resident immune cells (microglia and astrocytes) resulting in the increased release of inflammatory cytokines, such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, IL-13, IL-4, IL-10; chemokines like monocyte chemoattractant protein (MCP)-1, chemokine ligand (CCL)-5, CCL2, keratinocyte chemoattractant (KC); and other inflammatory mediators, which in acute TBI is necessary for host-defence and repair. The inflammatory mediators induce further breakdown of the BBB and recruitment of immune cells (leukocytes) from the blood into the brain to stimulate additional release of inflammatory mediators as well as other neurotoxic molecules such as reactive oxygen species (ROS). This neuroinflammatory response eventually results in neuronal damage and necrotic and apoptotic cell death. G-CSF, Granulocyte colony-stimulating factor; MIP-2, Macrophage inflammatory protein; GM-CSF, Granulocyte-macrophage colony-stimulating factor.

while the fibrous astrocytes are located all through the white matter and are characterised by multiple long fiber-like processes (118).

Under physiological conditions, astrocytes are involved in various CNS mechanisms and primarily function to maintain homeostasis and regulate the integrity of the BBB (119, 120). Astrocytes also support and control the development of neuronal synapses as well as supply energy in the form of glutamine and lactate to neurons (121). They decrease the effect of glutamate excitotoxicity to neurons and cells by regulating extracellular glutamate levels (122). Glial fibrillary acidic protein (GFAP) is an intermediate filament protein, predominantly expressed in mature astrocytes with its main function being to maintain structural integrity required to support the shape of astrocytes (123).

Activation of astrocytes, mostly in response to disease state or injury, is also referred to as astrogliosis. In addition to the characteristic of astrocytes under pathological condition, there is increased expression of GFAP, which is a common way to identify astrocytes, whose activities are known to peak within 3-7 days following experimental TBI (118).

Reactive astrocytes are often characterized by morphological changes including hypertrophy of cell bodies, thickened and shortened processes (118), increased cell proliferation, up-regulation of brain derived neurotrophic factor (BDNF), and increased expression of intermediate filament proteins glial fibrillary acidic protein (GFAP) and vimentin (124). Additionally, reactive astrocytes release inhibitory extracellular matrix consisting of chondroitin sulphate proteoglycans that contributes to the formation of a glial scar around the site of injury; this acts as a chemical and physical barrier for the protection of the intact/uninjured brain tissue against the toxic environment created as a result of the injury (125). However, sustained astrogliosis could obstruct or hinder tissue regeneration and repair (126). Overall, the initial effects of astrogliosis over an acute time period appears to be protective while over a long period of time, this effect becomes detrimental, suppressing regeneration and eventually hindering recovery.

1.6.5.2 Role of astrocytes in TBI

Astrocytes, which are one of the resident immune cells in the brain, play a vital role in the inflammatory response to injury – including TBI – and their role is dependent on the injury type and severity (125). Following an insult to the brain, astrocytes and microglia migrate to the lesion site, where they trigger numerous cellular and molecular mechanisms including the release of other mediators including metalloproteinases, endothelin, tumour necrosis factor-

alpha (TNF-α) and growth factors, to form glial scar as a part of a defence mechanism to suppress neuroinflammation, repair damaged BBB and stimulate neuronal regeneration (125, 127). Evidence suggests that the glial scar border, which is formed mainly by newly proliferated astrocytes, dependent on STAT3 signalling, eventually becomes a detrimental inflammatory response that hinders axonal regrowth (128, 129).

These beneficial and detrimental roles of astrocytes in neuroinflammation after trauma were further reinforced in the study by Wilhelmsson and colleagues (130) in which they demonstrated a significant loss in neuronal synapses at day 4 post-injury in a GFAP-/-Vim-/- injured mice, thereby indicating that, at early timepoint after injury to the brain, astrocytes are necessary for protection. However, contrary to this, in the same study, the authors reported that between days 4 and 14, they observed increased restoration of neuronal synapses, which were not ultrastructurally different from those pre-injury or compared to the uninjured mice. They attributed this response to the reduced hypertrophy of cellular processes demonstrated by reactive astrocytes in the GFAP/Vimentin knockout mice. Thus, suggesting that astrocytes, over a long period of time, could become harmful and damaging to the brain (130). Similarly, ablation of dividing reactive astrocytes revealed cortical degeneration after severe CCI (131). Together, these studies demonstrate the relevance of reactive astrocytes in protecting the brain and determining outcome after TBI while highlighting the heterogenous and extremely intricate role of reactive astrogliosis in trauma.

1.6.5.3 Microglia

Microglia are the resident macrophages of the brain accounting for 5-12% of all cells in the mouse brain and 0.5-16.6% in human brain depending on the anatomical region (132). They originate from microglial cell progenitors in the yolk sac during embryonic development, from where they migrate into the CNS to become microglia cells (133). Over a decade ago, it was discovered that in the healthy brain, microglia constantly survey the microenvironment for any disturbances to brain homeostasis (134). In addition to this function, they regulate synapse interactions and functions (135, 136), act to phagocytise debris during postnatal development and adult neurogenesis (137, 138), control neuronal activity and drive programmed cell death in the healthy brain (139, 140). The resting or surveillant microglia, which are typically found in the normal brain, exhibit morphological features of a small cell body (soma) with thin and highly ramified fine cellular processes (141). In a pathological state, this morphology quickly changes to one with an enlarged soma or amoeboid appearance accompanied with short and thick processes (142).

Microglia have previously been hypothesised to assume distinct M1 and M2 phenotypes with different physiological actions that could be either deleterious or neurorestorative, respectively (143). The M1 microglia are associated with the increased induction of pro-inflammatory cytokines like IL-6 and IL-1 as well as increased production of free radicals like ROS. Conversely, the M2 microglia generally promote the release of anti-inflammatory cytokines like transforming growth factor beta (TGF-β), IL-10 and IL-4 (143, 144) as well as other factors like arginase-1 (AG1), found in inflammatory zone-1 (FIZZ1) and Ym1, a macrophage protein (143, 145). These microglia are further sub-divided into M2a, M2b and M2c phenotypes with key roles in phagocytosis, tissue repair and regeneration (146).

The M1 phenotype could be beneficial in the short-term following an insult to the brain where it is involved with various mechanisms necessary to enhance host defence, however, over a prolonged period, M1 microglia could hinder cell repair (145, 147). Meanwhile, the M2 phenotype is associated with wound repair and neuronal recovery (148). However, this phenomenon of M1 and M2 polarization states has been proposed to be non-existent in recent years (149) as it's been suggested that due to the intricate signalling events in the brain following injury or disease, microglia display a mixed phenotype, where M1 and M2 are expressed simultaneously rather than existing as an exclusively polarized distinct subclass with specific functions in promoting cell toxicity or tissue repair (150-152).

1.6.5.4 Role of Microglia in TBI

Microglia play a key role in neuroinflammatory process, with evidence suggesting that microglia are the first line of responders to injury in the brain as they rapidly extend their processes towards the injury site to trigger the process of protecting the healthy brain tissue from the injured area (153). Like astrocytes, they exhibit dual but opposing roles. While on one hand, they initiate responses such as the phagocytosis of molecular and cellular debris, that are necessary to trigger tissue defence mechanisms, on the other hand, excessive activation of microglia can stimulate the release of neurotoxic mediators thereby hindering cell recovery (147, 154).

Following trauma, microglia become activated and quickly undergo profound morphological changes, as stated earlier, depending on the injury type and severity. As illustrated in Figure 1.2, these activated microglia migrate to the site of the injury and trigger various inflammatory responses including the secretion of inflammatory cytokines and chemokines (147, 155). Prolonged microglia activation initiates the release of additional

inflammatory mediators and neurotoxic molecules including pro-inflammatory cytokines like interleukin (IL)-6, tumour necrosis factor-α (TNF-α) and IL-1β, oxidative metabolites such as reactive oxygen and nitrogen species, nitric oxide, increased permeability of the BBB which allows the infiltration of leukocytes from the blood, secretion of proteases, and neurotrophic factors (110, 154). Also, activated microglia increase expression of major histocompatibility complex antigen (MHC), complement receptors and adhesion molecules, which further contributes in modulating the neuroinflammatory process after trauma (110).

Evidence suggests that in TBI, microglia M1/M2 states exist as mixed phenotypes and could shift from one state to the other (156-159). An in depth understanding of these states could be relevant in the development of pharmacological strategies that could modulate neuroinflammation by facilitating anti-inflammatory activities to promote neuronal survival and repair (160).

While the innate inflammatory response to injury has been described here, it is important to consider that adaptive immunity also contributes to neuroinflammation following TBI. For a detailed discussion of these processes, please refer to these excellent recent reviews (161, 162).

1.6.5.5 Inflammatory mediators: Cytokines/chemokines

Cytokines are small, pleiotropic proteins of 8–25 kDa in size that are produced by glial cells, lymphocytes and several other cell types (163, 164). They act as signalling molecules through their receptors found on many target cells, in various paracrine, endocrine and autocrine pathways to modulate inflammation and regulate many cellular activities, such as differentiation and proliferation (164-166). There are extensive numbers of different cytokines, including interferons, interleukins, tumour necrosis factors (TNFs), chemokines and growth factors (167). These cytokines are broadly classified into families according to their receptors, secretion or structural homology (163, 168). The seven cytokine receptor families are type I cytokine receptors, type II cytokine receptors, tumour necrosis factor (TNF) receptors, immunoglobulin superfamily receptors, TGF-β, G-protein coupled receptors, and interleukin-17 (IL-17) receptors (169).

Chemokines, a subfamily of cytokines are small, secreted protein molecules with a size of 8-14 kDa (170). They bind to specific seven-transmembrane G-protein coupled receptors to bring about their biological effects and are primarily involved in the chemoattraction of leukocytes and the migration of immune cells (171-174). As is typical of cytokines, the

receptors are expressed on a variety of cells and based on the type of chemokine they interact with, they are further subdivided into four classes namely CC, CXC, CX_3C or XC (175).

Cytokines and chemokines bring about their effect by binding to their specific receptors, which are expressed by neuronal and glial cells to induce the activation of signal transduction pathways (176, 177).

Pro-inflammatory cytokines/chemokines such as interleukin-6 (IL-6), IL-1, TNF-α, MCP-I, MIP-1α, MIP-1β and KC stimulate inflammation (178). Whereas anti-inflammatory cytokines inhibit inflammation (170). Some examples include IL-10, IL-4, IL-13 and interferon-gamma (IFN-γ). Although, it should be noted that most cytokines play opposing dual roles as they can elicit stimulatory and inhibitory effects to mediate numerous biological activities (170). These roles could be overlapping in some cases, where a specific cytokine may act as a pro-inflammatory cytokine and as well as an anti-inflammatory cytokine affecting different pathologies (178, 179).

1.6.5.6 Role of inflammatory mediators in TBI

Cytokines and chemokines are an integral component of the complex neuroinflammatory cascade that occurs after trauma. Besides the activation of astrocytes and microglia/macrophages, the increased release of multiple cytokines and chemokines can stimulate or inhibit neuroinflammation following experimental TBI. For instance, while on one hand, elevated levels of the pro-inflammatory cytokine, IL-6, are implicated in the mediation of neuroinflammation and poor outcomes (121, 180-182), on the other hand, an increased release of IL-6 has also been associated with neuroprotection and improved outcomes (183-189). Other cytokines/chemokines that are known to drive or modulate neuroinflammation after trauma include IL-1, tumour necrosis factor-alpha (TNF-α), macrophage inflammatory protein (MIP)–1 alpha, MIP–1 beta, monocyte chemoattractant protein-1 (MCP-1), granulocyte colony-stimulating factor (G-CSF) and IL-10 (190-192). The major sources of cytokines and chemokines following TBI include astrocytes, microglia, endothelial cells, neurons and leukocytes (190, 191, 193).

Most cytokines and chemokines peak within 4-12 hours after TBI (190, 194) and they are easily measured in serum, plasma, CSF or brain tissue. It has been proposed that the increased levels of cytokines and chemokines can predict the degree of cell damage in disease state and thus, could act as a biomarker for TBI (190, 195-198). Also, due to the broad effects

of cytokines and chemokines in the body, they could act as therapeutic targets for the treatment of various diseases including TBI (169, 199-201).

Some cytokines/chemokines that are known to drive or modulate neuroinflammation after trauma are further described in detail below:

1.6.5.6.1 Interleukin-1

Interleukin-1 (IL-1) is an essential mediator of neuroinflammation that has been extensively studied across various models of TBI as well as in spinal cord injury (202-204). IL-1 brings about its effects by binding to the receptor, IL-1R1, which is expressed on numerous cell types, including neurons and astrocytes in the brain (205, 206). However, in some cases, there is evidence indicating that some of IL-1 effects were elicited independent of its receptor, IL-1R1 (207, 208). IL-1 is subdivided into three isoforms: IL-1α, IL-1β and IL-1 receptor antagonist (IL-1ra) (209). While IL-1α and IL-1β are known to stimulate inflammatory effects, IL-1ra exerts inhibitory effects on inflammation (190, 192). Of the three isoforms, IL-1β is the most investigated isoform in TBI. Elevated levels of IL-1β induced by the activation of caspase-1 were demonstrated to cause neuroinflammation and cell death in both fluid percussion injury (FPI) and *in vitro* models of TBI, thereby suggesting that excess production of IL-1β could contribute to neuronal dysfunction or cognitive impairment following TBI (210). More evidence to support this was demonstrated by the study in which neutralization of IL-1β improved cognitive effects and conferred neuroprotection following TBI in mice (211). Furthermore, IL-1β stimulated the release of prostaglandins from astrocytes and induced the activation of cyclooxygenase-2 (COX-2) *in vitro* (212). Levels of both IL-1α and IL-1β were found to be significantly elevated at 3 hours post-TBI and resulted in a loss of hippocampal neurons. It was also observed in the same study that the TBI-induced increases in both cytokines occurred largely through their release from neurons and astrocytes (213).

In general, while on one hand in the healthy brain, low or moderate levels of IL-1 play significant beneficial roles in various biological processes including synaptic pruning, development, synaptic plasticity, memory and sleep, on the other hand, the same cytokine becomes neurotoxic, triggering detrimental effects following injury to the brain where their levels are elevated (214).

Although IL-1α and IL-1β have been associated with deleterious effects after trauma, transgenic overexpression of IL-1ra in a mice CHI model of TBI improved neurological

recovery (215). Recombinant IL-1ra treatment has also been implicated in neuroprotective effects after trauma (216, 217). Meanwhile, neuroinflammation and oedema were decreased following deletion of IL-1R in a mouse mild stroke model, hence implicating IL-1 as a pro-inflammatory cytokine in this mouse model. Inflammatory cytokines and chemokines have the capability to also induce the release of other cytokines which further aggravates the neuroinflammatory response after trauma (190, 218).

Contrary to the neuroprotective effects demonstrated in rodent studies above, IL-1ra treatment resulted in increased M1 and decreased M2 related cytokines and chemokines in the brain extracellular fluid following severe diffused brain injury in humans (219). These inconsistencies in the literature further reflects the possible pleiotropic effects of IL-1 as an inflammatory mediator post-injury.

1.6.5.6.2 Interleukin-6

Interleukin-6 (IL-6) is another cytokine that has been extensively studied in TBI. It is a multifunctional cytokine that plays a critical role in inflammation (220). It is also known to regulate various physiological and pathological processes including neural development, aging, bone metabolism, reproduction and haematopoiesis (189, 221, 222). In TBI, IL-6 is significantly elevated and contributes to TBI-induced neuroinflammation and poor functional outcome (180, 223). Although, the knockout of IL-6 has also proven to worsen outcomes at early timepoints following TBI, thus implying that IL-6 could be beneficial in acute phase inflammation (186, 224) as its been demonstrated to be crucial in the activation of glial cells and recruitment of myelo-monocytes following brain injury (187) . It has also been suggested that the conferred neuroprotective role of IL-6 may have been elicited via pathways regulating the expression of IL-1β and apoptosis as well as supporting the integrity of BBB (185, 225).

1.6.5.6.3 Tumour necrosis factor alpha

Tumour necrosis factor alpha (TNF-α) is a pleiotropic cytokine that is mostly produced by macrophages but can also be produced by a variety of other cells including lymphocytes and fibroblasts as well as in the brain by astrocytes and microglia (226, 227). It exists as a membrane-bound or soluble form with the 17 kDa soluble form cleaved from the 26 kDa membrane-bound protein by the metalloprotease TNF-α-converting enzyme (TACE) (226). TNF-α induces a broad range of biological effects which are mediated by binding to two cell

surface receptors, TNF-R1 (p55) and TNF-R2 (p75), which are each characterised by different signalling activities (228-230).

TNF-α plays a critical role in regulating the neuroinflammatory cascade after trauma. Although mostly referred to as a pro-inflammatory cytokine in TBI, in recent years, this has been contested as the role of TNF-α in neuroinflammation is controversial. While studies have associated the early expression of TNF-α with deleterious effects following TBI (231-233), TNF receptor knockout mice reported aggravated BBB disruption and tissue damage after TBI (234). Also, another study demonstrated increased neuronal injury at an early timepoint (1-2 days) in a TNF knockout mice but displayed neuroprotective effects by inhibiting neuronal injury at a later timepoint (2-4 weeks), thereby suggesting that the dual roles exhibited by TNF-α could be dependent on its expression level and time course (176). Likewise, TBI resulted in a significant neuromotor and memory deficits as early as 48h post-injury that was not recovered at 2-3 weeks in a TNF-α knockout mice when compared to wildtype post-injury (235). Furthermore, while a higher mortality rate was demonstrated in a TNF and lymphotoxin double knockout mice subjected to a closed head injury, an improved neurological outcome was observed with no change in the infiltration of leukocytes and BBB function (236). All these studies together imply that TNF-α could have a different role in acute inflammation phase compared to the role it exhibits over a long-term period.

1.6.5.6.4 Interleukin-10

Interleukin-10 (IL-10) is a 35 to 40 kDa cytokine that is produced in various cells including macrophages, dendritic cell, astrocytes, microglia and regulating T-cells (192, 237). It is an important anti-inflammatory mediator of inflammation primarily known to exhibit neurotrophic effects. IL-10 treatment improved neurological recovery and significantly attenuated the expression of proinflammatory cytokines, TNF-α and IL-1, in the injured cortex and hippocampus following experimental TBI, thus implying that the improved outcomes exhibited by IL-10 may be related to the decrease in the production of proinflammatory cytokines (199). Also, *in vitro* study demonstrated that IL-10 distinctly reduced microglia-induced inflammatory response (238). In addition, the intrathecal production of IL-10 in the CSF and serum of patients with severe TBI was suggested to control inflammatory responses including, IL-6, TNF-α, TGF-β1, the function of BBB (239) and indirectly by diminishing astroglia activities (240).

1.6.5.6.5 Monocyte chemoattractant protein (MCP-1)

Monocyte chemoattractant protein (MCP-1) which is also known as chemokine C-C ligand-2 (CCL2) belongs to the C-C chemokine family and interact with the C-C chemokine receptor, CCR2 (241). MCP-1 is expressed by multiple cells and attract monocytes, lymphocytes, macrophages, neutrophils, basophils and dendritic cells (241).

MCP-1 plays a crucial role in mediating post-traumatic secondary brain damage, and this was demonstrated in a CCL2 knockout mice where there was decreased astrogliosis, reduced lesion volume over time, improved neurological recovery and reduced neuronal loss in the ipsilateral cortex and thalamus following CHI (242). In the same study, TBI significantly elevated the levels of CCL2 which peaked within 24 hrs after injury in the CSF of severe TBI patients and in cortical homogenates of mice model of CHI. Additionally, poor outcomes including neurological dysfunction and increased brain damage have been reported to be associated with significantly elevated CCL2 expression following various models of brain injury (243-246).

Together, these inflammatory cytokines modulate the neuroinflammatory cascade after trauma and could act as possible therapeutic targets for TBI. Overall, neuroinflammation does play a very critical role in determining TBI secondary-induced injuries. Search for novel therapeutics could explore these various mechanisms with an aim to optimise the beneficial effects of neuroinflammation.

1.7 Potential therapeutics for TBI

Despite promising results from extensive preclinical studies, there is no effective treatment available for TBI to date. Translating preclinical findings to human injury has posed a major challenge, as clinical trials for potential TBI drugs have either failed or were discontinued due to undesirable effects (247). While different factors could account for the failures in clinical trials, one of the most important factors is the multi-faceted TBI pathophysiology and the fact that animal models do not truly reflect the heterogeneity of this disorder (248).

Multiple drugs with potential neuroprotective effects, some with known mechanisms of actions and others unknown or yet to be fully elucidated, have been investigated for the treatment of TBI. The efficacy of these drugs is often determined based on their ability to

attenuate pathologies associated with secondary injury mechanisms, such as levels of pro- and anti-inflammatory mediators, as well as reactive gliosis. Due to the heterogeneity of TBI pathophysiology, multipotent drugs simultaneously targeting several mechanisms have been suggested to be more likely to result in an effective treatment for TBI as against targeting a single injury mechanism (248).

Below are examples of drugs that have shown over the past few years to be potentially neuroprotective and have even made it to clinical trials. However, these drugs failed to translate the positive results from animal studies.

1.7.1 Statins

Statins are a family of drugs consisting of the 3-hydroxy-3-methylglutaryl (HMG) component which binds to HMG reductase coenzyme A (HMG CoA). They are also referred to as HMG CoA reductase inhibitors and include agents such as simvastatin, atorvastatin and rosuvastatin among others. They inhibit the biosynthesis of cholesterol as well as decrease low-density lipoprotein (LDL) levels and are generally used clinically for the treatment of cardiovascular diseases and dyslipidaemias (249, 250). Statins have a safe profile, a favourable adverse outcome and a wide availability. The efficacy of the various types of statins is determined by their degree of HMG-CoA reductase inhibition (249).

Statins regulate many mechanisms or processes after trauma, thus making them best suited as a potential drug for the treatment of TBI, which is often associated with varied pathologies (250). They have demonstrated anti-inflammatory, anti-excitotoxicity and anti-apoptotic effects and also promote neurogenesis and angiogenesis when administered at a low dose across experimental models of TBI (250) as well as in humans (251).

Rosuvastatin which is the most effective statin for reducing cholesterol and LDL levels, was shown in a randomized clinical trial to significantly reduce plasma levels of tumour necrosis factor (TNF)-α in patients with moderate to severe TBI (251). Rosuvastatin, also, in a mice CCI model of TBI, improved pathological outcomes in the hippocampus due to diminished activation of microglia, decrease inflammation and degeneration of neurons (252). A remarkable decrease in inflammatory cytokines, TNF-α and IL-1β following pre-administration of lovastatin led to a decrease in inflammation and neurological recovery (253). Similarly, Simvastatin have been reported to promote improvement in functional

outcomes following TBI. They selectively reduce the expression of IL-1β and impede the activation of microglial cells and astrocytes after TBI (254).

Statins as potential therapeutics for TBI have been very well researched however, a few controversies still exist (255, 256) and the mechanisms by which their neuroprotective effects are carried out remains unclear.

1.7.2 Progesterone

Progesterone, widely known as a female hormone due to the role it plays in menstrual cycle and pregnancy, is a steroid hormone that is produced primarily by the female ovary and in trace amount by the testes and adrenal cortex in the male. Progesterone which is enzymatically synthesized from pregnenolone, a derivative of cholesterol, is described also as a nuclear transcription factor that exerts its effects through the progesterone receptor (PR) for the regulation of gene transcription (257). PR exist in two major isoforms: PR-A, the N-terminally truncated form and PR-B (258, 259).

In addition to the regulation of gene transcription, progesterone exhibits neuroprotective effects across many experimental models of TBI and neurodegenerative diseases (260-263) including humans (264). Over the years, the idea of referring to progesterone as a sex hormone found only in females has changed immensely as research has shown that besides the areas mentioned earlier, they could also be synthesized in the brain of both males and females (265) and are thus referred to as neurosteroids. The presence of PR has been confirmed in the forebrain, mid-brain limbic system, hind brain and hypothalamic region of both the developing and postnatal brain (266, 267). Like Statins, the effects of progesterone are pleiotropic, that is, they could protect the brain against diseases and injuries through multiple mechanisms.

Following experimental TBI, progesterone decreased the levels of inflammatory mediators, nuclear factor kappa beta (NFκB) p65 and complement factor C3 in rats (268). Progesterone has further shown robust anti-inflammatory effects by reducing various inflammatory cytokines including IL-1β, TNF-α, IL-6, Toll-like receptor 2 (TLR-2), inducible nitric oxide synthase (iNOS), chemokine (C-C motif) ligand-2 (CCL2) and cyclooxygenase-2 (COX-2). as well as inhibited astrocyte activation (261, 269, 270) in both a time and dose dependent manner in acute TBI. Studies have also shown that progesterone decreases cerebral

oedema (271-273), apoptosis (274-276), oxidative stress (277) and inhibits excitotoxicity (278) to improve cognitive and neurological function following TBI.

Although, the mechanisms behind the neuroprotective effects of progesterone are yet to be fully understood, it has been suggested that progesterone exerts its protective effect on the brain by regulating the expression of aquaporin-4 (AQP4) in glial cells (272, 279), reducing lipid peroxidation (280) and decreasing the release of inflammatory cytokines and mediators (269, 275, 277, 281).

In early phases of clinical trials, progesterone was effective in the treatment of TBI patients as there were more TBI patients surviving in the treatment group than in the control group. It was also shown to be a safe drug and was well tolerated by these patients (264, 282). However, in later phase III trials, progesterone failed to show improved survival and neurological outcomes in patients with acute TBI (283, 284). It is important to note that, perhaps, the outcome of these clinical trials could have been different if further preclinical studies were undertaken as suggested by Gibson and colleagues in their systematic review. This systematic review, which evaluated the use of progesterone as treatment for experimental brain injury identified fundamental areas that required additional experimental evidence. One of such areas includes the optimisation of dose and timing of progesterone treatment following brain injury (285).

1.7.3 Erythropoietin

Erythropoietin (EPO) which is a hematopoietic hormone belonging to the cytokine family is extensively known for its role in regulating erythropoiesis (286). It is a 30.4kDa (165 amino acids) glycoprotein that acts through its receptor, EPOR (287). It is synthesized mainly in the adult kidney as well as in foetal liver (288) and is clinically used for the treatment of anaemia (289) in various disorders or health conditions.

In recent years, several experimental studies have reported the neuroprotective effects of EPO in different types of central nervous system (CNS) disorders and injuries (290-292), including TBI (293). EPO is a multi-functional drug targeting numerous mechanisms (294) that contribute to the pathophysiology of secondary injury after TBI.

In a combined rat model of diffused TBI and hypoxia, EPO decreased neuroinflammation by reducing the levels of IL-1β to the same as that of the controls and decreased *microtubule-associated protein-2* (MAP2) to enhance behavioural and cognitive

function as well as improve axonal pathology (295). Additionally, improved TBI outcomes and increase in the expression of anti-inflammatory cytokine IL-10 were demonstrated following a marked reduction in the infiltration of immune cells, decreased microglia activation and inflammatory mediators such as nuclear factor kappa light chain enhancer of activated B cells (NF-κB), IL-1β, IL-6, TNF-α and intercellular adhesion molecule-1 (ICAM-1) by EPO (296-298).

Recombinant human erythropoietin (rhEPO) treatment decreased the expression of the apoptotic enhancing BAX gene (299) and caspase-3 (300) as well as increased cortical cell survival by promoting the expression of phosphorylated Janus kinase-2/signal transducer and activator of transcription-3 (p-JAK2/p-STAT3) to increase mRNA expression, protein levels of B-cell lymphoma-2 (Bcl-2) and anti-apoptotic proteins (p-Akt and Bcl-xl) (301, 302).

Improved sensorimotor function, spatial learning performance, cognitive function, significant increase in cerebral blood flow (CBF), reduced oedema, decreased lesion volume and increase neurogenesis have also been associated with EPO treatment following TBI (303-307). Lu and colleagues showed a significant increase in the number of newly formed neurons using BrdU staining in a rat model of TBI that received EPO treatment daily for 14 days with the first dose at 24 hours (1day) post-TBI (305).

Together with the neuroprotective effects exhibited by EPO and its safety profile, EPO was considered a suitable potential target for TBI treatment in the future. However, sadly, recent clinical trials of EPO in moderate and severe TBI failed to improve outcomes (308).

1.7.4 Minocycline

Minocycline is a semi-synthetic antibiotic derived from tetracycline. It is widely used for the treatment of acne and some staphylococcal infections. It is safe, has a wide therapeutic window, highly lipophilic and can cross through the blood–brain barrier easily (309). More recently, minocycline has been found to exert potent anti-inflammatory, neuroprotective and anti-apoptotic effects in addition to their anti-microbial properties. The neuroprotective activities of minocycline have been reported in various experimental models of CNS disorders such as Parkinson's disease (310, 311), ischaemia (312), Huntington's disease (313), multiple sclerosis (314), Alzheimer's disease (315), amyotrophic lateral sclerosis (316), spinal cord injury (317), stroke (318) and TBI (319).

Minocycline treatment enhanced numerous secondary events including the decrease in microglial/macrophage activities, apoptosis and lesion size in experimental spinal cord injury (320, 321) as well as in experimental TBI (322, 323) to bring about an improvement in neuronal outcomes. Long-term neuroprotective effects of minocycline were observed in a weight drop model of TBI. These long-term effects resulted from the enhanced production of soluble forms of amyloid precursor protein-α (sAPPα), an endogenous neuroprotector (324). On the contrary, another study reported a short-term improvement in neurological outcome following minocycline administration in a closed head injury model of TBI (323).

In a rat model of mild blast-induced TBI (mbTBI), the activities of inflammatory markers, C-reactive protein (CRP), Monocyte chemoattractant protein-1 (MCP-1) and Toll-like receptor-9 (TLR-9) were either reduced or impeded after treatment with minocycline. Also, the behavioural abnormalities present in this model of TBI were rescued in the minocycline treated animals to almost the same levels as the uninjured animals (325). Minocycline decreased cerebral oedema and olfactory lesions following TBI (326, 327). However, it does not have a significant effect on neurogenesis (322). Despite these studies demonstrating beneficial actions, inconsistencies in the effects of minocycline following TBI exist in the literature. For example, a recent study demonstrated that minocycline did not improve TBI-induced impulsive and attentional deficits at both early and late timepoints in a rat CCI model. In the same study, minocycline did not decrease neuroinflammation following TBI (328).

Although, the precise mode of action for the neuroprotective effects of minocycline is unclear, a few possible mechanisms have been postulated; one of which is the inhibition of caspase 1 and 3 expressions (313, 319, 329, 330), involved in cell death pathways. Other suggested mechanisms include the suppression of reactive microgliosis (311, 322, 331) and blocking the release of mitochondrial cytochrome c which directly activates the caspase cell death pathway (316, 332).

Similar to studies in experimental models, clinical trials of minocycline as treatment for neurological disorders have shown mixed results. Improved neurological and functional outcomes were demonstrated in a clinical trial of minocycline in spinal cord injury (333) and likewise, in patients with acute TBI (334). However, previous phase III randomised trial of minocycline have shown harmful effects of the drug on patients with amyotrophic lateral sclerosis (335).

1.7.5 Peroxisome proliferator-activated receptor (PPAR) agonists

Peroxisome proliferator-activated receptor (PPAR) agonists are a group drugs that exhibits potent anti-inflammatory properties. They are ligand-activated transcription factors existing in three isoforms; PPAR-α, PPAR-δ and PPAR-γ which are highly expressed in most body tissues to control the process of adipogenesis and promote several metabolic activities particularly in maintaining physiologic levels of glucose and lipids (336).

The PPAR agonists are a part of the large family of nuclear hormone receptors (NRs) which when activated binds as heterodimers to retinoid X receptor thereby altering their conformation and by acting as agonist-dependent transcription factors, they bind to distinct promoter regions of target genes to result in the regulation of gene expression (337).

Activation of PPAR isoforms, α and γ have been associated with neuroprotection in various neurological disorders including TBI (338, 339). Treatment with PPAR-γ receptor agonist after TBI resulted in decreased ROS by reducing the expression of COX-2 and iNOS. They also reduced microglia activation, thereby promoting the activation of M2 microglia which is anti-inflammatory (340, 341). They have also been reported to improve neurological outcomes by attenuating cerebral inflammation, lesion size, inhibiting apoptosis as well as stimulate angiogenesis and neurogenesis (342, 343). However, they could play a dual role in apoptosis and neurogenesis where an excessive activation of PPAR-γ would result in the inhibition of cell proliferation eventually leading to cell death (342) and vice versa. Likewise, fenofibrate, an example of PPARα agonist decreased cerebral oedema, neurological deficit and brain lesion in a lateral fluid percussion injury rat model of TBI thereby resulting in neurological recovery (344).

Together, while all the above-mentioned drugs have demonstrated beneficial therapeutic effects for TBI in preclinical studies, no one has successfully made it through all the stages of clinical trials. Therefore, continued research into TBI therapeutics must be encouraged in the hope to discover and develop new therapies that could become effective treatments of TBI. One of such promising targets in the therapeutics of TBI is Heparin.

1.8 Unfractionated heparin and low molecular weight heparins

Unfractionated heparin (UFH) and low molecular weight heparins (LMWHs) such as enoxaparin, are potential neuroprotective drugs that have attracted the interest of researchers worldwide due to their non-anticoagulant biological effects.

UFH popularly known and used for years as an anticoagulant, is a naturally occurring highly sulphated endogenous polysaccharide belonging to the family of glycosaminoglycan's (GAGs). It was accidentally first discovered by Jay McLean in 1916 who was a medical student at the time in Johns Hopkins Medical School where he aimed to isolate thromboplastic agents (345). Structurally, heparin consists of recurring disaccharide units of uronic acid and D-glucosamine residues linked by 1, 4 glycosidic bonds. Heparin is produced by the mast cells of several tissues such as the intestine, liver, skin and lung of most mammals. It has a molecular weight ranging from 5-40 kDa and an average weight of 15 kDa which varies across different tissues (346, 347).

The established anticoagulant effect of heparin is associated with its unique pentasaccharide sequence (Figure 1.3) which comprises the antithrombin-binding domain. This sequence binds indirectly to antithrombin III, a serine protease inhibitor which in turn enhances the inhibition of thrombin or factor IIa and factor Xa (activated factor X) (348, 349). The distinctive feature on the pentasaccharide sequence, which is the 3-0-sulfate group on the glucosamine unit found at position three in the sequence, is crucial for activating the heparin-antithrombin III complex. In addition, sulphate groups (6-O and N-sulphate groups) are necessary to facilitate high binding affinity (348, 350).

In recent years, LMWHs have replaced the clinical use of heparin due to the pharmacokinetic and pharmacological limitations that heparin presents. These limitations include drug-induced adverse effects like osteoporosis, low bioavailability associated with subcutaneous route of administration, high risk of bleeding and an unreliable dose response (351).

LMWHs are fragments of unfractionated heparin produced by chemical or enzymatic depolymerisation, which display an average molecular weight of about 4-6kDa and constitute both anticoagulant and non-anticoagulant oligosaccharides. Like heparin, LMWHs bind and activate antithrombin III through the pentasaccharide sequence to exert their anticoagulant activity, but unlike heparin, they have a reduced risk of bleeding and a better safety profile with more predictable pharmacokinetics properties and efficacy. These improvements are possibly

due to their decreased tendency to interact with macrophages, plasma proteins and endothelial cells (352). Each LMWH has distinct pharmacokinetic properties and anticoagulant profiles probably because they are prepared by different methods of depolymerisation. While on one hand, the LMWH enoxaparin, inhibited the release of inflammatory cytokines from peripheral blood mononuclear cells (PBMCs) of asthmatic individuals by more than 48%, on the other hand, dalteparin, another type of LMWH, increased their release by more than 25% in an *ex vivo* study comparing both LMWHs (353).

UFH and LMWHs which are administered via parenteral route (354) are currently used clinically for the treatment of thrombosis and thromboembolism.

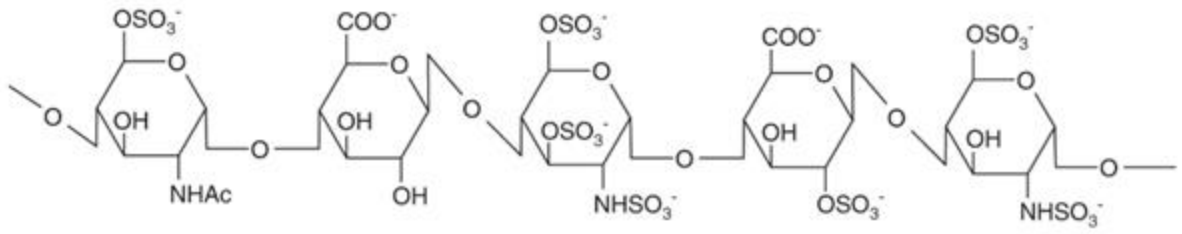


Figure 1.3: Pentasaccharide sequence of Heparin and LMWHs (355).
The pentasaccharide sequence with the distinctive feature, 3-0-sulfate group on the glucosamine unit binds to antithrombin III to bring about the anticoagulant effect of heparin.

1.9 Non-anticoagulant effect of Heparins/Low molecular weight heparins

Extensive studies have shown that UFH and LMWHs also have therapeutic potential for treating other disorders without an underlying clotting pathology, including ulcerative colitis (356), lichen planus (357, 358) and asthma (355, 359), as well as neurodegenerative disorders such as Alzheimer's disease (360). Enoxaparin was reported to inhibit syndecan-1 shedding and demonstrated a notable decrease in the expression of inflammatory cytokines, IL-1β and IL-10 in the intestinal mucosa of a dextran sulfate sodium (DSS)-induced mouse model of experimental colitis (361). Low doses of UFH and enoxaparin also decreased inflammation by reducing the activities of mucosal myeloperoxidase and nitric oxide synthase in a rat model of experimental colitis which was associated with decreased colonic weight and improved mucosal pathology as early as the first day in this study (362). Similarly, heparin and

enoxaparin were reported to significantly improve the pathology of ulcerative colitis in human patients (363, 364).

The common underlying pathology in most of these disorders is inflammation and therefore it has been proposed that UFH and LMWHs possess potential anti-inflammatory properties, which could have resulted in the improved effects observed in the various experimental models of inflammation mentioned above including attenuating neuroinflammation following brain injury (365-367). The anti-inflammatory effects of heparin are proposed to be mediated by binding and blocking the activities of the vascular cell adhesion molecules, L and P – selectin. The selective O-desulfation (368) and the 6-*O*-sulfation groups on the glucosamine residues plays a significant role in this process (369, 370). Heparin inhibited inflammation by decreasing IL-13-dependent eotaxin-1 production in human airway smooth muscle (ASM). The expression of IL-13 and the chemokine, eotaxin-1 are both markedly increased by ASM cells in asthma. This study also identified the importance of sulfation in this process (371).

Other biological effects such as anti-metastatic properties (372, 373) and anti-tumour properties (374) have also been demonstrated by non-anticoagulant heparin derivatives in various experimental models including human melanoma metastasis models (375). Although several studies have shown the anti-proliferative effect of heparin in cancer (376), a few other studies have reported an opposite effect. For instance, Uzun and colleagues reported that heparin had no significant anti-proliferative effect on colon cancer cells *in vitro* (377). Additionally, following administration of LMWHs or its derivatives, cancer patients (378) and patients with advanced malignancy (379) showed improved rate of survival, thereby further confirming the effects of the non-anticoagulant fragments of UFH and LMWHs besides their anti-inflammatory and anticoagulant activities (380-382). The blocking of L and P selectin have also been postulated to be the possible mechanism by which anti-metastasis activity is effected (383, 384).

1.10 Heparin and LMWHs in TBI

Heparin and LMWHs have shown promising neuroprotective effects (385) across different models of TBI, however, their use/application is limited due to their risk of bleeding. Both enoxaparin, a LMWH, and low doses of heparin enhanced neurologic function following TBI by decreasing brain contusion (386, 387), cerebral oedema, inflammation, microvascular

permeability, recruitment and accumulation of leukocytes (365, 388), and also reduced infarct size in ischaemia (389). Significant decrease in the expression of COX-2, hippocampal thiobarbituric acid-reactive substances, reactive gliosis and oxidized protein levels resulting in the amelioration of inflammation, oxidative stress and astrocytosis was observed following the administration of enoxaparin in a rat lateral fluid percussion brain injury model (390). Also, enoxaparin displayed dose-dependent neuroprotective effect on a cold-induced model of TBI reducing infarct volume and cell apoptosis in mice cortex (366).

These enoxaparin effects seen were reported to be associated with blocking the signalling of high mobility group box 1 (HMGB1) protein (391), a protein known to interact with toll like receptor 4 (TLR4) which is expressed by multiple cells in the brain (392-394). There is evidence to demonstrate that HMGB1 activates the nuclear factor kappa B (NF-κB) signalling pathway, which is implicated in exacerbating neuroinflammation via TLR4 (395). However, more studies are required to fully elucidate this. A high dose of heparin was shown to aggravate functional recovery caused possibly from increased bleeding in the injured brain (365, 396), whereas, on the other hand, it reduced polymorphonuclear neutrophil (PMN) accumulation and was linked with the propensity to lower systemic blood pressures as well as diminished levels of haemoglobin (396). The damage from administering high doses of heparin far outweighs the benefits.

Once more, heparin was shown to confer neuroprotective effect at a low dose incapable of initiating anticoagulation. Here, heparin reduced neuroinflammation, trans-synaptic apoptosis and demyelination in the hippocampus of model of subarachnoid haemorrhage and transient focal cerebral ischaemia (367).

Although, heparin and LMWHs have induced a remarkable decline in neuroinflammation in experimental models of TBI, the exact mechanisms behind these effects remain unclear. One thing that seems to be quite clear is that the non-anticoagulant fragments of heparin and LMWHs possess other biological effects that needs to be explored and studied in detail for their therapeutic potential in the treatment of TBI and perhaps other neurological disorders.

1.11 Separation of the non-anticoagulant oligosaccharides

The non-anticoagulant and anticoagulant oligosaccharides of enoxaparin are commonly separated by chemical or enzymatic depolymerisation. However, the depolymerisation process could lead to structural modifications and physical changes of the oligosaccharides especially during very high temperatures and freeze-drying processes. For example, freezing and thawing resulted in the physical changes of some oligosaccharides present in LMWHs and altered their biological effects (397). Similarly, when exposed to heat that is typically used in desulfation process, LMWHs oligosaccharides undergo chemical modifications which is associated with a gradual loss of activity (398).

In recent years, a variety of separation techniques that does not require initial chemical or enzymatic digestion of oligosaccharides were developed to isolate and identify different fragments of the complex LMWHs oligosaccharides, which were mostly based on their molecular weights. These techniques include reversed-phase ion pair high-performance liquid chromatography (RPIP-HPLC) (399, 400), capillary electrophoresis (CE) (401, 402), and high-performance size exclusion chromatography (HP-SEC) (402, 403). However, limitations such as from the inability to separate oligosaccharides with masses greater than octasaccharides as well as structurally different oligosaccharides with the same or similar molecular masses limit the usefulness of these techniques for the separation of the complex oligosaccharides' chains in LMWHs. Ion-exchange chromatography (IC) is another analytical technique that fractionates oligosaccharides based on their ion charge. Various oligosaccharides fractions of enoxaparin were identified by this method and their molecular weights were established (404).

1.12 Heparin derivatives- Degree of Polymerization 2 & 4

Degree of polymerization (Dp) is simply the number of monomeric units in a polymer or macromolecule. Therefore, Dp2 and Dp4 are disaccharide and tetrasacharide units, respectively of structurally undefined macromolecules that are extremely sulphated. They are not associated with bleeding risks because they do not contain the pentasaccharide sequence (Figure 1.3) which binds anti-thrombin III to bring about the anticoagulant activity of heparin. Therefore, this could make their use a much better therapeutic approach compared to enoxaparin in the treatment of disease or disorders with underlying inflammatory pathologies, as long as they retain enoxaparin's anti-inflammatory actions.

Shastri and colleagues successfully separated the non-anticoagulant and anticoagulant oligosaccharides with each fraction named according to its degree of polymerization and the length of their chain. For example, enoxaparin's Dp4 chain is composed of two disaccharide units: that is, four sugars, hence its name. This study further showed that the separated enoxaparin non-anticoagulants oligosaccharides (Dp2 and Dp4) have no anticoagulant activity, using a modified low volume microtitre plate anti-factor Xa (AFXa) assay (404). Furthermore, as a measure to determine anti-inflammatory effects, Dp4 inhibited the release of nitric oxide (NO), whose production by activated macrophages, which is involved in enhanced inflammation (405).

Dp2 and Dp4 fractions have shown robust anti-inflammatory activity across various studies of inflammation. The release of TNF–α was supressed by Dp2 and Dp4 in cultures of peripheral blood mononuclear cells (PBMCs) of asthmatic patients/subjects (353). Both fractions also inhibited the release of IL-6 and IL-8, two main pro-inflammatory mediators involved in the pathogenesis of asthma, in an *in vitro* study of human pulmonary epithelial cells (406).

While the oligosaccharide fragments of heparin/LMWHs are associated with both pro- or anti-proliferative effects, the smaller oligosaccharides, for example Dp2, display anti-proliferative effects by the significant inhibition of proliferation in human colon carcinoma epithelial cells *in-vitro.* Importantly, this effect was reported to be independent of their anticoagulant activity, whereas, oligosaccharides with minimal or more anticoagulant property, enhanced proliferation (407). Likewise, Kazi and colleagues (408) reported that heparin derivatives with absent or reduced anticoagulant activity suppressed the proliferation of smooth muscle cell (SMC) after arterial injury in rats. In this study, the anti-proliferative capacity of chemically altered heparin derivatives increased with decreased anti-coagulant activity especially for the smallest derivative that showed no anti-coagulant activity and consisted of tetrasaccharides with a molecular weight of 1,400 Da (408).

The Dp4 fraction was also associated with anti-cancer activity. In a Severe Combined Immunodeficient mouse model that was intravenously injected with human melanoma cells, Dp4 reduced the formation of lung metastasies. (409).

While the anti-inflammatory effects of Dp2 and Dp4 have been assessed in several tissues such as lung and gut, it has never been assessed in the brain, which forms the basis for the present thesis.

1.13 Hypothesis

Unfortunately, to date, there is still no effective treatment available for TBI despite extensive research into identifying and developing potential pharmacotherapies targeting various aspects of TBI pathophysiology. Considering the central role that neuroinflammation plays in promoting secondary TBI-induced damage, drugs targeting the inflammatory cascade are being investigated as potential new therapies. With increasing evidence linking the beneficial effects of enoxaparin to its non-anticoagulant properties, for the first time, to our knowledge, disaccharide and tetrasaccharide fragments of enoxaparin, Dp2 and Dp4, were assessed for anti-inflammatory activity in the brain following TBI. Unlike their parent drug, enoxaparin, Dp2 and Dp4 are not associated with bleeding risk and they have shown robust anti-inflammatory effects in *in-vitro* and *in-vivo* models of peripheral inflammation. Therefore, we hypothesise that treatment with Dp2 and Dp4 will also decrease neuroinflammation and improve behavioural outcomes in a mouse model of focal TBI.

1.14 Aims

1. 1a. To determine if treatment with enoxaparin and Dp4 decreases gliosis in the pericontusional cortex at 3 days post-TBI.

1b. To determine the optimal dose and best mode of administration of Dp4 and Dp2.

2. To determine if treatment with enoxaparin and Dp4 decreases the levels of inflammatory cytokines in the pericontusional cortex at 6 hours post-TBI

3. To determine if treatment with Dp2 and Dp4 improves long-term functional outcomes following TBI.

4. To systematically evaluate and meta-analyse the available literature on the potential neuroprotective effects of heparin and LMWH as treatment for TBI.

CHAPTER 2

GENERAL METHODOLOGY

2.1 Overview

This Chapter presents the general materials and methods that was employed for this thesis. Here, details of the TBI model used, the drug types, modes of drug delivery and the various doses of drug used were highlighted. Furthermore, detailed description of the different outcome measures as well as their analysis were also outlined.

2.2 Animals

All experimental procedures were approved by the University of Tasmania (UTAS) Animal Ethics Committee under approval numbers A0016233 and A18665. These experimental procedures were conducted in accordance with the code of practice for the use and care of animals for scientific purposes of the National Health and Medical Research Council. Adult C57BL/6 male mice were purchased from the UTAS breeding facility at Cambridge and were allowed to acclimatise for 7 days prior to experimentation. The mice were housed with food and water ad libitum under a 12 h dark/light cycle. Mice were subjected to trauma at age 10-14 weeks, weighing 25–32 g.

2.3 Controlled Cortical Impact model of TBI.

Controlled cortical impact (CCI) injury model, a type of unilateral moderate focal brain injury, was performed based on a previously described protocol (410). Mice were weighed and then placed into a chamber filled with 4% isoflurane for approximately 2 minutes or until their breathing rate was observed to be deep with approximately 60 breaths per minute. The depth of anaesthesia was assessed by monitoring the respiration rate and by checking the pedal withdrawal reflex. Then, the scalp fur was shaved, and the mouse's head was positioned onto the stereotaxic frame (Figure 1) where anaesthesia was maintained at 2% throughout the entire procedure. Eyes were coated with lacri-Lube to prevent them from drying out and mice were administered 5 mg/kg of Meloxicam as general analgesia, while Lidocaine (~5 mg/kg at 0.25%) and Bupivicaine (~1 mg/kg at 0.06%) were co-administered subcutaneously as local analgesia (total ~40 ul was injected into the scalp region). A 1cm midline scalp incision was made using a scalpel blade to expose the skull. Then, the precise coordinates for trauma induction, which is centred at 2 mm posterior and 2 mm lateral to bregma, were identified (Figure 2.1), a craniotomy (~5 mm diameter) was performed at these coordinates with a dental drill and the

exposed brain was then subjected to a controlled cortical impact using a flat surfaced cylindrical rod (3.0mm diameter tip) driven by a computer controlled electromagnetic impactor (Leica MyNeuroLab Impact One™ Stereotaxic impactor, Leica Biosystems, Inc.) whose tip was aligned with the dura at an angle of approximately 20° to allow for the curvature of the brain at this location. The impact parameters for trauma induction were 5m/s velocity, 1.0 mm depth and 500 ms dwell time (see set-up in Figure 2.2). Lastly, the incision was sutured, and mice were removed from the stereotaxic frame and placed in a heated recovery box (37°C) where they were monitored until they regained consciousness. Then mice were transferred to a clean cage with food and water ad libitum. Mice were housed individually after trauma.

The control sham operated mice underwent all anaesthetic and surgical procedures described above, except for the impact. They were returned to a heated recovery box and moved to a clean cage with food and water ad libitum. The mortality rate for this procedure is less than 1%.

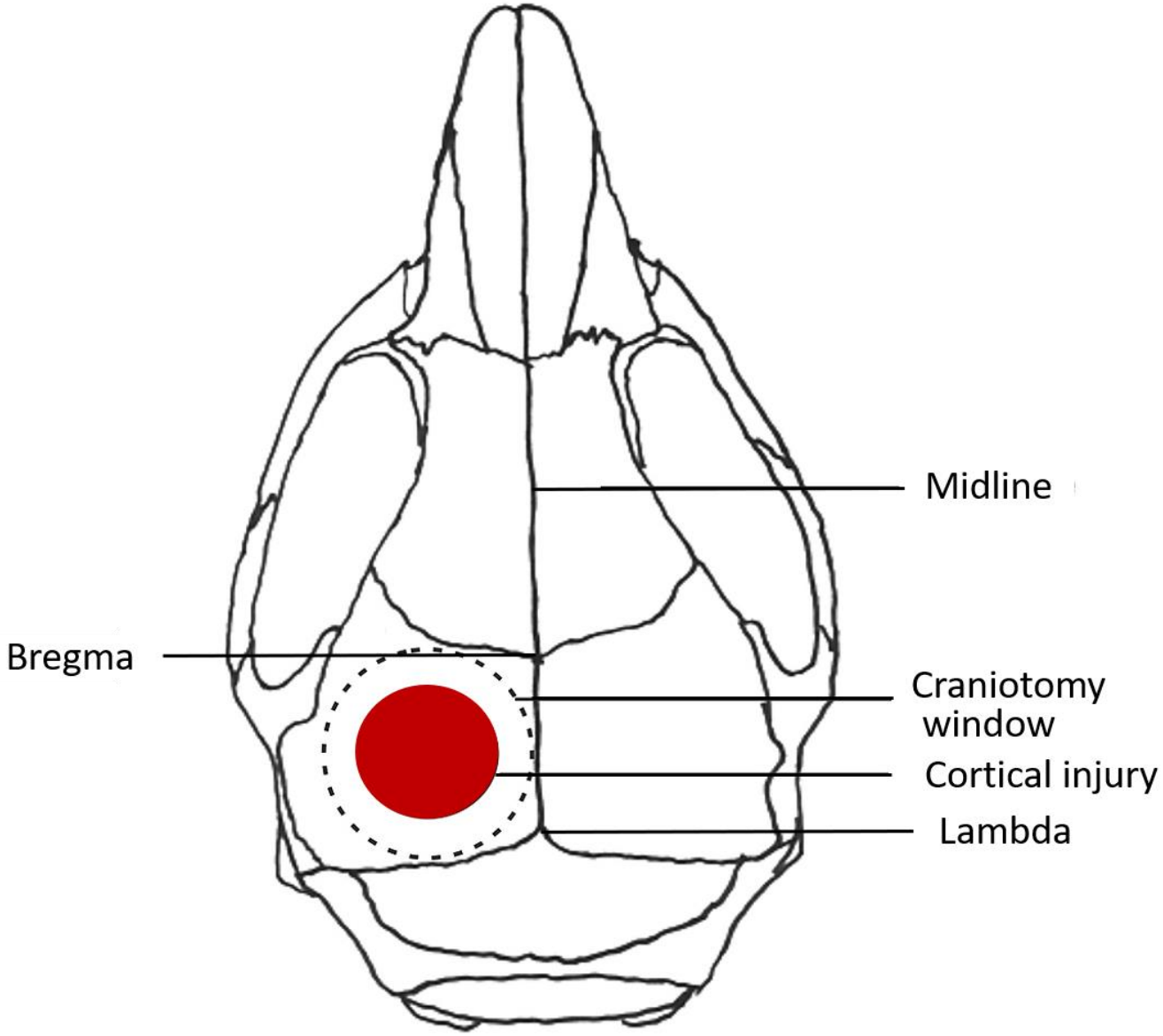


Figure 2.1: Schematic representation of the position of cortical injury (Adapted) (411).

The site of injury as depicted by the red circle was centred at 2 mm posterior and 2 mm lateral to bregma. The craniotomy performed was approximately 5 mm diameter.

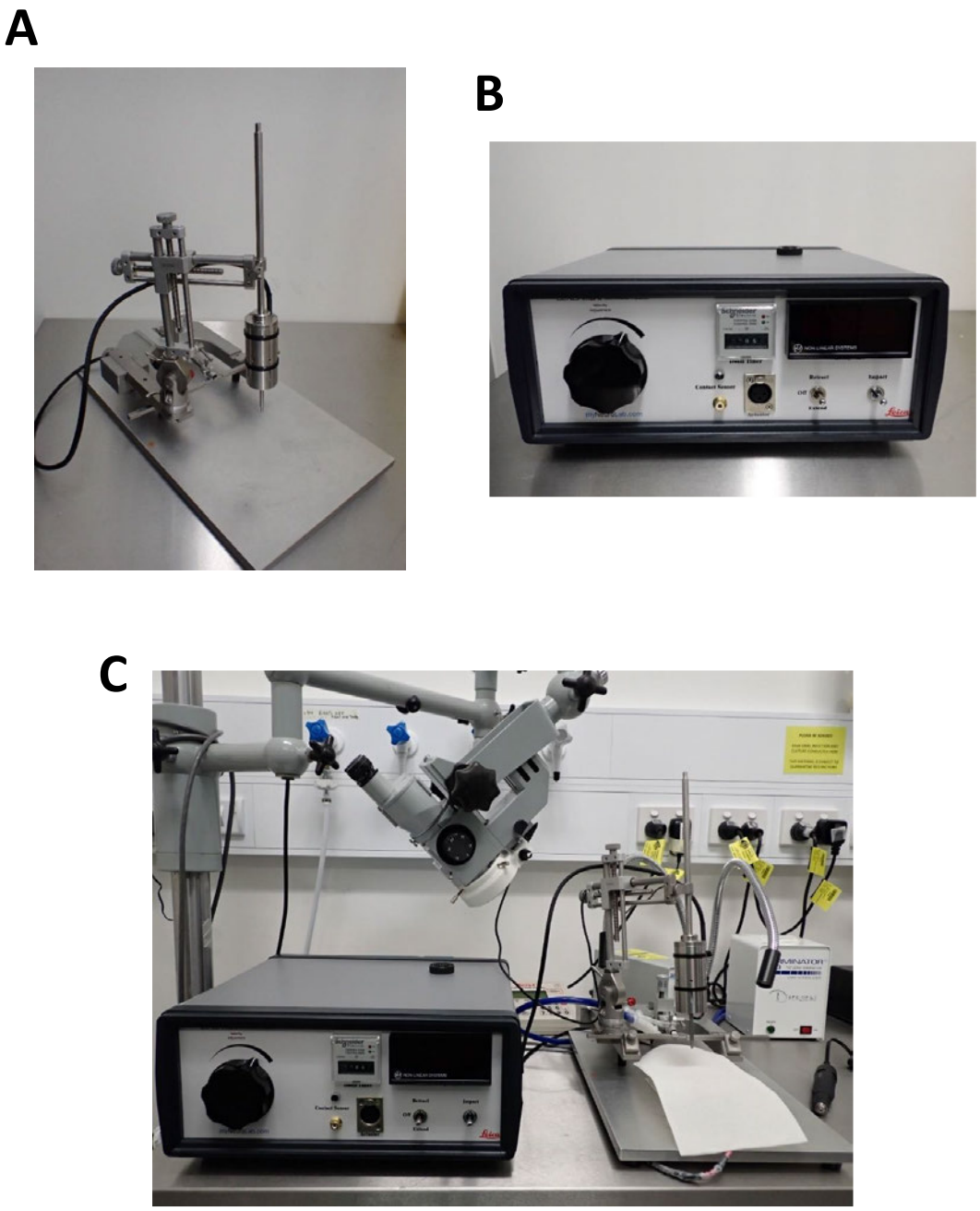


Figure 2.2: Electromagnetic controlled cortical impact device for trauma induction. *Controlled cortical injury in mice following craniotomy was induced using the flat surfaced cylindrical rod with 3.0 mm diameter tip on the stereotaxic frame* ***(A)*** *which was driven by a computer controlled electromagnetic impactor* ***(B).*** *The 3.0 mm diameter tip was aligned with the dura at an angle of approximately 20° to allow for the curvature of the brain at this location and the impact parameters for inducing trauma were 5 m/s velocity, 1.0 mm depth and 500 ms. Full device set up is shown in the third image* ***(C).***

2.4 Drug administration

Enoxaparin, Dp2, Dp4 and a combined therapy of Dp2 and Dp4 were administered by subcutaneous infusion (SC) via mini–osmotic pumps, intraperitoneal (IP) bolus injections or by bolus subcutaneous injections (Table 2.1). Vehicle control TBI and sham groups received sterile saline. Dp2 and Dp4 dose were based on their equivalent amount in enoxaparin.

2.4.1 Implantation of osmotic minipumps for drug infusion

The osmotic minipumps (Alzet model 2006) were pre-filled with drug solution as per the manufacturer's instructions and were implanted subcutaneously at the time of CCI surgery. For pump placement, the skin below the shoulders of the mouse was loosened by inserting a pair of small sterile haemostats through the scalp incision to make a pocket for the subcutaneous insertion of the minipumps. The minipumps were then inserted into the pocket following the CCI and skin was sutured. Mice were then placed in a heated recovery box and monitored till they regained consciousness.

Table 2.1: Summary of all drugs and dosage

Drugs	**SC infusion**	**IP injections**	**SC injections**
Enoxaparin (dose based on published studies)	2.5 mg/kg/24 hrs	1 mg/kg/injection	1 mg/kg/injection
$Dp4_{LOW}$ (equivalent to amount of Dp4 fraction in enoxaparin dose)	129 µg/kg/24 hrs	50 µg/kg/injection	-
$Dp4_{HIGH}$ (Twice $Dp4_{LOW}$)	258 µg/kg/24hrs	100 µg/kg/injection	104 µg/kg/injection
Dp2	-	-	104 µg/kg/injection
Dp2 + Dp4 Combined	-	-	104 µg/kg Dp4 + 104 µg/kg Dp2 per injection

2.5 Tissue collection and processing

Animals were euthanised at different timepoints post-TBI or sham procedure for the different experiments. Brains were collected at 3- and 30 days post-TBI or sham procedure for cryosectioning and subsequent immunohistological labelling and analysis of glia and immune cells. Brains collected at 30 days were not analysed in this study. They were extracted from the animals that were assessed for behaviour for future studies. Brains were also collected at 6 hours post-TBI or sham procedure for protein extraction to quantify cytokine and chemokine levels.

2.5.1 Perfused tissue and cryosectioning

Animals were terminally anaesthetised with an intraperitoneal injection of 110mg/kg body weight of sodium pentobarbitone and when animals became deeply anaesthetized, they were pinned to a board in a supine position. A bilateral thoracotomy was performed to expose the heart and a 23G needle connected via a tube to the perfusion pump was then inserted into the left ventricle. 0.01M phosphate buffered saline solution (PBS) was first flushed through the mice for approximately 1-2 minutes to clear the blood from the vasculature, followed by 4% paraformaldehyde (PFA) for tissue fixation. At the same time, the jugular veins and aorta were incised to allow for the evacuation of the blood vessels. 4% PFA was infused at a flow rate of 2.5 ml/min for four minutes and approximately 10ml was flushed through each animal.

Brains were removed and placed in 4% PFA overnight, followed by cryoprotection in 30% sucrose for 48 – 72 hours at 4°C. The olfactory bulb and cerebellum were removed using a brain matrix and brains were then placed in a cryomold and embedded in optimal cutting temperature (OCT). Brains were frozen using a Leica CM 1850 cryostat and were stored at -80°C until sectioning.

Brains were removed from -80°C to -20°C at 24 hours prior to sectioning and this is to allow the brains to equilibrate before cutting takes place. Serial coronal sections were cut at 12 μm or 30 μm thick for slide mounted and free-floating tissues respectively, spanning across the entire lesion area using a Leica CM 1850 cryostat at a cutting temperature of -18°C. It is important to note that the different thicknesses were used in different experiments and was not mixed up in one single experiment. Sections were collected in a 1:8 series for free floating frozen brain sections (approximately 20 sections/brain) or mounted onto microscope slides in 1:25 series with approximately 3-4 tissues per slide totalling between 200 to 320 sections per

brain (approximately 16-22 sections/brain). Free floating frozen brain sections were stored at 4°C in PBS azide (0.01% azide/200 ml PBS) until use while slide mounted sections were stored at -80°C until needed. Collection of brain sections started from approximately coronal level 44 (bregma 1.05 mm) to about coronal level 97 (bregma −4.38 mm) (412). This brain region was collected because, beside it being the area around the injury, it is the sensorimotor area, linked to motor output which will also allow for the assessment of motor function deficit after trauma.

2.5.2 Tissue homogenization and protein quantification

Mice were also anaesthetised with intraperitoneal injection of 110mg/kg of sodium pentobarbitone as described above and once deep anaesthesia was established by pedal reflex and tail pinch, mice were then transcardially perfused with 0.01M PBS in order to clear the blood from the vasculature. Brains were immediately removed, dissected on ice and snap frozen in 0.5 ml Eppendorf tubes on dry ice. Brains were dissected into left and right cortex (with a 5 mm diameter punch containing the lesion or equivalent region of the uninjured side and shams, collected separately to the remaining cortex), hippocampus, mid-brain, and cerebellum. Frozen brain samples were stored at -80°C until protein extraction.

For protein extraction, frozen brain samples (cortex punches) were added to ~300ul of ice-cold extraction buffer (w/w) containing 50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1% triton-100, and protease inhibitor cocktail tablets (complete EDTA-free, Roche diagnostics) using a dilution ratio of 1:4 parts tissue to buffer respectively. Tissue-buffer samples were homogenised at high speed with an IKA ultra TURRAX T10 homogenizer for 20 seconds and was stopped for 10 seconds (this process was repeated twice), then all samples were left to shake on ice for 90 minutes and centrifuged at 12,000g for 15 minutes at 4°C. Tubes were gently transferred from the centrifuge into ice, supernatants were collected, and pellets were discarded. Bradford assay (Bio-Rad Laboratories) was used on the supernatants to determine the total protein concentrations. Samples were aliquoted (50µl) and stored at -80°C until cytokine / chemokine analysis.

2.6 Immunohistochemistry

Immunohistochemistry (IHC) is an important tool that employs the use of specific antibodies to identify target antigens in cells or tissues. Due to the multiple steps involved in IHC, it is important that every step be optimised to enhance signal detection. The basic

principle of IHC that requires the appropriate detection and binding of primary antibody to target cell or tissue antigen is the most critical element in the process of IHC. Afterwards, a suitable secondary antibody is used to bind the primary antibody for signal detection. While the process of IHC could vary depending on the experiment and target antigen, for this study, the target cells, astrocytes and microglia that were immunostained as detailed in chapter 3 were visualized by conjugating secondary antibody to a fluorophore that was detected by fluorescent microscopy following primary antibody incubation. I was blinded to the experimental groups throughout the period of performing immunohistochemistry and analysis. Details of all antibodies used are summarised in table 2.2.

2.7 Quantification of immunepostive cells

Percentage area positive for GFAP staining was calculated using the commonly used quantification method, thresholding in order to compare results with the newly adopted automated image analysing tool imageSURF (see appendix A). For this analysis, images from 17 mice (n=5-6 of three groups) were converted to greyscale using imageJ software, then all images were adjusted to similar brightness and contrast. Thereafter, the threshold cut-point was consistently adjusted until a good number of the positive cells were identified, then, the same threshold cut-point was applied to the rest of the images. GFAP immunofluorescence was determined in the ROIs (described above) of only the injured cortex and was expressed as mean %GFAP staining. To minimise bias, I was blinded to the experimental groups throughout the entire process of analysis.

2.8 ImageSURF analysis

One of the major challenges in biomedical research is the lack of effective tools for quantifying immunohistochemical and immunofluorescent labelling. Most of the available tools such as the commonly used thresholding method requires the investigator to choose a thresholding limit that best distinguish between the actual positive signal and background noise which is then applied to the rest of the images (413). This is subjective, especially for an investigator who is not blinded. Due to this subjectivity, the thresholding method is not reliable, as it does not take into account the individual characteristics of the images, variability in pixel intensity across the entire image set, screen brightness and ambient light thereby leading to

increased bias and poor result reproducibility (414). Hence, the need for a more reliable automated quantification tool that will improve the accuracy of image analysis with minimum bias, increased repeatability, and better computational time.

ImageSURF is a newly developed automated trainable image segmentation plug-in on Image-J (Fiji). It segments images based on well-defined, pre-trained pixel details (415) which could be further enhanced to identify image artifacts and intensity shifts by considering the entropy, texture, edges, and intensity of each pixel. For instance, to analyse the images that were captured at 20x with Olympus-VS120-L100-W virtual slide-scanner, ROIs from a set of images representing the overall characteristics of all the images to be quantified were selected (800 x 800 pixels or 1200 x1200 pixels depending on the series in which the image was processed on imageJ) from both the injured and uninjured hemisphere. These images totalling approximately 21 to 30 images were then converted to 8-bit RGB (red, green, and blue channels) with brightness and contrast automatically adjusted. This was simply done to visibly annotate the various features on the image. Then, using adobe photoshop element 15, the immunolabelling on these images was manually annotated into different classes of features (positive signal, tissue background, slide background and out of focussed positive signal) that were to be segmented (Figure 2.3B). Afterwards, a specialised algorithm within the ImageSURF tool studied the annotated pixels while comparing it with the unannotated (Figure 2.3A) and raw (no brightness/contrast adjustments made) versions of the same images to create a classifier model that clearly distinguished between the classes of features identified above (Figure 2.3C). The classifier was saved and was applied to rest of the identically processed images of ROIs that was obtained. This segmentation method is consistent, unbiased, and more reproducible than thresholding.

It is important to state that as a new tool that was being used for the first time to quantify immunofluorescent labelling of glial cells in TBI mouse brain, each of the steps mentioned above had to be optimised and as with all systems, there were challenges involved with using imageSURF. One of the major challenges was that it was prohibitively, egregiously, and extraordinarily time consuming. It took approximately 12-16 hours ±4 hours or more to run one image. Secondly, the feature files saved during the analysis are extremely large (~100 GB) and oftentimes a run was interrupted due to lack of storage space.

ImageSURF will be an excellent tool for the quantification of immunohistochemical and immunofluorescent labelling if the issues stated above are addressed.

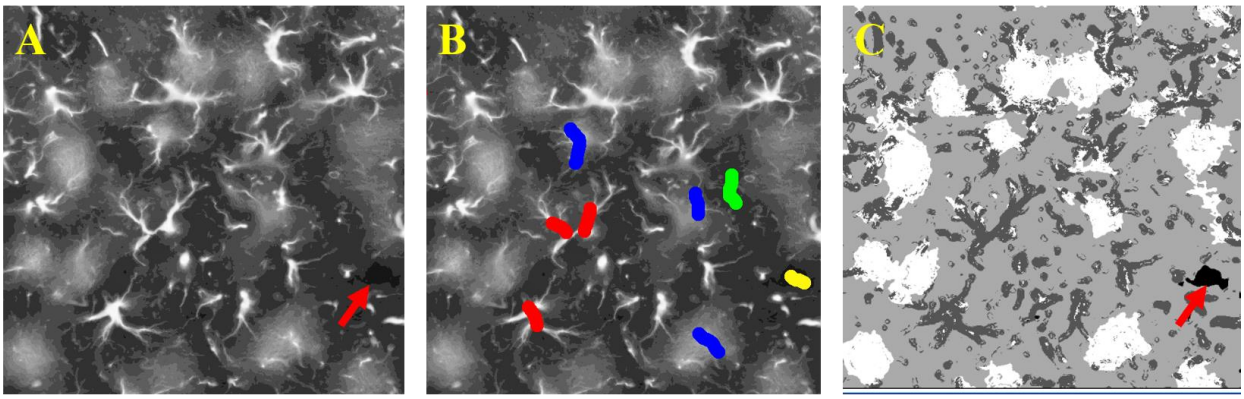


Figure 2.3: Image segmentation by imageSURF plugin on imageJ (fiji)

ImageSURF is an automated pixel – based image segmentation tool. Images were first converted to 8-bit RGB with brightness and contrast adjusted ***(A)****. Thereafter, image features were identified following manual annotations (positive signal – red, slide background – yellow, tissue background – green, out of focussed positive signal – blue) indicating the different classes to be segmented* ***(B)****. A machine derived algorithm studied the marked pixels in image B and created a classifier that clearly distinguished the identified classes* ***(C);*** *dark grey – positive signal, light grey – tissue background, white– out of focus signal. The classifier was then applied to the rest of the images to be quantified. The red arrow on image A and C shows a region of slide background, which is not included in the quantification.*

2.9 Group numbers and statistical analyses

Power analysis calculations was performed to determine the minimum number of animals required to obtain a strong, statistically significant effect of treatment. All data were presented as mean ± standard deviation (SD) for each treatment group and the n's for each group is outlined in table 2.3.

Group comparisons were made with either unpaired *t*-test, one- or two-way analysis of variance (ANOVA) or by repeated measures ANOVA. Tukey's post-hoc test was used for comparisons between and within the different factors. A p-value of $p < 0.05$ was considered statistically significant. All calculations were performed with GraphPad prism (version 9) software.

Table 2.2: Details of antibodies used for immunohistochemistry

Primary antibody	**Target antigen**	**Cell type identified**	**Dilution**	**Host**	**Company and catalogue #**	**Secondary antibody/conjugate**	**Dilution**	**Host**	**Company**
Anti GFAP	GFAP, an intermediate filament protein.	Astrocytes	1:1000	Rabbit	DAKO #Z0334	Anti-rabbit IgG Alexa fluor 488nm	1:250	Donkey	Invitrogen #LTSA21206
Anti IBA-1	IBA-1, a calcium binding protein.	Resting and activated microglia/macrophage	1:500	Rabbit	WAKO #019-19741	Anti-rabbit IgG Alexa fluor 488nm	1:500	Donkey	Invitrogen #LTSA21206
Anti CD68	CD68, a type-1 transmembrane glycoprotein.	Activated and phagocytic microglia / macrophage	1:500	Rat	BioRad #MCA1957	Anti-rat IgG Alexa fluor 594nm	1:500	Donkey	Invitrogen #A-21209

Abbreviations: GFAP - Glial fibrillary acidic protein; IBA-1 – Ionized calcium-binding adaptor protein-1; CD68 – Cluster of differentiation 68

Table 2.3: Summary of total number of animals used and analysed.

Experiment	Total animals used	Final number of animals in each group (n)								Outcome measures	Number of animals statistically analysed	Excluded animals	Reason for exclusion
		Grp1	Grp2	Grp3	Grp4	Grp5	Grp6	Grp7	Grp8				
Chapter 3: Brain immune cells (SC* infusion)	29	-	6	6	5	6	-	-	-	GFAP immunostaining	23	6	Poor sham surgery
Chapter 3: Brain immune cells (Repeated IP* injections)	16	-	3	4	3	3	-	-	-	GFAP immunostaining	13	3	Damaged tissue
Chapter 3: Brain immune cells (Repeated IP* injections)	16	-	4	4	4	4	-	-	-	IBA-1/CD68 immunostaining	16	-	N/A

Chapter 4: Cytokines (SC infusion)	27	-	5	-	6	-	-	-	-	23 cytokines multiplex kit	11	16	Poor shams (n = 4). Limited available sample wells in multiplex plate for Enoxaparin and Dp4low groups (n = 6 each).
Chapter 4: Cytokines (repeated IP injections)	30	6	6	-	6	6	-	-	-	23 cytokines multiplex kit	24	6	Limited sample wells in multiplex Dp4low group.
Chapter 5: Behavioural outcomes	75	11	11	-	-	12	8	10	11	Ledged beam and hanging wire tasks	63	12	Culled (n = 2), incomplete trauma from blown fuse (n = 4) and missed data (from one day of no testing, n = 6).
Chapter 5: Behavioural outcomes	75	12	11	-	-	12	10	12	12	Open field task	69	6	Culled (n = 2), incomplete trauma from blown fuse (n = 4)
Total	**268**	**29**	**46**	**14**	**24**	**43**	**18**	**22**	**23**		**219**	**49**	

Keys: Grp1 – Sham, Grp2 – TBI+Vehicle, Grp3 - TBI+Dp4Low, Grp4 - TBI+Dp4High, Grp5 - TBI+Enoxaparin, Grp6 - TBI+Dp2, Grp7 - TBI+Dp4, Grp8 - TBI+(Dp2+Dp4)

*SC – subcutaneous; IP – intrapetritoneal

Note: The same animals but different sections were used for the GFAP and IBA-1/CD68 experiment. The same animals were used for all behavioural assessments.

CHAPTER 3

EFFECT OF ENOXAPARIN AND DP4 ON GLIOSIS

3.1 Introduction

Neuroinflammation is a complex pathophysiology of TBI that contributes to ongoing neuronal damage and the activation of astrocytes and microglia plays a very crucial role in this process (reviewed in Chapter 1). While the importance of gliosis in protecting the brain and enhancing tissue repair and recovery has been demonstrated in studies that have reported exacerbated TBI-induced damage following the acute ablation of astrocytes and microglia (130, 131, 416), prolonged glial activation becomes detrimental, worsening outcomes and eventually resulting in neuronal cell death (110). Hence, all these studies together suggest that a successful therapeutic intervention for treating TBI may be to regulate the neuroinflammatory process, rather than abolish it (417, 418).

Consequently, potential new therapies targeting specific aspects of the neuroinflammatory process appear to be of interest as several studies have indicated that attenuating gliosis could improve recovery outcome. For instance, minocycline was previously shown to reduce lesion volume and improve neurological outcome by reducing microglial activation in a closed head injury model of TBI (323). Similarly, astragaloside reduced TBI-induced cerebral contusion, neuronal apoptosis and neurological motor dysfunction by attenuating microglial activation in a rat model of TBI (419). Carprofen, also resulted in improved functional outcomes by decreasing gliosis following TBI (420). Collectively, the drugs from these studies exhibited potential anti-inflammatory effects leading to beneficial outcomes after trauma.

As detailed in chapter 1, enoxaparin, a LMWH, has shown numerous potential neuroprotective benefits improving outcomes following TBI (385). However, treatment with enoxaparin is often delayed or withheld from TBI patients due to an increased risk of cerebrovascular haemorrhage (421). Dp4 is a tetra-saccharide fragment of enoxaparin with anti-inflammatory properties, but no anticoagulant activity (353).Therefore, with no risk of bleeding and the robust anti-inflammatory effects of Dp4 in the peripheral system (406, 422, 423), as well as considering that there is evidence that enoxaparin improves TBI-induced outcomes (386-388, 390) including decreased reactive gliosis (390) and decreased leukocyte accumulation (386, 388), we hypothesise that enoxaparin and for the first time, Dp4, will attenuate the deleterious effects of neuroinflammation and associated secondary brain damage in a control cortical injury model of TBI. Specifically, we aimed to investigate whether enoxaparin and Dp4 will affect astro- and microgliosis, since evidence suggests that these drugs

may exert anti-inflammatory effects by blocking toll like receptors. For example, UFH, LMWHs or chemically modified heparins inhibit the activation of nuclear factor kappa B (NF-κB) signalling pathway (424, 425), an inflammatory pathway that induces the release of pro-inflammatory genes and mediators such as cytokines and chemokines (426). In the brain, activation of NF-κB signalling pathway is mediated via toll like receptor 4 (TLR4), which is expressed by most brain cells (393) including astrocytes (427) and microglia (428). Therefore, enoxaparin and Dp4 could possibly inhibit the activation of the NF-κB signalling pathway by blocking TLR4, resulting in decreased gliosis following TBI.

This is likely to be a successful therapeutic strategy for the treatment of TBI, since there is evidence that following TBI, activation of TLR4 induced neuroinflammation and exacerbated secondary injury mediated brain damage including neurological impairments and cerebral oedema (429-432). Suppressed astrocyte activation following TBI was demonstrated in TLR4 knockdown rats (431). Likewise, following TBI, a shift in inflammatory response towards microglia M2 phenotype promoting tissue repair was exhibited in TLR4 knockout mice (433). Together, these studies confirm that the TLR4 pathway plays a crucial role in driving neuroinflammation post-TBI, and that blocking its activation has beneficial effects.

Despite the beneficial effects demonstrated by enoxaparin treatment following TBI, there seem to be inconsistencies in the literature on its most effective dose and route of administration. Formulation of an appropriate dosing regimen including identifying a suitable route of drug administration is a critical process in the preclinical testing of novel therapeutics which contributes to determining the safety and efficacy of the drug as well as its overall performance in clinical trials (434, 435). To address this challenge, in this study, drugs were administered as either a low or high dose intraperitoneally or subcutaneously so as to determine which dose yields the most maximal effect following trauma.

Additionally, this chapter aimed to evaluate the therapeutic effect of enoxaparin and Dp4 on gliosis in a focal model of TBI. Also, I aimed to validate our CCI model of TBI, which was being used by our group for the first time in these studies, by comparing the glial activation I observed to similar studies from the TBI literature.

3.2 Methods

To address the aims of this study, C57Bl/6 mice were subjected to controlled cortical impact (CCI) as described in section 2.3. Enoxaparin or Dp4 drugs were administered either by subcutaneous (SC) infusion or by repeated intraperitoneal (IP) injections beginning at 30 minutes post-TBI, and at 3-days post-TBI, mice were killed (Figure 3.1). Mouse brains were collected and processed for immunohistochemistry to identify microglia and astrocytes, which were then quantified as percent area covered by immunolabelling using imageSURF software, or by counting the number of cells in a defined region of the cortex and/or hippocampus manually.

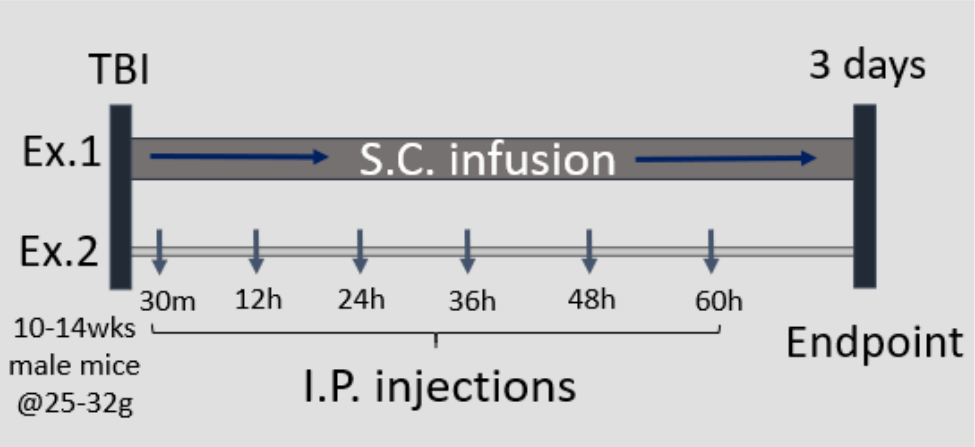


Figure 3.1: Schematic representation of experimental timeline

Two modes of drug delivery were employed in this study. SC infusion of the drugs were administered via mini-osmotic pumps for 3 days post-TBI and multiple IP injections were given for up to 60 hours post-TBI.

The SC and IP experiments were performed separately, with the sections for the two experiments collected differently: either as free-floating sections or as slide mounted sections, respectively. In the IP experiment, sham mice were not included as a control. While data from sham-controls can provide important information regarding the extent of a drug's beneficial effect, our main research question was focused on whether treatment reduces the amount of glial activation relative to untreated-TBI mice. Based on extensive literature exploring the effect of anti-inflammatory drugs on glial activation following experimental TBI, we would not anticipate that our treatment would be able to eliminate gliosis, thus returning the extent of

glial staining to sham levels; therefore, statistical comparisons to this group are not required for this particular analysis.

For the experiment in which drugs were administered via SC infusion, tissue sections were immunolabeled for microglia markers by a master's student in our group, and therefore the results for this are not included here.

3.2.1 Drug Administration

Enoxaparin, low dose Dp4 (Dp4Low), high dose Dp4 (Dp4High) and vehicle (saline) were administered either by subcutaneous infusion (SC) via mini–osmotic pumps or by repeated intraperitoneal (IP) injections (Table 3.1). Vehicle control TBI and sham groups

Table 3.1: Drug dosage for each mode of delivery

Drugs	**SC infusion**	**IP injections**
Enoxaparin (dose based on published studies; used as a benchmark for anti-inflammatory activity).	2.5 mg/kg/24 hrs	1 mg/kg/injection (Delivered as ~60 ul of 500 ug/ml solution in saline).
$Dp4_{LOW}$ (equivalent to amount of DP4 fraction in Enoxaparin dose)	1.29 mg/kg/24 hrs	50 µg/kg/injection (Delivered as ~60 ul of 25 ug/ml solution in saline).
$Dp4_{HIGH}$ (Twice $Dp4_{LOW}$)	2.58 mg/kg/24hrs	100 µg/kg/injection (Delivered as ~60 ul of 50 ug/ml solution in saline).

received sterile saline. Drugs were subcutaneously infused for 3 days post-TBI or administered as multiple IP injection every 12 hourly starting at 30 minutes and finishing at 60 hours post-TBI.

3.2.2 Immunohistochemistry

Refer to section 2.5 and 2.7 for details of tissue collection and processing.

3.2.2.1 Glial fibrillary acidic protein (GFAP)

Activation of astrocytes was evaluated by immunostaining brain sections with anti-GFAP antibody, an intermediate filament protein marker specific for astrocytes. A complete 1:8 series for free-floating sections and a complete 1:25 series for slide-mounted sections were used (approximately 16-25 sections per mouse). Prior to the start of the experiment, slide mounted frozen brain sections were removed from -80°C and aired dried for 5 to 10 minutes. Both free floating and slide mounted frozen brain sections were washed in 0.1M Phosphate Buffered Saline (PBS) (3 times, 5 minutes each) and blocked with 4%w/v milk for 60 minutes at room temperature. Then, all sections were incubated with rabbit anti-GFAP (1:1000 in 0.1M PBS, DAKO, cat #Z0334) primary antibody overnight at 4°C. The negative control was not incubated with primary antibody but with PBS. Sections were incubated with donkey anti-rabbit IgG Alexa fluor 488nm fluorescent secondary antibody (1:250 in 0.01M PBS, Invitrogen, cat #LTSA21206) for 60 minutes following washing in PBS (3times, 5minutes each) after primary antibody incubation. Then, the sections were washed again in PBS for 4 times at 10 minutes each and on the third wash, 4',6-diamidino-2-phenylindole (DAPI; 1:10,000) was added to stain the nuclei. Following the final wash, the floating sections were mounted on microscopic slides and all slides were cover slipped with DAKO fluorescent mounting media. Negative controls were incubated in secondary fluorescent antibody and were included in each batch of GFAP IHC run.

3.2.2.2 Microglia/Macrophages

The activation and accumulation of microglia/macrophages was determined following immunostaining with anti IBA–1 antibody, a cytoskeletal protein marker (436) and the cell surface marker, anti CD68, which is predominantly an intracellular protein marker (437, 438). The calcium binding protein, Iba-1, is a general marker of microglia/macrophages (resting + activated) while the type-1 transmembrane glycoprotein, CD68, marks activated phagocytic microglia/macrophages (439, 440). Here, only the brain sections from the IP injection cohort of mice were labelled as another researcher in our lab had stained the sections from the SC infusion cohort of mice for microglia/macrophages.

All antibodies used were diluted with 0.1M PBS. Slide mounted brain sections were removed from -80°C and allowed to air dry for 5 to 10 minutes. Sections were blocked with 4%v/v normal horse serum for 60 minutes at room temperature following 3 times washing with PBS for 5 minutes each. Then, primary antibodies, rabbit anti-IbA-1(1:500, WAKO, cat #019-

19741) and rat anti-CD68 (1:500, BioRad, #MCA1957) were diluted in 1% blocking solution (normal horse serum) and allowed to incubate overnight at 4°C. Afterwards, sections were washed 3 times in PBS for 5 minutes and incubated in donkey anti-rabbit Alexa fluor 488nm (1:500 in 1% blocking solution, Invitrogen, cat #LTSA21206) or anti-rat Alexa fluor 594nm (1:500 in 1% blocking solution, Invitrogen, cat #A-21209) secondary antibodies for 60 minutes. The slides were washed again with PBS for 40 minutes (4times, 10minutes each). Thereafter, Sudan Black B (SBB) 0.1%w/v in 70% ethanol was applied onto the sections for 2 minutes to quench autofluorescence. Slides were again washed with PBS (3times, 5minutes each) and DAPI (1:10,000) was added into the last but one wash to stain the nuclei. Finally, all slides were cover slipped with DAKO fluorescent mounting media. Negative controls were only incubated with the secondary antibodies and were included in each run.

3.2.3 Quantification of immunopositive cells

Different methods were used to quantify immunopositive cells after immunohistochemistry, but for all experiments, images from all labelled sections were captured at 20x using an Olympus-VS120-L100-W virtual slide-scanner with a monochrome camera (Olympus-XM10) and were viewed using OlyVIA software.

For immunohistochemistry with GFAP labelling, images were processed using imageJ (Fiji) software and then region of interests (ROIs) were cut using adobe photoshop element 15. A total of four ROIs, which include the cortex and hippocampus of the left hemisphere (injured hemisphere) as well the cortex and hippocampus of the right hemisphere, were selected for quantitative analysis (Figure 2.3A) from images obtained from 29 mice (n=5-6) brains for the SC drug infusion study and 16 mice (n =4) brains for the IP bolus injection study. Images were first processed on image-J as series 3 of bio-format series options for three planes split. However, subsequent analysis was carried out on images that were opened in series 1 as this were of much higher resolution and provided a better image quality for analysis. The selected ROIs for cortex analysis were consistently mapped out by measuring a fixed length of either 3500 pixels (series 3) or 16,000 pixels (series 1) along the outer edge of the cortex including the entire injury site, then a line was drawn from the border of the cortex through the midline separating the hemispheres to the corpus callosum in order to create an enclosed anatomically fixed area spanning the length of the pixels drawn which included the injury cavity. The entire defined anatomical area of the hippocampus was selected as the ROI for hippocampal analysis.

GFAP immunolabelling was quantified using ImageSURF, a newly developed automated quantitative imaging plug-in on Image-J which consistently and reproducibly segments marked features on an image based on the details of its pixels (415). The mean percentage (%) area positive for GFAP immunolabelling was calculated for the middle 15-18 cortical sections for the SC drug infused study and 10-12 cortical sections for the IP bolus injection study from approximately B0.62 to -3.45mm. While shams (6 mice) were excluded from the analysis in SC drug infused study due to errors in sham surgeries, there were no shams performed in the IP injection study, as described above. In addition, 3 mice were excluded from the cortex analysis of the IP bolus injection study due to badly damaged tissue resulting from the large size of injury produced at the time of impact. Furthermore, for the SC drug infused study, approximately 5-8 coronal sections of the hippocampus were analysed for GFAP immunolabelling, while hippocampal analysis for the IP bolus injection study was not performed due to badly damaged hippocampus resulting from the substantial injury produced following TBI induction.

For immunohistochemistry with IBA-1 and CD68, two ROIs (left and right cortex) were selected from images obtained from 16 mice (n = 4) based on the area of healthy tissue left after injury. As previously stated, the selected ROIs for the left and right cortex analysis were consistently mapped out by measuring a fixed length of 16,000 pixels along the outer edge of the cortex including the entire injury site (Figure 3.2A), then, an enclosed anatomically fixed area spanning the length of the pixels was drawn to exclude the injury cavity (Figure 3.2B). While IBA-1 positive cells were quantified using ImageSURF and expressed as percentage area positive for IBA-1 immunolabelling, every CD68 positive cells were manually counted using image-J multi-point tool and was expressed as average number of positive cells per section. Also, for the same reason stated above, IBA-1 and CD68 analysis was not performed on the hippocampus. As for the SC drug infused study, a master student in our lab had previously analysed the IBA-1 / CD68 data for the free-floating sections. A summary of the total number of animals used an analysed is detailed in table 2.3.

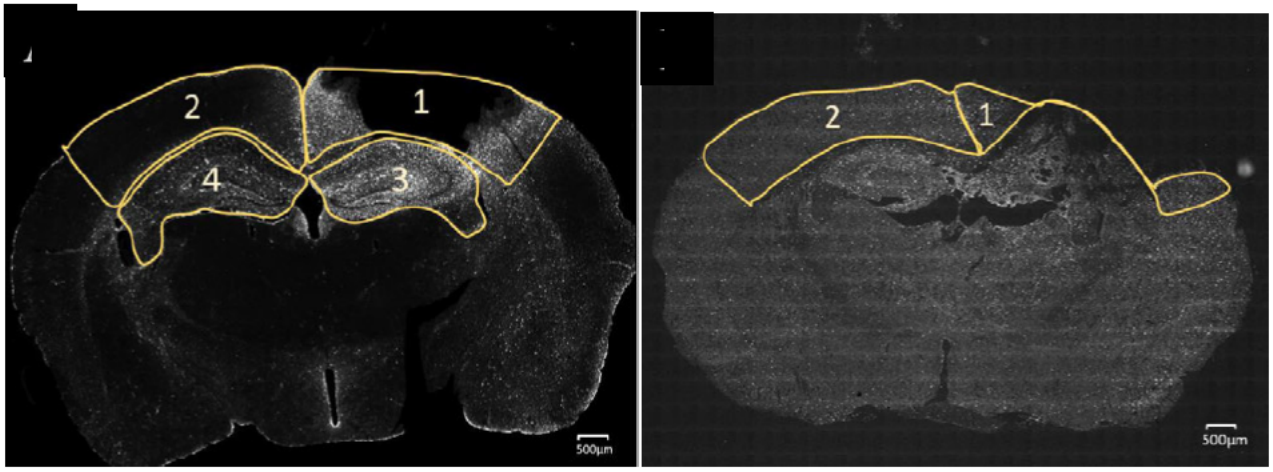


Figure 3.2: Representative images with ROIs selected for quantitative analysis.

Four ROIs including the injury cavity from the injured (left cortex and hippocampus) and uninjured hemisphere (right cortex and hippocampus) were selected ***(A)*** *for image analysis of GFAP immunohistochemistry (IHC), however, hippocampal analysis was not done for the IP bolus injection study due to the severe damage caused by trauma to the hippocampus. The region of cortex selected as shown in the image was either 3,500 pixels or 16,000 pixels depending on the series in which the image was opened. Meanwhile the entire anatomical hippocampus was selected for analysis for the SC drug infusion study. To determine microglial activities, one cortical ROI each from the uninjured cortex and the injured cortex excluding the injury cavity* ***(B)*** *were identified for IBA-1 and CD68 analysis. The ROIs selected were consistently measured at 16,000 pixels. Also, due to the significant damage to the left hippocampus following trauma induction, quantitative analysis for IBA-1 and CD68 was not conducted in the hippocampus of mice from the IP bolus injection study.*

3.2.4 Statistical analysis

All data were presented as mean ± standard deviation (SD) for each treatment group. Group comparisons were made with two-way analysis of variance (ANOVA). Tukey's post-hoc test was used for comparisons between and within the different factors. A p-value of $p < 0.05$ was considered as statistically significant. All calculations were performed with GraphPad prism (version 9) software.

3.3 Results

3.3.1 Qualitative analysis of GFAP immunostaining shows increased astrogliosis in the injured hemisphere at 3 days post-TBI.

To investigate the response of astrocytes at 3 days post-TBI, brain tissue was immunostained with anti-GFAP antibody. On qualitative examination of the immunostained tissue, it was observed that despite using similar parameters to induce injury by CCI, the sizes of the injuries were very different between the two experiments. While the IP injection cohort of mice predominantly had a much larger size of injury with most of their hippocampi severely impacted, the SC infusion cohort of mice still had their hippocampi intact (Figure 3.3B and C).

Furthermore, it was observed that there was increased accumulation of GFAP positive cells in the cortex and hippocampus of the sham controls of the SC drug infused experiment following craniotomy (Figure 3.3A). Seeing that the procedure generated an inflammatory response), shams were excluded from the quantitative analysis as they were not meaningful 'basal' controls. Injury response of the ipsilateral (injured) hemisphere was compared to that of the uninjured (contralateral) hemisphere, while comparisons regarding the effect of treatment were made relative to the TBI+saline group. Subsequently, sham controls were not included in the IP bolus injection experiment. After trauma, GFAP-labelled cells were clearly more abundant within the injured hemisphere than in the uninjured hemisphere. As shown in Figure 3.3, increased accumulation of GFAP-stained astrocytes was found in the cortex ipsilateral to injury compared to the contralateral cortex (Figure 3.3B and C). Similarly, GFAP immunoreactivity was higher in the hippocampus ipsilateral to injury compared to the contralateral hippocampus of the SC infusion cohort of mice at 3 days post-TBI (Figure 3.3B). In the IP bolus injection cohort of mice, since the hippocampus ipsilateral to injury was hugely impacted, the GFAP immunoreactivity could not be compared to the contralateral hippocampus (Figure 3.3C).

Also, there appear to be more GFAP-labelled cells in the uninjured cortex and hippocampus of the IP experiment than in the SC experiment, which could probably be a response to the overall larger lesion in this experiment (Figure 3.3B and C).

The morphology of the astrocytes in the uninjured cortex was different from the astrocytes around the lesioned cortex. Resting astrocytes, which were more commonly seen in the uninjured cortex and in regions farther from the injured cortex, distinctly possessed thin, long processes and small cell bodies (Figure 3.3D and E) compared to the reactive astrocytes,

mostly found around the lesion, which are characterised by hypertrophic cell bodies with short and thick processes (Figure 3.3F and G).

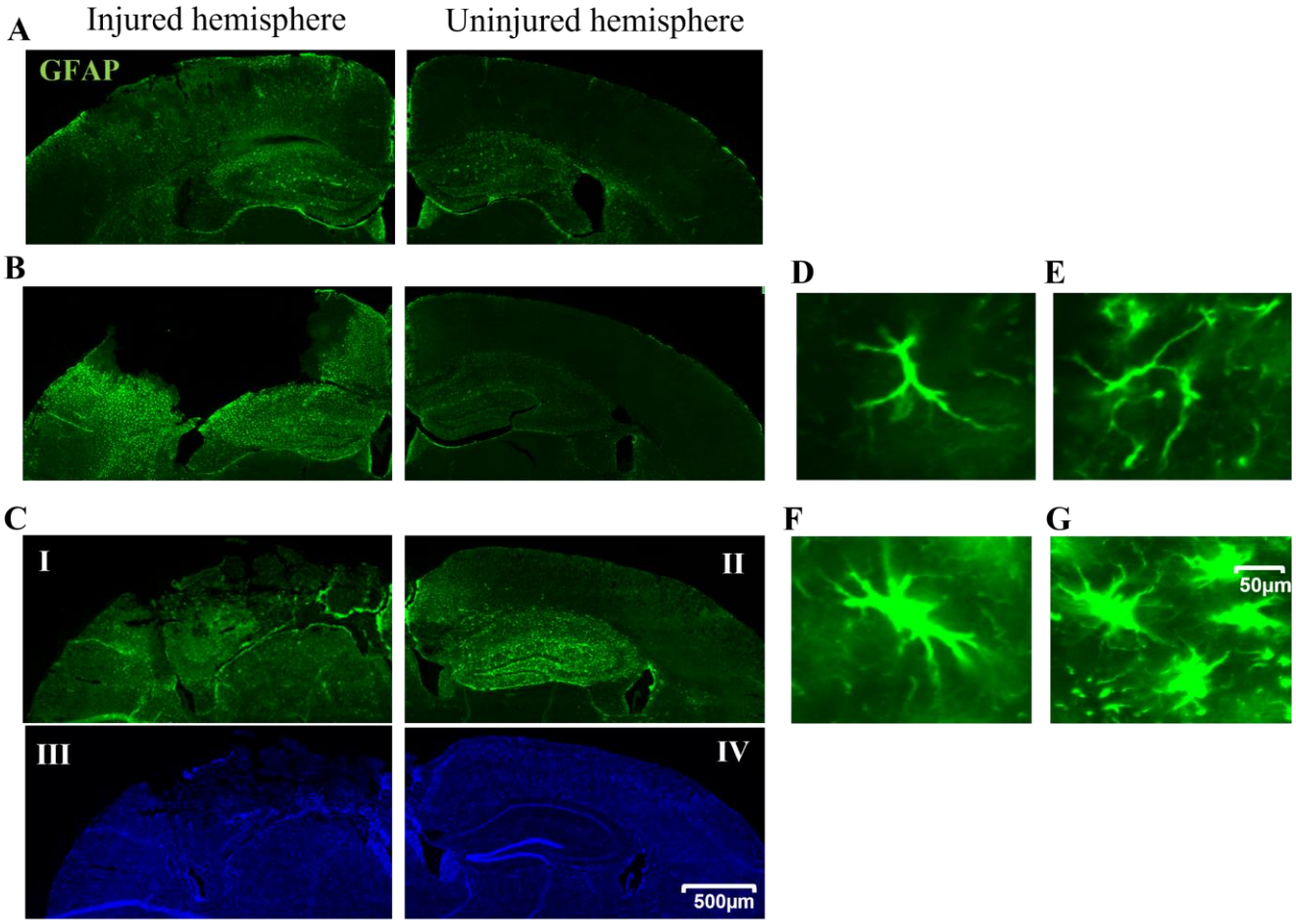


Figure 3.3: High GFAP immunoreactivity in the injured hemisphere at 3 days post TBI.

Representative brain tissue sections from the SC infusion ***(A and B)*** *and IP bolus injection* ***(C)*** *experiments labelled with GFAP antibodies to mark astrocytes. Representative DAPI images for C(i) and C(ii) are also shown* ***(C(iii) and C (iv)).*** *The anatomical location of all images shown is approximately between Bregma -1.455 mm and Bregma -1.655 mm). Sham controls of the SC experiment generated an inflammatory response following craniotomy (A). GFAP immunostaining was visibly greater in the injured brain hemisphere (cortex and hippocampus) than in the uninjured hemisphere of both experiments at 3 days post-TBI. Also, the size of injury in the experiment where drugs were administered by IP injection (C) was notably larger with severely damaged hippocampus compared to the injury in the experiment with drug administration by SC infusion (B). Enlarged images show the different morphologies of astrocytes at 3 days post-TBI. Resting astrocyte, seen mostly in the cortex contralateral to injury have small cell bodies with long processes* ***(D, E)*** *whereas, reactive astrocytes, seen around the lesion site were characterised by hypertrophic cell bodies with thick and short processes* ***(F, G).*** *Scale bar; 500 um (A, B & C), 100 um (D & E), 50 um (F & G).*

3.3.2 Dp4 and enoxaparin do not inhibit astrocyte activation in the pericontusional cortex at 3 days post-TBI.

To determine the response of astrocytes to SC or IP treatment with Dp4 or enoxaparin at 3 days following TBI, GFAP immunoreactivity was quantified in both the injured and uninjured cortex. The results show that following trauma, the percentage area positive for GFAP immunostaining significantly increased in the injured cortex, approximately 3-4 fold compared to the uninjured cortex, irrespective of treatment, in both the SC infusion and IP injection cohorts of mice (Figure 3.4 and 3.5; two-way ANOVA, effect of side relative to injury, $P < 0.0001$, $P = 0.0001$, respectively). However, Dp4 and enoxaparin did not alter

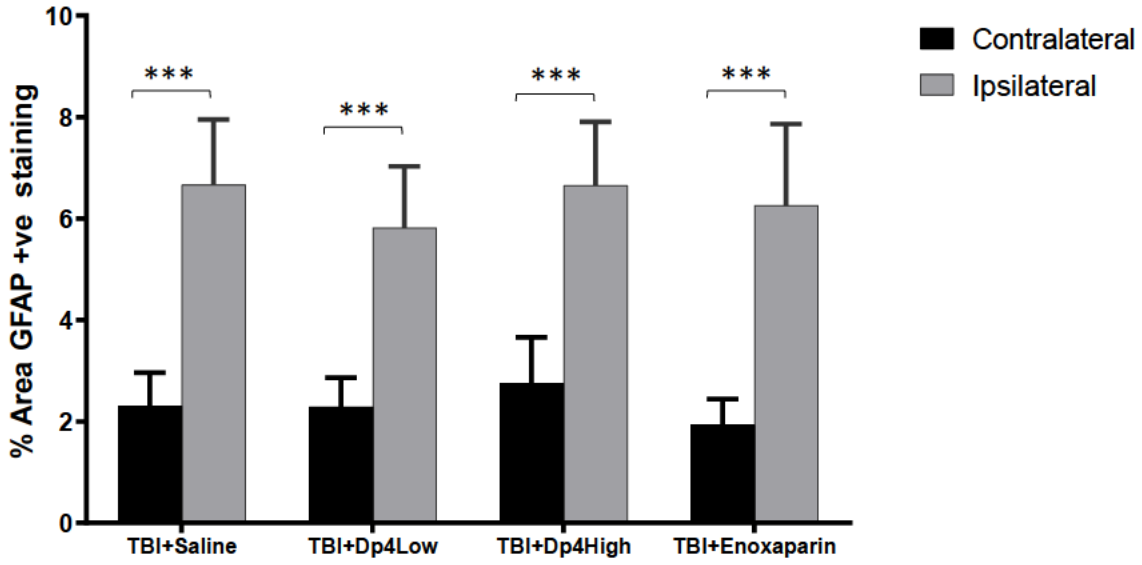


Figure 3.4: Subcutaneous infusion of Dp4 and enoxaparin did not affect astrocyte activation in the pericontusional cortex at 3 days post TBI.

*At 3 days post TBI, there was significant increase in the percentage area positive for GFAP immunolabelling in the cortex ipsilateral to injury compared to the contralateral cortex (Two-way ANOVA, effect of side relative to injury, ***P < 0.0001). However, treatment with Dp4 and enoxaparin did not have any effect on GFAP immunolabelling (Two-way ANOVA, effect of treatment, P = 0.4276). Data is expressed as mean ± standard deviation; n = 5-6.*

the accumulation of GFAP immunostained cells in the cortex at 3 days post-TBI, following either SC infusion (Figure 3.4; two-way ANOVA, effect of treatment $P = 0.4276$) or IP injection (Figure 3.5; two-way ANOVA, effect of treatment, $P = 0.8436$). Also, there was no

significant interaction between the effect of treatment and side relative to injury (Figure 3.4 and 3.5; two-way ANOVA, effect of interaction, P = 0.7499, P = 0.9865, respectively).

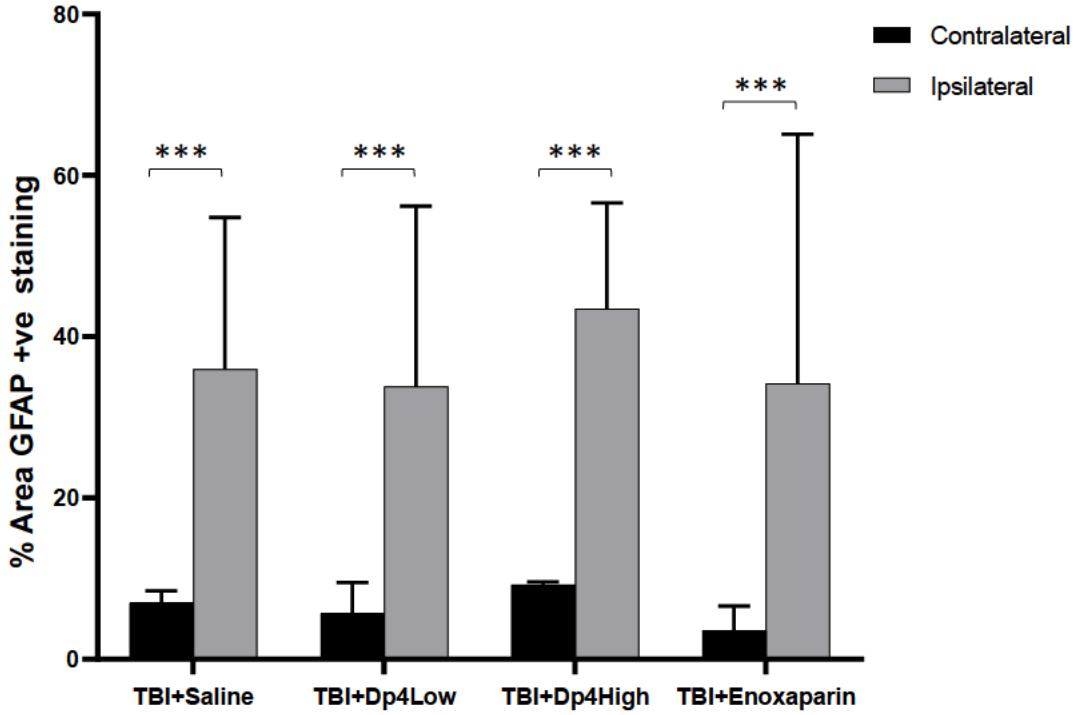


Figure 3.5: Intraperitoneal injection of Dp4 and enoxaparin did not affect astrocyte activation in the pericontusional cortex at 3 days post TBI.

*Quantification of GFAP immunostaining demonstrated a 3-4 fold increase in the cortex ipsilateral to injury compared to the uninjured cortex at 3 days post-TBI (Two-way ANOVA, effect of side relative to injury, ***P = 0.0001). However, IP bolus injection of Dp4 and enoxaparin did not alter the activation of astrocytes following trauma. Data is expressed as mean ± standard deviation; n = 3–4.*

3.3.3 Subcutaneous infusion of Dp4 and enoxaparin did not inhibit astrocyte activation in the hippocampus at 3 days post-TBI.

GFAP immunolabelling was also quantified in the hippocampi of both hemispheres at 3 days post-TBI in the SC infusion cohort of mice, but not in the IP injection cohort because due to the size of injury in this experiment, most of the hippocampi ipsilateral to injury were extensively damaged. In contrast to the cortex, there was no significant change in GFAP immunostaining in the hippocampus ipsilateral to the cortical lesion compared to the

contralateral hippocampus (Figure 3.6A and B; Two-way ANOVA, effect of side relative to injury, P = 0.0931). Furthermore, SC infusion of Dp4 and enoxaparin also had no effect on the level of GFAP immunostaining (Two-way ANOVA, effect of treatment P = 0.5375), irrespective of side (Two-way ANOVA, effect of interaction P = 0.3537).

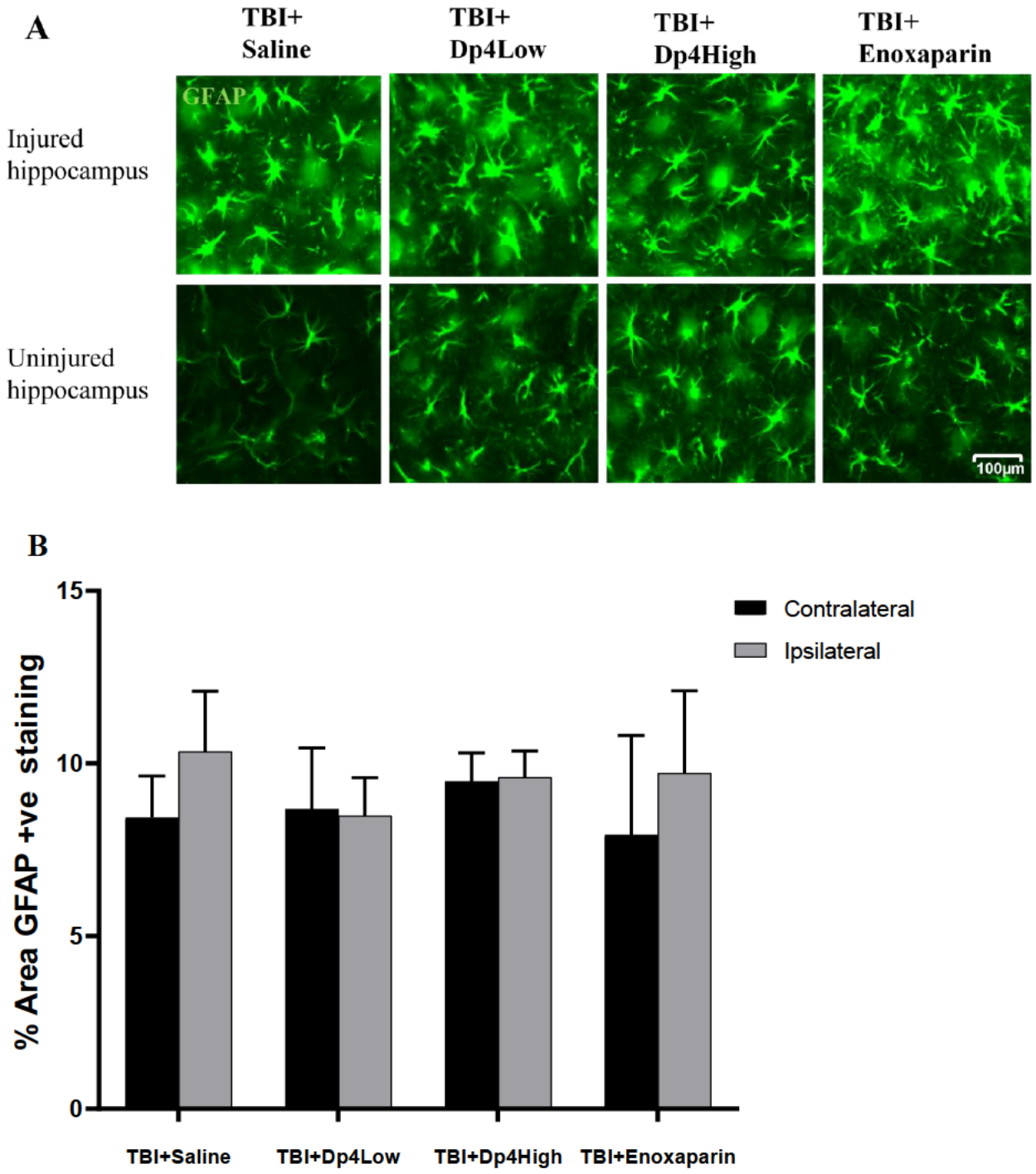


Figure 3.6: Subcutaneous infusion of Dp4 and enoxaparin did not affect GFAP levels in the hippocampus at 3 days post-TBI.

Representative images show GFAP immunolabelling in the injured and uninjured hippocampus ***(A).*** *Quantification of GFAP immunostaining at 3 days post-TBI* ***(B)*** *showed no significant difference in the hippocampus ipsilateral to cortical injury compared to the contralateral hippocampus. Likewise, there was no effect of treatment on the percentage area positive for GFAP immunostaining following drug delivery by SC infusion. Data is expressed as mean ± standard deviation (SD); n = 5-6.*

3.3.4 Qualitative analysis of IBA-1 and CD68 immunolabelling at 3 days post-TBI

Accumulation of microglia/macrophages at the lesion site was assessed in the brains of mice from the IP bolus injection study. As mentioned earlier, I did not perform similar analysis for the SC infusion study because this was done by a master's student in our group.

To qualitatively evaluate microgliosis in the injured brain following trauma, brain sections were double labelled with anti-IBA-1 and anti-CD68 antibodies. Regardless of treatment, the accumulation of IBA-1 and CD68 positive cells was visibly greater in the injured cortex than in the uninjured cortex. Furthermore, since IBA-1 is a general marker for resting and activated microglia/macrophages, there were more IBA-1 positive cells present throughout the brain than CD68 positive phagocytic microglia/macrophages, which were mainly situated around the lesion area at 3 days post-TBI (Figure 3.7A-D).

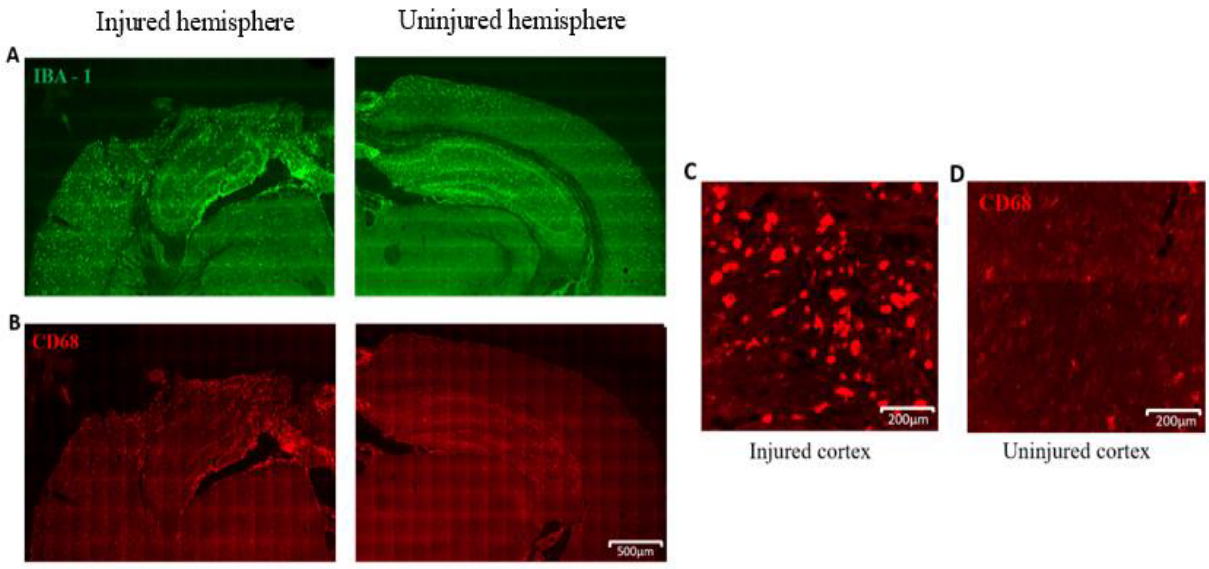


Figure 3.7: High accumulation of IBA-1/CD68 positive cells in the injured cortex

Immunohistochemical analysis using IBA-1 and CD68 was used to assess microglia/macrophages in the brain after trauma. IBA-1 ***(A)*** *and CD68 positive cells* ***(B)*** *were predominant in the injured cortex compared to the uninjured cortex at 3 days post-TBI. Overall, IBA-1, a general microglia marker (ramified + activated), was shown to be more widespread throughout the brain than CD68 immunolabelling which marks activated phagocytic microglia. There was significantly more CD68 positive cells in the injured cortex* ***(C)*** *than in the uninjured cortex* ***(D)****. Due to the severity of the injury, the hippocampus ipsilateral to injury was shown to be severely squashed or torn compared to its contralateral side which was still intact. Scale bar; 500 um (A & B), 200 um (C &D).*

Microglia/macrophages exhibited different striking morphologies upon further observation of the IBA-1/CD68 immunostained tissues. Present within the lesioned site are microglia/macrophages which are activated with circular cell bodies that have no processes (Figure 3.8A). There were also activated microglial characterised by enlarged, ameboid cell bodies and short processes mostly found near the lesion (Figure 3.8B). Reactive microglia with thick processes were seen within the injured cortex (Figure 3.8C) and further away from the pericontusional area, increasing numbers of resting microglia with small cell bodies and long thin processes were spotted (Figure 3.8D). These resting microglial morphologies were also commonly present in the uninjured cortex.

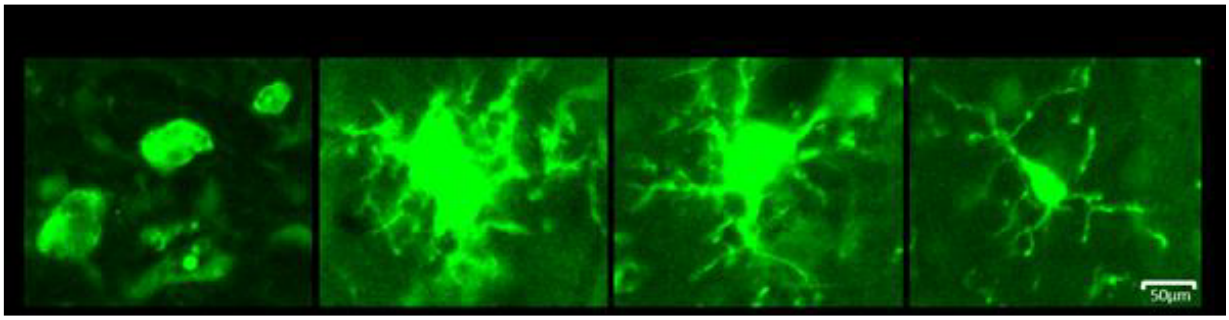


Figure 3.8: Distinct microglial morphologies in the brain after trauma.
Following IBA-1 immunolabeling, four distinct morphologies were identified after trauma, including activated microglia/macrophages ***(A),*** *and activated* ***(B),*** *reactive* ***(C)*** *and resting* ***(D)*** *microglia. Scale bar; 50 um.*

3.3.5 Dp4 and enoxaparin do not inhibit microglia/macrophage activation in the pericontusional cortex at 3 days post-TBI.

To determine if treatment with IP injection of Dp4 and enoxaparin affects the activation/accumulation of microglial/macrophages in the pericontusional cortex at 3 days post-TBI, immunolabelling of IBA-1 and CD68 was quantified in the cortex of the hemispheres ipsilateral and contralateral to the site of injury. Quantitative analysis showed that there was a significant increase in both the percentage area positive for IBA-1 staining and the number of CD68 positive cells in the injured cortex compared to the uninjured cortex at 3 days post-TBI (Figure 3.9A and B; two-way ANOVA, effect of side relative to injury, $P = 0.0007$, $P < 0.0001$ respectively). However, Dp4 and enoxaparin did not affect the expression of IBA-1 in the cortex of the treated groups compared to the untreated animals, irrespective of side (Figure

3.9A; two-way ANOVA, effect of treatment, P = 0.7172, effect of interaction, P = 0.9021). Interestingly, there was a significant effect of treatment on the number of CD68 positive cells

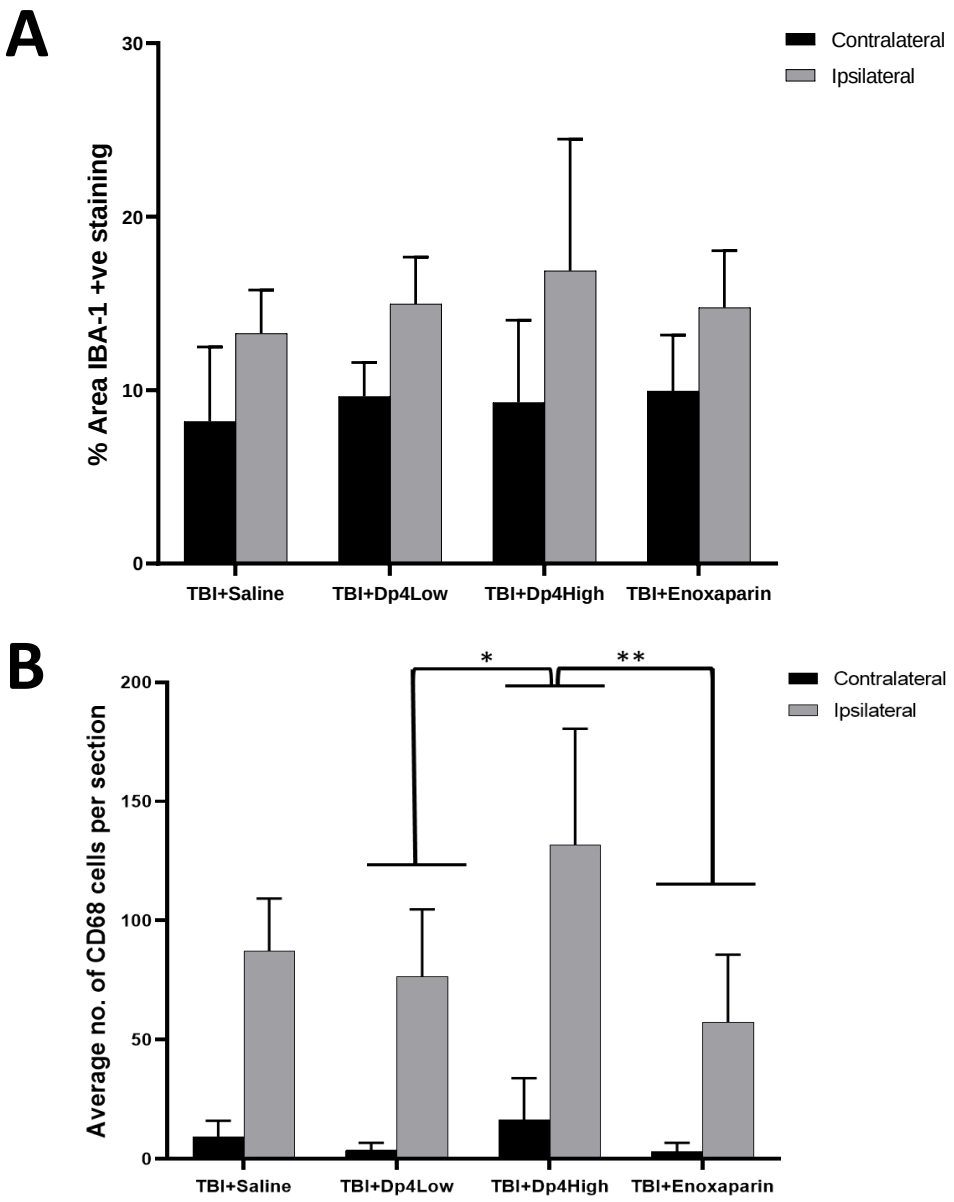


Figure 3.9: Intraperitoneal bolus injection of Dp4 and enoxaparin did not attenuate activation/accumulation of microglia/macrophages in the pericontusional cortex at 3 days post TBI. *The %Area of IBA-1immunolabelling* ***(A)*** *and numbers of CD68 positive cells* ***(B)*** *were significantly increased in the cortex at 3 days post TBI, (Two-way ANOVA, effect of side relative to injury IBA-1, P = 0.0007; CD68, P < 0.0001, respectively). Dp4 and enoxaparin had no effect on Iba1 immunoreactivity; however, the Dp4high group had significantly greater numbers of CD68+ cells than Dp4low and enoxaparin groups (Tukey post-hoc, *P < 0.05 and **P < 0.01, respectively). All data is expressed as mean ± standard deviation (SD); n = 4.*

in the cortex, irrespective of side (Figure 3.9B; two-way ANOVA, effect of treatment, $P = 0.0106$; effect of interaction, $P = 0.1151$), with the Dp4high group having greater numbers than the Dp4low and enoxaparin groups.

3.4 Discussion

Deleterious consequences have been associated with increased activation of astrocytes and microglia, a common characteristic of the neuroinflammatory response in both animal models (205, 441-445) and patients with TBI (154, 446). Attenuating gliosis and possibly promoting recovery with the anti-inflammatory drug Dp4 was the primary aim of this study. However, we demonstrated in this present study for the first time that Dp4 treatment did not significantly reduce astro- and microgliosis in the pericontusional cortex, regardless of the mode of administration. Other sub-aims were to validate our injury model and determine the optimal dose and best mode of administration of enoxaparin and Dp4. Overall, our CCI model exhibited TBI-induced gliosis and treatment, irrespective of dose and mode of delivery, did not attenuate gliosis following experimental TBI.

3.4.1 Upregulation of glial cells in the pericontusional cortex after trauma

TBI triggers astrogliosis and induces glial scar formation which together with other toxic inflammatory mediators could eventually lead to neurodegeneration (118, 441, 447). In the current study, as expected, there was a 3–4 fold increase in the accumulation of reactive astrocytes in the injured cortex at 3 days post-TBI, the time course at which glial activation begins to peak (448, 449). This finding was consistent with studies that have previously reported significant increase in astrocytes in the cortex at 3 days after trauma (448, 450, 451). Several previous studies have also shown increased activation of astrocytes in the cortex from as early as 4 hours up till 30 days with significant increase mostly at day 4 or day 7 post-TBI across different models of TBI including CCI (449, 452, 453), weight drop injury (205) closed head concussion injury (454), juvenile mTBI (455), CHI (456), FPI (457), mild and severe cortical contusion injury (124) as well as in other CNS injuries like intracerebral haemorrhage (458), ischemia (459, 460) and spinal cord injury (461). Furthermore, this present study was consistent with previous studies in that the morphology of astrocytes in the ipsilateral hemisphere of injured animals was predominantly hypertrophic cell bodies with short, thickened processes, indicative of an activated state, while in the contralateral hemisphere (cortex and hippocampus) after trauma and in sham operated animals, astrocytes displayed a more ‘normal’ morphology of long, thin processes with defined cell bodies (449, 453, 462-466).

Upregulation of reactive astrocytes in the ipsilateral hippocampus following trauma has also been extensively studied and reported across various models of TBI over different

timepoints after injury (451, 467), meanwhile, there is not as much evidence in the literature showing astrocytes activities in the contralateral hippocampus. Like the cortex, the hippocampus is known to undergo neuropathological processes after trauma (450, 466, 468, 469) and while some studies have shown elevated astrogliosis in the ipsilateral hippocampus with peak expression at 3-7 days after trauma (124, 450, 451, 464, 466, 467), a few other previous studies have reported a decrease in the number of reactive astrocytes in the ipsilateral hippocampus when compared to the contralateral hemisphere or sham-operated animals after trauma. This observed decrease was associated with the early loss of astrocytic cells which was often accompanied by neuronal loss after trauma (462, 463, 465, 470-472). None of these studies were consistent with our findings that demonstrated no differences in the number of accumulated astrocytes in the ipsilateral hippocampus compared to the contralateral hippocampus after trauma. These discrepancies observed in the hippocampal response to injury could be attributed to factors including assessed timepoint or hippocampal region that may contribute to sub-regional sensitivity to injury (467, 469, 473). For instance, GFAP immunoreactivity was reported to be extremely more in the dentate/hilar sub-regions of the hippocampus compared to the cornu ammonis-1 region (467).

Microglia and macrophages, like astrocytes, play a key role in mediating the inflammatory processes that are initiated following injury to the brain and contribute to the myriad of secondary injury processes that lead to poor outcomes and eventually to neuronal cell death (154, 474, 475).

In this study, we demonstrated that activated microglia/macrophages were significantly elevated in the pericontusional cortex compared to the uninjured cortex at 3 days post-injury, thus reproducing what was previously observed in studies by others following CCI in rodents (448, 476). Moreover, an extensive body of literature has demonstrated significantly high numbers of activated microglia/macrophages at various timepoints in the cortex and hippocampus of different experimental models of TBI (205, 323, 456, 466, 477, 478) as well as in other brain regions such as the thalamus (445), corpus callosum and optic tract (441) and even years after in the brains of humans that have undergone fatal or severe TBI (114, 446, 479).

Importantly, microglia undergo major morphological changes which differ depending on the type of CNS insult. For instance, the morphologies of microglia in ageing are very different from those exhibited by activated microglia in CNS injuries such as in trauma or

compared to those *in vitro* (323, 480-484). These cells may acquire a rod-shaped, ameboid, bushy, spindle or bi– and tripolar morphologies (485-487). Consistent with previous studies, we observed four distinct morphologies, indicative of different states of activation, exhibited by microglia following trauma (323, 488), which together with the other glial responses to injury observed in this study, validates our TBI model.

3.4.2 Effect of enoxaparin and Dp4 on activated astrocytes and microglia/microphages.

In this study, we demonstrated that irrespective of the mode of drug delivery, low and high doses of Dp4 did not attenuate the CCI-induced accumulation of activated astrocytes and microglia in either of the assessed brain regions at 3 days post-injury. A similar result was surprisingly observed following treatment with enoxaparin, which had previously been shown to reduce reactive gliosis by significantly decreasing GFAP staining intensity in the hippocampus of a rat lateral fluid percussion injury model of TBI (390), although, interestingly, the authors reported no effect of enoxaparin on the area of astrocytes' perikarya. It is possible that the differences in results from this study and our study could be attributed to the differences in methodology. For example, while they specifically evaluated reactive gliosis in the dentate hilus region of the rat hippocampus, in our study, both the cortex and the entire hippocampal region were assessed for astrogliosis in a CCI mouse model of TBI. This species difference could potentially have affected our results, since a previous *in vitro* study had demonstrated that the expression of GFAP in rats following a scratch injury was substantially different from that in mice at 24, 48 and 72 hours post-injury. In the same study, the authors showed that the observed differences in GFAP expression between both species post-injury was because the rat cells were more proliferative at all evaluated timepoints (489). Moreover, with evidence suggesting that sub-regions within the hippocampus have differential astrocyte expression to injury (490, 491), it is possible that perhaps a different analytical approach such as targeting a specific area of the hippocampus, may have revealed an effect of treatment. An additional important difference between our study and the one by Zupan et al., 2011 was that they used a different mode of delivery. For example, they subcutaneously injected enoxaparin, while in this study, enoxaparin was given via SC infusion and multiple IP injections. While SC infusion is a much slower release of the drug, SC injections are faster. Additionally, unlike the IP mode of delivery which is subject to first pass liver metabolism, drugs administered via the SC route does not undergo first pass liver metabolism and so, this could have perhaps resulted in more

bioavailability of the drugs, leading to the positive effect exhibited in the study of Zupan and colleagues (390, 492).

Considering that the neuroinflammatory process is a complex one, it could be that enoxaparin or Dp4 may produce a potent effect on a different aspect of neuroinflammation, such as on the expression of inflammatory mediators like cytokines and chemokines that either stimulate or inhibit cerebral inflammation (493).

Although treatment did not decrease the number of accumulated astrocytes and microglia in this study, it is possible that enoxaparin or Dp4 treatment could have resulted in a shift in the inflammatory profiles of these cells to a more neuroprotective phenotype that may promote tissue restoration and eventually recovery (417, 494). Astrocytes and microglia can exist in states that can either be neurotoxic or neuroprotective (495, 496). The dual expression of microglial responses to neuroinflammation for many years was associated with its M1 and M2 phenotypes, proposed to have well-defined roles that could be either deleterious or neurorestorative respectively, when activated (143). This concept has now been remodelled in support of both phenotypes (M1/M2) existing as a continuum rather than as exclusively distinct polarised states (149-152). Like microglia, two distinct phenotypes of reactive astrocytes have recently been characterised: the A1 phenotype which is neurotoxic and contributes to neuronal cell death and the A2 phenotype with neurotrophic effects (495, 497). A likely mechanism in which enoxaparin and Dp4 could affect this shift in neuroinflammatory profiles will be by inhibiting TLR4 pathway. It had previously been suggested that following TBI, a shift in inflammatory response towards microglia M2 phenotype promoting tissue repair was exhibited in TLR4 knockout mice (433). Therefore, if indeed enoxaparin and Dp4 does inhibit TLR4 as hypothesised in the introduction, then it may shift the inflammatory profiles as shown previously. However, I did not assess this using immunohistochemistry in this chapter.

Another way of exploring this shift in inflammatory phenotypes would be to look at the profile of inflammatory mediators being released in the damaged tissue, where I would expect to see a decrease in pro-inflammatory ‘M1’ cytokines/chemokines, such as IL-1, IL-6, TNF-α and an increased production of ‘M2’ anti-inflammatory cytokines, including IL-4, IL-13, IL-10. This hypothesis will be discussed in the next chapter.

3.4.3 Study Limitations

There are a few limitations of this study. Firstly, a quantitative morphological assessment of microglia was not performed. This is important as it could have provided additional information on the degree of inflammation, both after TBI alone and following treatment (488, 498). In this study, I chose instead to quantify microglia/macrophage activation and accumulation by measuring the percentage of the area of a region of interest that contains immunolabelling for Iba1. Although lacking the ability to detect more subtle shifts in microglial morphology, this powerful technique is commonly applied in TBI research to successfully quantify changes in microglial reactivity (for example: (499-501).

This study specifically explored the effects of treatment with enoxaparin and Dp4 on neuroinflammation; however, to fully evaluate the therapeutic potential of these drugs it would also be important to quantify neurodegeneration, using measures such as lesion volume (502). In this study, lesion volume analysis was not performed as I could not do a reliable measure of the area of the brain tissue in the SC study. The sections from this experiment were collected as free floating, and for many sections some of the delicate, damaged tissue surrounding the injury site was lost. Hence, for the IP study, brain sections were collected as slide mounted to preserve the integrity of the tissue. Given this limitation, the neuroprotective effects of the SC and IP studies could not be quantitatively compared. In addition to lesion volume, other measures that could have been employed to explore neuroprotective activities following treatment include apoptotic cell death in the hippocampus and cortex, but for lack of time, I did not do these types of analysis as it was outside the scope of my study. Determining whether the drugs were neuroprotective in the absence of the hypothesised decrease in neuroinflammation is important for the broader question about the overall value of these drugs as treatment for TBI. Therefore, neuroprotective outcomes in these experiments will be assessed prior to publication of this study.

Another, limitation of this study was not including a sham control group that could be compared with the untreated TBI group to determine the effect of TBI. As described in section 3.3.1, this group wasn't included in the data analysis for this experiment because since we were learning to do craniotomies with this early experiment, we often damaged the underlying cortical tissue, causing an inflammatory response, and thus preventing these mice from being the intended 'uninjured' controls. (However, our skill noticeably improved with time.) Having sham or naïve control data is valuable for both confirming that there has been an effect of TBI

and determining whether the extent of a treatment effect is great enough to approach uninjured values; so in future studies, either one of these control groups, or perhaps both, will be incorporated as we had intended for this study. We did include in this study data from measurements of the contralateral (uninjured) brain hemisphere. This is not a true baseline control since there were likely low-level pathological changes in the contralateral hemisphere post-TBI. Therefore, this data was included to allow a comparison of the effect of treatment between a high-injury environment (injured hemisphere) versus a low-injury environment (uninjured hemisphere), as mentioned above.

3.4.4 Conclusion

Altogether, the results from this study demonstrated that treatment with enoxaparin or Dp4 did not attenuate the accumulation of activated astrocytes and microglia in the injured cortex at 3 days after trauma. Therefore, from this study, I concluded that more work to investigate the potential therapeutic effects of enoxaparin and Dp4 on neuroinflammation should be carried out to explore other aspects of the inflammatory cascade.

CHAPTER 4

EFFECT OF ENOXAPARIN AND DP4 ON INFLAMMATORY CYTOKINES/CHEMOKINES AT 6 HOURS POST-TBI

4.1 Introduction

As detailed in chapter one, the intricate inflammatory cascade following TBI involves the excessive release of inflammatory mediators, including cytokines and chemokines. The pro-inflammatory cytokines are implicated in the upregulation of inflammatory responses that ultimately drive neuroinflammation to a more toxic state, whereas the anti-inflammatory cytokines regulate and inhibit neuroinflammation, essentially, promoting tissue repair and restoration (143, 170, 503).

Pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α, and chemokines like MCP-1 and MIP-1beta, are elevated early following TBI, usually peaking in animal models between 4-12 hours, and are linked to neuronal dysfunction and poor outcomes in both animal models (190, 216, 231, 242, 504-509) and in humans (223, 242, 510, 511). Due to the integral role of inflammatory cytokines and chemokines in modulating inflammation, targeting the inflammatory cytokine and chemokine cascade may be a promising therapeutic strategy as several studies have demonstrated this (512). For example, treatment of mice with MW151, a small CNS penetrant molecule administered by IP injection, reduced CHI-induced cognitive impairment by suppressing IL-1β, a pro-inflammatory cytokine known to significantly contribute to TBI-induced secondary brain damage (513). Similarly, the anti-inflammatory drug, minocycline, significantly reduced the levels of IL-1β, which was associated with a decrease in activated microglia and lesion volume, as well as improved functional outcomes within 24 h after CHI (323). Furthermore, treatment with IL-10, an anti-inflammatory cytokine, improved neurological recovery in a rat lateral fluid percussion model of TBI, which was potentially mediated by the decreased levels of pro-inflammatory cytokines, TNF-α and IL-1 in the injured brain (199).

As discussed in the previous chapter, I hypothesised that enoxaparin and Dp4 will decrease pro-inflammatory cytokines and increase anti-inflammatory cytokines in the injured brain, as both drugs could potentially shift the inflammatory profile of glial cells from a neurotoxic phenotype to a reparative phenotype. While there is a paucity of research on the effect of enoxaparin and Dp4 on inflammatory cytokines following TBI, enoxaparin previously reduced inflammasome proteins, IL-1β and caspase-1, in the cortex at 24 hours post-TBI using a CCI mouse model (514). Besides, there are a few more studies in which enoxaparin significantly reduced pro-inflammatory cytokines, including TNF-α, IL-1β and caspase-3, in cardiac inflammation (515) and inflammation of the gut (423). Furthermore, some cytokines such as TNF-α, IL-13, IL-4 and IL-5, which are included for analysis in this study, had

previously been reduced by enoxaparin in the peripheral blood mononuclear cells of asthmatic patients. Further investigations by the authors revealed that Dp4 also brought about the decreased levels of the abovementioned cytokines (353, 422). Similarly, Dp4 reduced the levels of IL-6 and IL-8 in human pulmonary epithelial cells *in vitro* (406).

In this chapter, I aimed to explore the effect of enoxaparin and Dp4 administration on the levels of inflammatory cytokines and chemokines in the pericontusional cortex at 6 hours following a CCI injury in mice. The inflammatory cytokines that were evaluated in this study were selected mainly based on two criteria, which are, firstly, cytokines that are known to be elevated after trauma (IL-1, IL-6, MCP-1, MIP-1a, MIP-1b, KC, G-CSF, RANTES, IL-12p40 and IL-12p70), and secondly, cytokines that were reduced by enoxaparin and Dp4 in models of peripheral inflammation (TNF-α, IL-13, IL-4, IL-5).

4.2 Methods

To address the aims of this chapter, similar drug regimen as in chapter 3 was employed on the same TBI model, however, mice were killed at 6-hours post-TBI (Figure 4.1) and protein extracts from the pericontusional cortex were prepared and analysed using a BIO-RAD Bio-Plex Pro assay kit, to measure the levels of twenty-three cytokines and chemokines. These were assessed in the experimental groups (Sham, TBI+Saline, TBI+Enoxaparin, TBI+Dp4High and not TBI+Dp4Low) of mice that received drug by intraperitoneal (IP) injection, while only the TBI+Saline and TBI+Dp4High (excluded sham, Dp4+enoxaparin and Dp4+low) experimental groups from the mice cohort that received drugs by subcutaneous infusion were assessed. Although, protein was extracted from all the experimental groups, some groups were excluded due to the limited number of samples that could be read in the assay. I was blinded to the experimental groups, while performing this analysis.

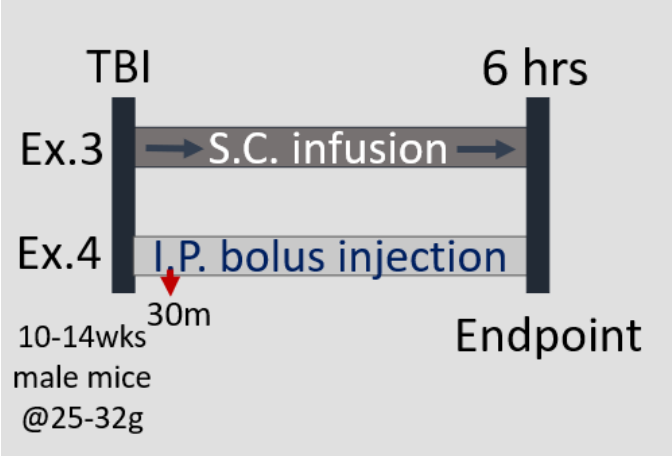


Figure 4.1: Schematic representation of experimental timeline.

Two modes of drug delivery were employed in this study. SC infusion of the drugs were administered via mini-osmotic pumps for 6 hours post-TBI and a single IP injection was given at 30 minutes post-TBI.

4.2.1 Drug administration

The details of the drugs, dose and mode of delivery utilised for this study are outlined in the table below. As in Chapter 3, drugs were administered via SC infusion or as a single IP injection given at 30 minutes post-TBI. Vehicle control TBI and sham groups received sterile saline. See table 4.1 for detailed dosage for both modes of delivery.

Table 4.1: Drug dosage for each mode of delivery

Drugs	**SC infusion**	**IP injection**
Enoxaparin (dose based on published studies; used as a benchmark for anti-inflammatory activity).	2.5 mg/kg/24 hrs	1 mg/kg/injection (Delivered as ~60 ul of 500 ug/ml solution in saline).
$Dp4_{LOW}$ (equivalent to amount of DP4 fraction in Enoxaparin dose)	129 µg/kg/24 hrs	50 µg/kg/injection (Delivered as ~60 ul of 25 ug/ml solution in saline).
$Dp4_{HIGH}$ (Twice $Dp4_{LOW}$)	258 µg/kg/24hrs	100 µg/kg/injection (Delivered as ~60 ul of 50 ug/ml solution in saline).

4.2.2 Measurements of cytokines and chemokines in the injured cortex

Brain homogenate samples from the injured cortex were prepared as described in section 2.5.2. Samples were thawed on ice and the levels of 23 cytokines and chemokines (IL-1β, IL-6, IL-12p70, G-CSF, IL-12p40, IL-1α, IL-4, IL-3, IFN-γ, IL-2, TNF-α, IL-17(A), GM-CSF, eotaxin, KC, MCP-1, RANTES, MIP-1α, MIP-1β, IL-5, IL-10, IL-9, IL-13) were quantified in 200 ug total protein using Bio-Plex Mouse Cytokine Array kit (Bio-Rad Laboratories, Catalogue number #M60009RDPD) according to the manufacturers' instructions. Briefly, the antibody-coupled beads were vortexed and diluted in assay buffer prior to use. Then 50 ul of vortexed beads were added to each well of the 96-well assay plate and were washed twice with wash buffer using a hand-held magnetic washer (Bio-Rad Laboratories). All standards, samples and blanks were vortexed and 50 ul of each was added to the plate. The plate was covered with a sealing tape and allowed to incubate on a shaker at 850 rpm for 30 minutes at room temperature, then the plate was washed three times with wash buffer and 25 ul of vortexed detection antibody mix was added to each well. The plate was again covered with a sealing tape and incubated on a shaker at 850 rpm for 30 minutes at room temperature. Following three times wash with wash buffer, 50 ul of streptavidin-PE was vortexed and added to each well. The plate was covered and incubated by shaking at 850 rpm for 10 minutes at room temperature. The plate was washed again for the last time with wash

buffer and beads were resuspended in 125 ul of assay buffer, then the plate was covered and incubated by shaking at 850 rpm for 30 seconds. The sealing tape was removed, and the plate was immediately read using a MAGPIX Luminex device (MAGPX09274304) with Luminex xPONENT software that was pre-set to include dilution factor prior to starting the experiment.

4.2.3 Statistical analyses

Data are presented as mean ± standard deviation (SD) for each treatment group. Group comparisons were made with one-way analysis of variance (ANOVA) and unpaired *t*-test with $p<0.05$ considered as statistically significant. All calculations were performed with GraphPad prism (version 9) software.

4.3 Results

The effect of enoxaparin and Dp4 on the levels of inflammatory cytokines and chemokines in the pericontusional cortex was assessed at 6 hours post-TBI, a time at which many cytokines and chemokines are beginning to elevate (190).

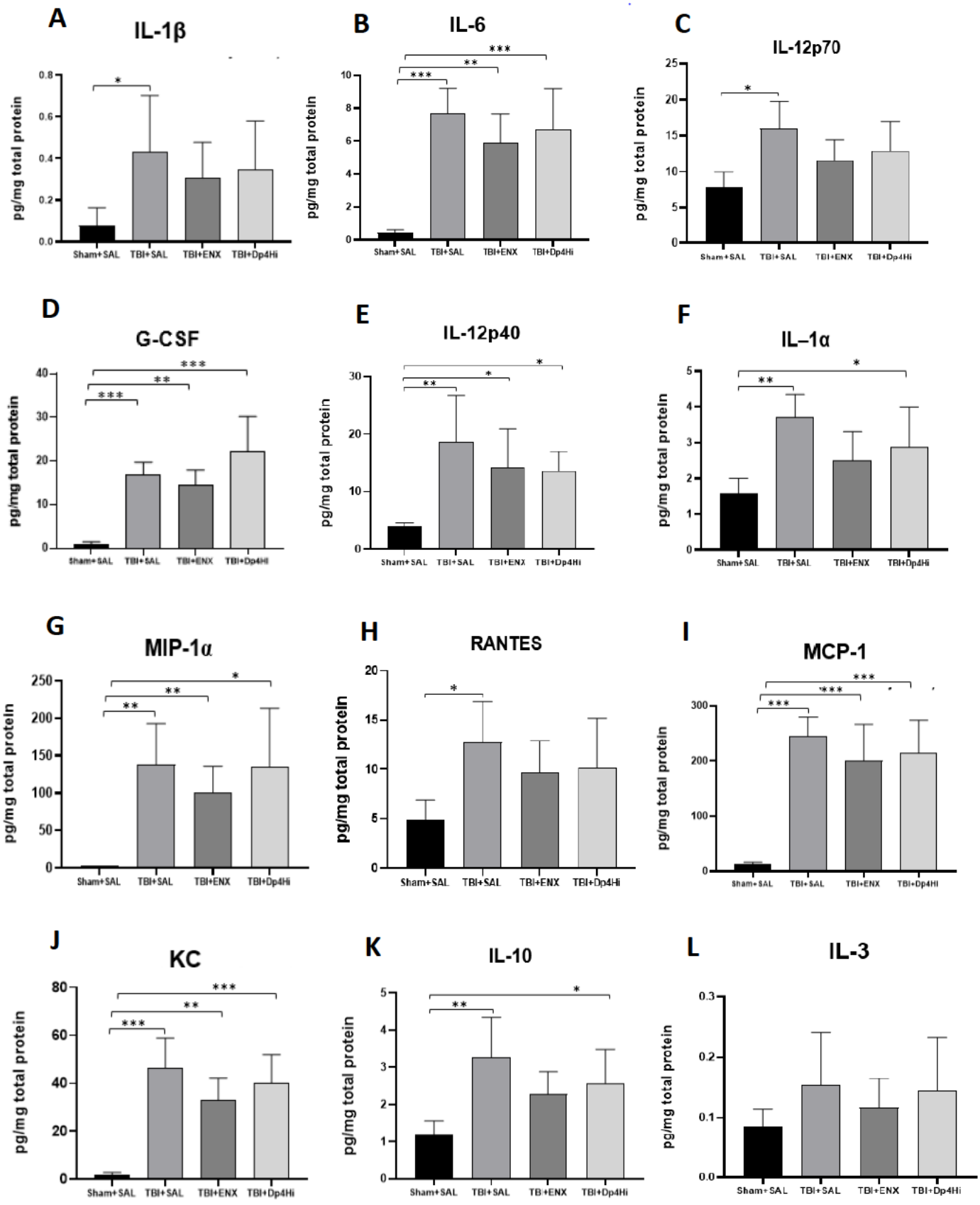
Overall, in the cohort of animals that received treatment by IP injection, of the 23 cytokines and chemokines assessed, 14 were significantly elevated at 6 hours post-TBI and there was no change in the levels of 9. Interestingly, the levels of 2 of the 23 cytokines/chemokines measured were significantly reduced following the IP injection of enoxaparin.

4.3.1 No effect of treatment on many inflammatory cytokines and chemokines in the injured cortex at 6 hours post-TBI in mice receiving IP injections.

As expected, the levels of pro-inflammatory cytokines, IL-1β, IL-6, IL-12p70, G-CSF, IL-12p40, IL-1α and IL-5, were all significantly elevated in TBI+saline group compared to uninjured sham controls (Figure 4.2A-F, 4.4D; one-way ANOVA, P = 0.0393, <0.0001, 0.0038, <0.0001, 0.0014, 0.0014 and <0.0001 respectively; refer to figures for Tukey post-hoc p-values). Similarly, the levels of chemokines, MIP-1α, RANTES, MCP-1, KC, MIP-1β were also significantly increased in the injured cortex of the TBI+saline mice compared to the uninjured sham controls at 6 hours post-TBI (Figure 4.2G-J, 4.3A; one-way ANOVA, P = 0.0005, 0.0140, < 0.0001, < 0.0001, and 0.0022, respectively; refer to figures for Tukey post-hoc p-values). Interestingly, the levels of both anti–inflammatory cytokines, IL-4 (Figure 4.3C) and IL-10 (Figure 4.2K) were significantly upregulated in the cortex at 6 hours post-TBI (one-way ANOVA, P 0.0013 and 0.0016, respectively; refer to figures for Tukey post-hoc p-values).

In contrast, the levels of IL-3, IFN-γ, IL-2, IL-17(A), GM-CSF, eotaxin and TNF-α were not altered in the cortex ipsilateral to injury compared to the uninjured sham controls at 6 hours post-TBI (Figure 4.2L-Q, 4.4B; one-way ANOVA, P = 0.3271, 0.0522, 0.1506, 0.8758, 0.0870, 0.0404 and 0.0550 respectively).

Furthermore, there was no effect of drug treatment on any of these cytokines/chemokines at 6 hours post-TBI.


A
IL-1β
pg/mg total protein
Sham+SAL
TBI+SAL
TBI+ENX
TBI+Dp4Hi
B
IL-6
C
IL-12p70
D
G-CSF
E
IL-12p40
F
IL–1α
G
MIP-1α
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RANTES
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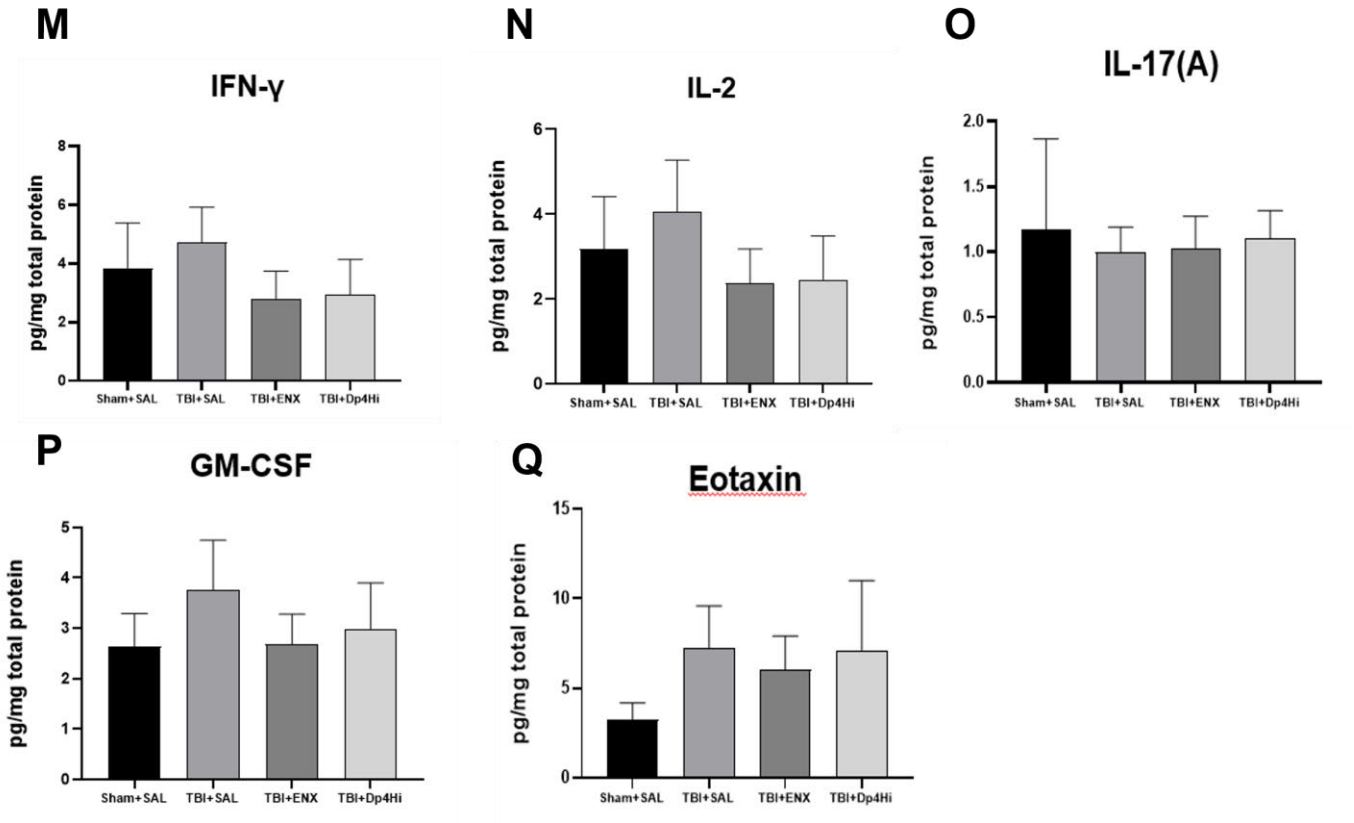


Figure 4.2: Levels of inflammatory cytokines and chemokines in brain homogenates at 6 hours post-TBI.

*Pro-inflammatory cytokines, IL-1β, IL-6, IL-12p70, G-CSF, IL-12p40, IL-1α (A-F) and chemokines, MIP-1α, RANTES, MCP-1, KC (G-J) were significantly upregulated in the pericontusional cortex compared to uninjured sham control after trauma. While the anti-inflammatory cytokine, IL-10 (K) was also significantly elevated in the injured cortex compared to the sham operated control at 6 hours post TBI, the levels of other inflammatory mediators, IL-3, IFN-γ, IL-2, IL-17(A), GM-CSF and eotaxin (L-Q) were not altered in the pericontusional cortex compared to the uninjured sham control at 6 hours post-TBI. For all these cytokines/chemokines, intraperitoneal injection of enoxaparin and Dp4 did not significantly alter the elevated cytokines and chemokines in the injured cortex after trauma. Data are expressed as pg/mg total protein (mean ± SD); Tukey's post-hoc analysis, *P < 0.05, **P < 0.001, ***P < 0.0001; n = 6.*

4.3.2 Intraperitoneal administration of enoxaparin attenuated the expression of MIP-1β and IL-9 in the pericontusional cortex at 6 hours post-TBI.

Of all the cytokines and chemokines assessed, IP injection of enoxaparin led to significant decreases of 50% and 43% in the levels of macrophage inflammatory protein-1beta (MIP-1β) and interleukin-9 (IL-9), respectively, when compared to the saline-treated group of mice (Figure 4.3A and B; one-way ANOVA, P = 0.0022 and 0.0363, respectively; refer to figures for Tukey post-hoc p-values). On the contrary, while there appeared to also be decreases of 39% and 38%, respectively, in the levels of these mediators following treatment with Dp4 compared to TBI+saline, post-hoc analysis revealed that these were not statistically significant.

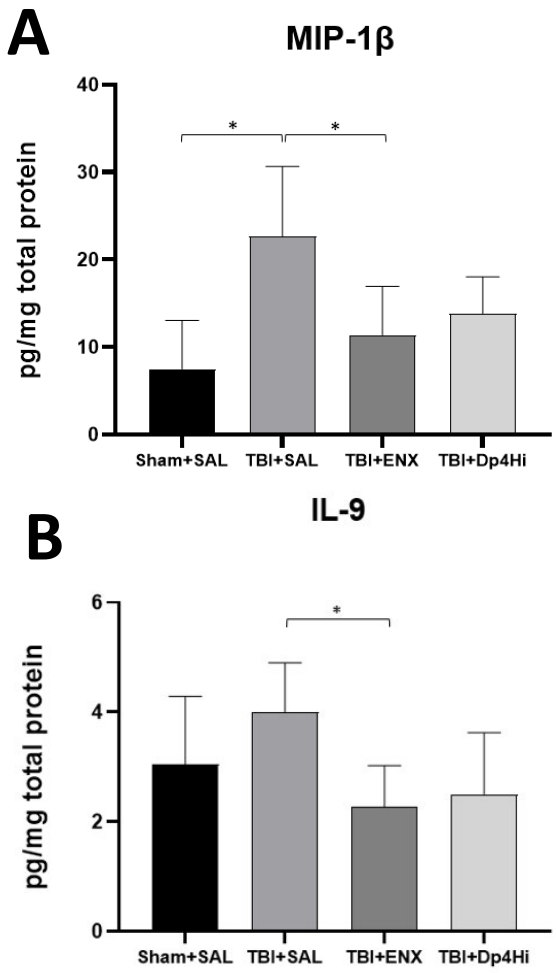


Figure 4.3: Enoxaparin decreased the levels MIP-1β and IL-9 in the pericontusional cortex. *At 6 hours post TBI, the level of the chemokine MIP-1β (A) was significantly higher in the injured cortex as compared with the uninjured sham control. Intraperitoneal administration of enoxaparin significantly reduced the levels of MIP-1β and IL-9 (B). Data are expressed as pg/mg total protein (mean ± SD); Tukey's post-hoc analysis, *P < 0.05; n = 6.*

4.3.3 Enoxaparin and Dp4 had no effect on those inflammatory cytokines previously shown to be reduced in peripheral inflammation.

After trauma, IP injection of enoxaparin or Dp4 had no effect on the levels of IL-13, TNF-α, IL-4 and IL-5 as compared with the saline-treated TBI group (Figure 4.4A–D; one-way ANOVA, P = 0.0815, 0.1506, 0.0013 and <0.0001, respectively).

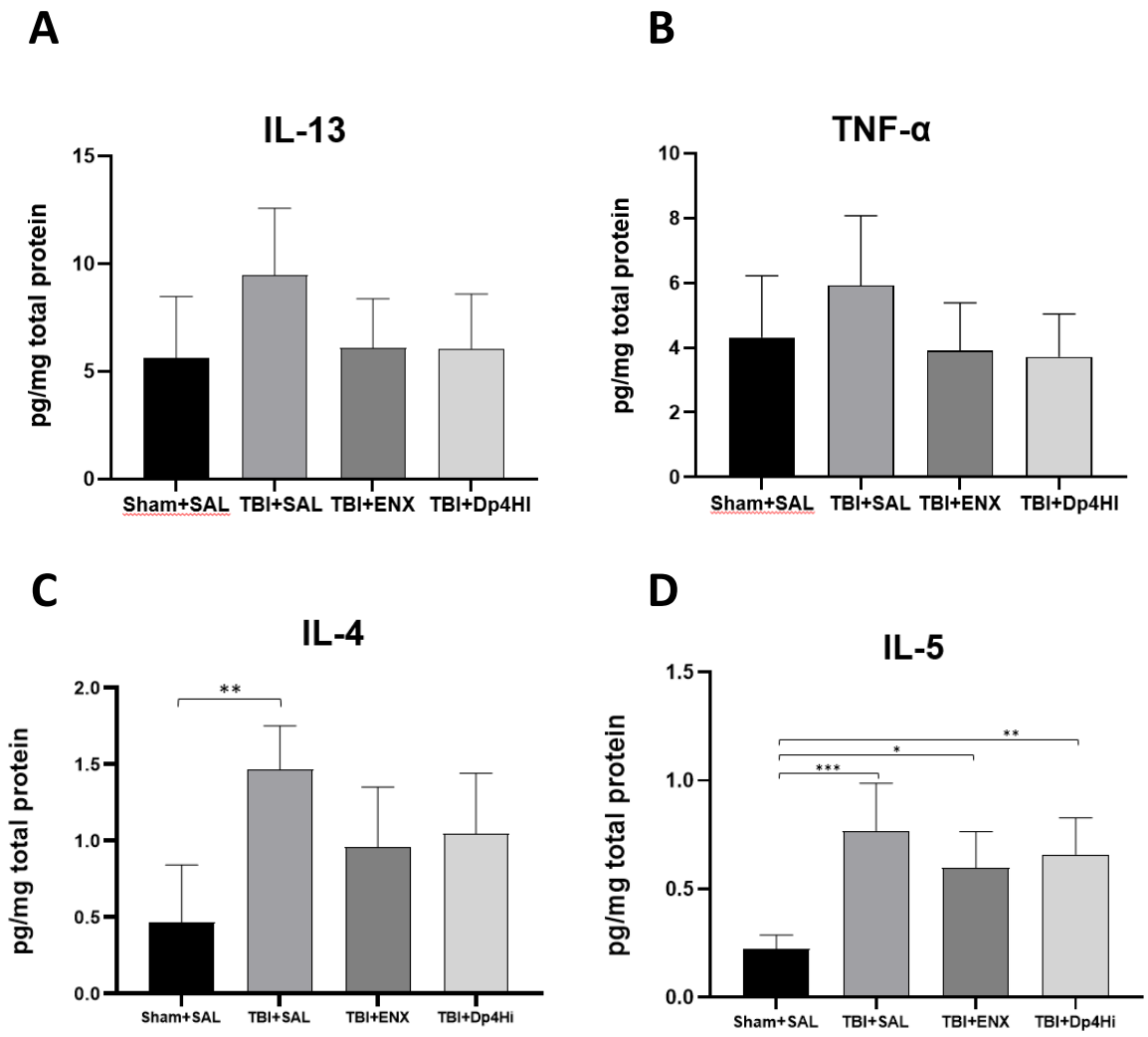


Figure 4.4: No effect of enoxaparin and Dp4 on the levels of IL-13, TNF-α, IL-4 and IL-5 in brain homogenates at 6 hours post TBI.

While the levels of inflammatory cytokines IL-13 ***(A)*** *and TNF-α* ***(B)*** *were not significantly altered in brain homogenates at 6 hours post-TBI, IL-4* ***(C)*** *and IL-5* ***(D)*** *increased significantly after trauma as compared with the sham operated control. However, IP bolus injection of enoxaparin and Dp4High had no effect on all four cytokines at 6 hours post-TBI. Data are expressed as pg/mg total protein (mean ± SD); Tukey's post-hoc analysis, *P < 0.05, **P < 0.001, ***P < 0.0001; n = 6.*

4.3.4 Subcutaneous infusion of Dp4 did not affect the levels of inflammatory mediators in the pericontusional cortex at 6 hours following TBI.

For the SC drug infusion experiment, shams were excluded from the final data analysis, as it was observed that the sham levels of all inflammatory mediators assessed were similar to the levels of untreated injured animals (TBI+SAL). I interpreted this as an inflammatory response to the craniotomy in the sham controls, rather than a lack of inflammatory response following TBI, since the levels in these sham mice were also substantially higher than the sham levels of the IP bolus injection cohort. The SC drug infusion experiment was among the first experiments conducted with our TBI model when our lab was set up and so our sham procedure was still being optimised. Therefore, there was no appropriate uninjured control for the SC drug infusion experiment. Instead, comparison was made between the Dp4High treated mice and the untreated injured mice to assess the effect of treatment post-TBI only.

Overall, in this experiment, there were no changes in the levels of any cytokines/chemokines measured between the two experimental groups at 6 hours post-TBI (Table 4.2).

Table 4.2: Levels and p–values of t-test analysis of the 23 cytokines measured at 6 hours post-TBI following subcutaneous infusion of enoxaparin and Dp4 (mean±SD; n = 4-6).

Cytokines/Chemokines	**TBI+SAL**	**TBI+Dp4**	**Unpaired *t*-test**
	(pg/mg)	**(pg/mg)**	**(p-values)**
Pro – inflammatory cytokines			
IL-3	0.13±0.03	0.14±0.05	0.686
IL-1β	0.40±0.11	0.43±0.22	0.759
TNF-α	5.17±1.11	6.04±2.43	0.483
IL-9	2.81±0.75	2.89±0.80	0.864
IFN-γ	3.34±0.97	3.44±1.00	0.875
IL-2	3.00±0.77	3.16±0.92	0.761
IL-13	8.60±2.41	10.46±4.15	0.401
IL-6	3.98±0.92	4.23±1.69	0.777
IL-4	1.24±0.32	1.54±0.53	0.297
IL-1α	3.25±1.11	3.35±1.33	0.889
G-CSF	11.99±2.85	11.48±2.90	0.775
IL-17(A)	0.81±0.21	0.74±0.11	0.501
GM-CSF	2.83±0.71	3.09±0.96	0.630
IL-12p40	18.15±4.36	24.91±16.12	0.390
IL-12p70	12.94±3.20	15.40±5.14	0.379
Anti – inflammatory cytokines			
IL-5	0.65±0.16	0.64±0.22	0.932
IL-10	2.52±0.74	3.00±1.20	0.452
Chemokines			
MCP-1	232.05±43.95	238.57±82.88	0.878
RANTES	12.09±1.50	11.06±3.39	0.549
KC	31.62±4.58	39.43±18.01	0.373
Eotaxin	8.43±2.99	7.56±3.15	0.651
MIP-1β	13.12±5.23	21.72±15.15	0.260
MIP-1α	140.07±56.88	143.18±79.29	0.943

4.4 Discussion

Upregulation of inflammatory cytokines and chemokines is a common pathological feature present in most neurological disorders (168). It is one of the crucial components of neuroinflammation after trauma that contributes to additional neuronal damage and progressive secondary injury processes (503, 511). The primary objective of this present study was to determine the effect of enoxaparin and Dp4 on the levels of select inflammatory cytokines/chemokines in the pericontusional cortex at 6 hours after trauma. These results also served to further validate neuroinflammation in our TBI model. Overall, while the SC infusion of Dp4 did not have an effect on the levels of inflammatory mediators after trauma, there were some significant changes in the levels of inflammatory mediators in the IP study and these are discussed below.

4.4.1 Increased production of inflammatory cytokines and chemokines after trauma

As expected, 14 of the 23 inflammatory mediators, including seven pro-inflammatory cytokines (IL-1β, IL-6, IL-12p70, G-CSF, IL-5, IL-12p40, IL-1α), two anti-inflammatory cytokines (IL-10, IL-4) and five chemokines (MCP-1, RANTES, KC, MIP-1α, MIP-1β), were significantly elevated in brain homogenates at 6 hours following a controlled cortical injury. These inflammatory cytokines/chemokines are well established from previous studies to increase after trauma in the brain, in both animal models of TBI and in CSF of humans (417, 443, 504, 506, 510, 516-521). Therefore, our finding corroborates these. Interestingly, in this study, there was no change in the levels of IL-13 and TNF-α at 6 hours following TBI. While this was not consistent with previous studies that have shown elevated levels of TNF-α after trauma (231, 505), our finding does support an earlier study that showed no significant difference in the levels of TNF–α over a 24-hour period using a closed head injury model of moderate focal TBI in mice (242). These differences in studies could be attributed to the multifunctional role of TNF–α, where in acute neuroinflammatory phase following TBI, TNF–α could mediate deleterious effects. However, it could be neuroprotective in chronic timepoints (235, 522). Meanwhile, IL-13 is an anti-inflammatory cytokine associated with the expression of M2a microglia phenotype (521), a subtype of the microglia M2 polarization state involved in tissue repair and growth stimulation (418, 494). In contrast to our study, elevated levels of IL-13 were detected in the rat brain at 24 hours post-TBI (158). It is possible that our CCI model did not induce this cytokine at 6 hours post-TBI, because it was still yet to peak as

evidence has shown significantly elevated levels of IL-13 at 8 hours, peaking at 7 days after injury (523).

4.4.2 Treatment with enoxaparin and Dp4 had no effect on the levels of most cytokines and chemokines assessed in this study

In this study, I assessed the effect of treatment with enoxaparin and Dp4 post-TBI on the levels of IL-4, IL-5, IL-13, and TNF–α because these cytokines were reduced in cultures of Peripheral Blood Mononuclear Cells (PBMC) from asthmatic patients following treatment with enoxaparin, Dp2 and Dp4 (422). However, since the levels of IL-13 and TNF-α were not affected by TBI, as discussed above, the effect of treatment on the production of these cytokines is not a relevant question. However, we did find that, contrary to our hypothesis, neither enoxaparin nor Dp4 had any significant effect on the levels of IL-4 and IL-5 in the injured cortex at 6-hours post-TBI. Aside from methodological considerations, which will be discussed in Chapter 7, there are a few possibilities as to why enoxaparin and Dp4 may not have affected the levels of these cytokines in the injured brain, is that different cell types and multiple stimuli are involved in the inflamed brain compared to the *in vitro* experiments, and it's likely that the drug is not able to block all pathways (524).

Although enoxaparin and Dp4 did not decrease the elevated cellular activation that was observed in the previous chapter, we hypothesised that both drugs may have shifted the inflammatory phenotypes of astrocytes and microglia, ultimately influencing the release of inflammatory cytokines in favour of tissue recovery after trauma. SC infusion or a single IP injection of enoxaparin and Dp4 were administered to C57Bl/6 mice after trauma to evaluate their effect on the levels of inflammatory cytokines/chemokines.

Having hypothesised from the previous chapter that treatment could have shifted the M1/M2 or A1/A2 phenotypes, pro-inflammatory cytokines and chemokines like IL-1, IL-6, TNF-α, MCP-1 and KC were expected to decrease, while anti-inflammatory cytokine like IL-10, IL-4, IL-13 were expected to increase (525). However, my hypothesis was not supported as treatment did not have an effect on the levels of the abovementioned inflammatory cytokines, thereby, implying that there was no effect of treatment on microglia or astrocytes afterall following experimental TBI.

4.4.3 Enoxaparin reduced the levels of MIP-1beta and IL-9 at 6 hours post-TBI.

This study shows for the first time that IP injection of enoxaparin attenuated the levels of MIP-1β and IL-9. This is consistent with the study in which oral administration of enoxaparin reduced the levels of pro-inflammatory cytokines including MIP-1β, in a mice model of acute ulcerative colitis (423).

MIP-1β/CCL4 is a chemokine belonging to the supergene CC chemokine family. It acts through the CCR5 receptor and plays a major role in leukocyte regulation and migration (172, 526-528). Elevated expression of MIP-1β/CCL4 has been demonstrated in the inflammatory processes of various neurological diseases including TBI (507, 509), Alzheimer's disease (529, 530), mice model of cerebral amyloid deposition (530) and cerebral ischemia (531) as well as in other forms of inflammation including osteoarthritis (532), chronic bronchitis (533) and type 1 diabetes (534). Together, these studies show that increased MIP-1β/CCL4 contributes to neurodegeneration, tissue damage and poor outcomes. Additionally, a study that used neutralizing anti-MIP-1β antibodies in a rat model of acute lung inflammatory injury further described the role of MIP-1β/CCL4 in inflammation and tissue damage (535). While T-cells have been reported to secrete MIP-1β, *in vitro* and *in vivo* studies have shown that microglia and astrocytes are also sources of MIP-1β, with predominant expression by a subpopulation of reactive astrocytes (507, 536-538), as demonstrated by their upregulated mRNA expression of the chemokine within the first hour following experimental spinal cord injury in mice (202). In the brain, MIP-1β specifically acts as an attractant for neutrophils and together with other chemokines such as MIP-1α (CCL3), MCP-1 (CCL2), RANTES (CCL5), they promote the recruitment of T-cells/leukocytes into the site of injury (171, 507, 539, 540). Therefore, the decrease observed in the levels of MIP-1β from our study could indicate that treatment with enoxaparin may reduce neutrophil and/or T-cell infiltration, which could ultimately attenuate ongoing secondary damage associated with neuroinflammation after trauma (541, 542).

IL-9, a pleiotropic cytokine which exhibits multiple effects on numerous cell types, is primarily produced by the Th2 subset of T-cells (543), although, there is evidence that IL-9 is also expressed in the brain by microglia and macrophages (544, 545) and its receptor IL-9R, which is expressed on T-cells, has been reported to be also present on astrocytes (544, 546). Interestingly, unlike MIP-1β, not much is known about IL-9 in the context of TBI. Elevated levels of IL-9 were shown in patients with mild TBI from admission up to 12 months post-injury (547). Also, IL-9 was significantly upregulated in the brain and spinal cord

following experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis. Here, the IL-9 receptor was highly expressed in astrocytes, oligodendrocyte progenitor cells, oligodendrocytes and microglia cells (548). Besides the brain, IL-9 has also demonstrated pro-inflammatory activities in airway inflammation where the selective expression of IL-9 within the lungs of transgenic mice led to hypertrophy of airway epithelium, mast cell hyperplasia, infiltration of eosinophils and lymphocytes, mucus hypersecretion and bronchial hyperresponsiveness (549). Similar results were observed following the induction of IL-9 transgene by doxycycline (550). Additionally, treatment with IL-9 attenuated inflammation in a mouse model of arthritis (551). These together not only show the pleiotropic capabilities of IL-9, but also display the typical characteristic of neuroinflammation exhibiting dual roles.

Since MIP-1β and IL-9 are both predominantly produced by T-cells, our finding that enoxaparin reduced their levels following TBI supports previous suggestions that enoxaparin could be exerting its effect via the suppression of T-cells (422, 552). Furthermore, previous studies have reported that treatment with enoxaparin and heparin decreased leukocyte recruitment following TBI (365, 388, 396, 553). Together, these studies and my present data showing a decrease in the levels of both MIP-1β and IL-9, suggest that T-cell recruitment is likely being suppressed in my study. Attempts to investigate this by immunostaining for T-cell subsets on brain tissue sections of the enoxaparin and Dp4-treated TBI mice used in my previous chapter failed.

Another interesting observation from this study is that Dp4High treatment reduced the levels of MIP-1β and IL-9 almost to the same extent as treatment with enoxaparin (i.e. 50% and 43% decrease with enoxaparin vs 39% and 38% decrease with Dp4High, for MIP-1β and IL-9, respectively), however these apparent decreases did not reach statistical significance (Tukey post-hoc, Dp4 vs saline, $P = 0.08$ and $P = 0.08$ for MIP-1β and IL-9, respectively). This potentially similar but weaker effect of Dp4 relative to enoxaparin could indicate that the other active fragments of enoxaparin may be working in addition to Dp4 to generate a more potent anti-inflammatory effect. For example, the non-anticoagulant fragment, Dp2, but also the longer anticoagulant fractions Dp6, Dp10, Dp14 of enoxaparin, also inhibited the release of cytokines from activated peripheral mononuclear blood cell cultures (353, 422). Therefore, in subsequent study, I tested the therapeutic potential of Dp2 and Dp4 combined.

4.4.4 Study Limitations

The variability in our data was greater than what was expected based on earlier studies in our lab (242, 323) and our group size (based on power calculations using the earlier data) was not large enough to achieve the statistical power we had intended, thus limiting the possibility of a more definitive interpretation of our results. Therefore, negative results need to be interpreted with caution, and using larger group sizes (based on new power calculation with current data) could potentially reveal additional group differences to those seen here.

4.4.5 Conclusion

Altogether, our findings demonstrated that both pro- and anti-inflammatory cytokines and chemokines were produced in the pericontusional cortex at 6-hours post-injury, thereby validating our model of TBI. I have also shown that enoxaparin reduced the levels of the chemokine MIP-1β/CCL4, and the cytokine, IL-9, both of which are mainly produced by T-cells. However, these findings did not support my hypothesis that enoxaparin and Dp4 treatment did not decrease the activation of astrocytes and microglia after trauma as proinflammatory cytokines were not downregulated.

For the future, it remains to be seen if a higher bolus dose of the drug will have more effect on inflammatory cytokine/chemokine levels after trauma and if the drugs bring about their effect by suppressing the release of T-cell mediated cytokines/chemokines or they perhaps act through a completely different pathway.It is of great importance that to develop effective therapeutics for TBI, a deeper understanding of inflammatory mediators is achieved, since a significant change in cytokine/chemokine levels can significantly shift neuroinflammation to a more deleterious or beneficial process.

While the aim of this study was to explore markers of inflammation, there is the possibility that treatment may have neuroprotective effects that were not detected with the parameters we have measured. For example, heparin and LMWHs significantly decrease infarct volume in a rat model of ischemic injury and lesion volume following trauma (385, 554). Although this was beyond the scope of my research questions, in order to more fully understand the therapeutic potential of these drugs, it would be valuable to further explore whether enoxaparin and Dp4 were neuroprotective in these studies, either via quantifying lesion volume or evaluating cell death in the cortex or hippocampus.

CHAPTER 5

LONG-TERM EFFECT OF NON-ANTICOAGULANT FRACTIONS OF ENOXAPARIN ON BEHAVIOURAL OUTCOMES POST-TBI

5.1 Introduction

Traumatic Brain Injury (TBI) remains one of the most prevalent causes of disability in individuals aged ≤45 years including in Australia, where in 2004-2005, over two thirds of TBI-related disabilities were reported in people aged under 65 years (29, 62). Disabilities associated with TBI are often complex, resulting in lifelong behavioural dysfunction that remarkably contributes to decreased quality of life in survivors of trauma and who could become a burden to their families as they are unable to return to work in most cases (2, 555-559).

Several studies have linked TBI to debilitating neurobehavioural sequelae including cognitive and neurological impairment (560-563) motor function deficits (564, 565), anxiety and/or depression (566-568) and social abnormalities (569). While these deficits can manifest early after brain injury, in most instances, and depending on injury severity, they become evident at a much later time or worsen over time (570, 571). Although the underlying physiological and neurobiological mechanisms are yet to be fully understood, some of these outcomes observed in trauma patients have been more fully characterised using animal models that can replicate some of the pathological changes associated with TBI in humans (572, 573).

There are many different behavioural tasks that have been utilised for the assessment of different aspects of TBI pathologies across different injury severities and animal models (574-577). Due to the diverse nature in which TBI outcomes present, multiple tasks testing a range of different behavioural aspects are suggested to be incorporated in experimental designs, as these could serve to complement one another for a more thorough or extensive analysis (574). For example, a single test cannot adequately assess the different processes involved in TBI-related behavioural deficits such as in motor, social, cognitive, pain and anxiety and/or depression. For this reason, in the study presented in this chapter, TBI-induced deficits in motor function, muscular strength and locomotion activity (hyperactivity) were assessed using the ledged beam, hanging wire and open field tasks. While the ledged beam task had previously been used successfully in our lab to access the beneficial effects of anti-inflammatory drugs, hanging wire and open field tests were both introduced to further explore other aspects of behavioural abnormalities.

As research into TBI therapeutics has significantly increased over the years, it is important that potential new therapies improve functional impairments resulting from TBI. Several drugs including enoxaparin have aptly demonstrated improved neurobehavioural outcomes following TBI. For instance, treatment with heparin and its derivatives including

enoxaparin improved cognitive impairment, motor function deficits, neurological function with learning and memory in experimental TBI (365, 386, 388, 396, 553).

Considering that enoxaparin had beneficial effects on behavioural outcomes previously, we speculated that perhaps treatment with enoxaparin and Dp4 might be improving function through a mechanism not related to attenuating gliosis, as we had originally hypothesised. For example, improved function could result from decreased infiltration of T-cells and other leukocytes, as suggested by my results in the previous chapter and earlier work by others (365, 385, 388, 389, 396), or from actions separate to inflammation, such as decreased apoptosis (366, 387, 578) and brain oedema (386, 579). Importantly, other treatment strategies targeting these pathologies have been associated with improved functional recovery in TBI models (580, 581).

In this chapter, therefore, I aimed to investigate the long-term effects of enoxaparin, Dp2, Dp4 and the combination of Dp2+Dp4 administration on functional impairment and behavioural outcomes following a CCI-induced model of moderate TBI. Here, Dp2 was introduced for the first time as a treatment group in this thesis. Dp2 is a disaccharide fraction of enoxaparin, which like Dp4 has demonstrated robust anti-inflammatory effects in peripheral inflammation. Dp2 was found to inhibit release of the same inflammatory cytokines (IL-4, IL-5, IL-13 and TNF-α) as Dp4 in the PBMCs. While Dp4 inhibited this release by more than 68%, Dp2 inhibited the release of these cytokines by more than 57%. Therefore, we proposed that they could be different, possibly acting through different pathways to bring about their effect. Thus, we decided to explore the potential anti-inflammatory effect of Dp2 in the brain post-TBI and also the combination of Dp2 and Dp4 was investigated to determine if together, they could have an addictive effect following trauma.

5.2 Methods

For this study, a single dose of Dp4 was used (equivalent to the high dose used in earlier experiments) and two additional treatment groups were introduced: Dp2 and Dp2+Dp4 combined. Other groups included sham controls, TBI vehicle controls and enoxaparin treated group as in the previous experiments.

5.2.1 Drug administration

Repeated subcutaneous injections of Dp2, Dp2+Dp4, enoxaparin, or Dp4, were administered to mice at 1, 6, 24 and 30 hours post-TBI. TBI vehicle controls and sham operated mice received equal volume injections of saline at the same timepoints. In this study, a new method of drug delivery was used. Since changes in cytokine levels were observed following bolus injections but not with continuous, low-concentration infusions, we opted to administer the drugs in this experiment via repeated injections. We chose SC rather than IP, since this is the clinically relevant route for heparins, and is also more directly comparable to the earlier preclinical studies that have demonstrated a beneficial effect of enoxaparin following TBI. Doses of drugs used are detailed in the table below (Table 5.1).

Table 5.1: Drug dosage for each mode of delivery

Drugs	**Repeated Subcutaneous injections**
Enoxaparin (Dose based on published studies; used as a benchmark for anti-inflammatory activity).	1 mg/kg/injection (Delivered as ~200 ul of 150 ug/ml solution in saline and administered at 1, 6, 24 and 30 h post-TBI).
Dp2	104 µg/kg/injection (Delivered as ~200 ul of 16 ug/ml solution in saline and administered at 1, 6, 24 and 30 h post-TBI).
Dp4	104 µg/kg/injection (Delivered as ~200 ul of 16 ug/ml solution in saline and administered at 1, 6, 24 and 30 h post-TBI).
Dp2+Dp4 (Combined therapy)	104 µg/kg Dp4 + 104 µg/kg Dp2 per injection (Delivered as ~200 ul solution of 16 ug/ml Dp4 + 16 ug/ml Dp2 in saline and administered at 1, 6, 24 and 30 h post-TBI).

5.2.2 Mice welfare assessments post-injury

Mouse wellbeing was monitored after trauma to assess unanticipated ongoing pain and distress. Two scoring systems were employed for this assessment. The first welfare assessment was carried out based on the observations as detailed in table 5.2. This uses a three-point score to assess the general condition, activity/posture, spontaneous behaviour, breathing and movement of mice after trauma. A score of one represents normal responses across all assessment criteria and a score of three represents a poor response across the various assessment parameters mentioned earlier. Animals with a score of three that did not respond to treatment were to be euthanised.

Table 5.2: Welfare score criteria

Score Assessment	**Score 1**	**Score 2**	**Score 3**
General Condition	Normal	Changed coat. Dull fur	Piloerection Fur unkempt
Activity / Posture	Normal activity undisturbed and disturbed. Responds to handling.	Mildly inactive Possible posture change. Respond to handling.	Inactive or active only when stimulated. Response to handling is reduced significantly.
Spontaneous behaviour	Walking, Rearing, Grooming, Sniffing	Showing reduced level of behaviour	No display of behaviours
Breathing	Normal	Change to rate	Change in rate, rhythm depth noted / irregular shallow agonal or laboured.
Movement	No aberration in movement pattern.	Slowed, lameless, tiptoe changed gait but animal can move around.	Immobile, only moves when stimulated.

The second monitoring system used to evaluate mouse wellbeing was the mouse grimace scale (MGS). The MGS is an effective and reliable tool for assessing pain intensity in mice (582, 583). Like the welfare score, it also utilizes a three-point scale to determine the intensity of the following MGS parameters / features: orbital tightening, nose bulge, cheek bulge, ear position and whisker change. Like the welfare scoring system, a value of zero depicts the absence of MGS feature, a value of one represents the moderate presence of a feature and a value of two indicates the severe presence of a feature. A total score of 10 on the MGS indicates the highest pain/distress level.

Both welfare assessment scoring systems were conducted using a monitoring sheet (Figure 5.1) prepared by our lab to assess mice on days 1, 2, 4 and 7 post-TBI.

Daily Monitoring Sheet

Mouse #:

Date TBI:

Welfare score

Mouse#	D1	D2	D4	D7
General Condition				
Activity / Posture				
Spont. behaviour				
Breathing				
Movement				
Weight (g)				
Δ Weight from D0				

Mouse grimace scale

Mouse#	D1	D2	D4	D7
Orbital Tightening				
Nose Bulge				
Cheek Bulge				
Ear Position				
Whisker Change				

Figure 5.1: A mice wellbeing assessment sheet.

Observation and assessment of mice wellbeing were recorded on this sheet at day 1, 2, 4 and 7 post TBI. The sheet contains the various observed testing parameters / features of the welfare score and mouse grimace scale.

5.2.3 Functional assessments

Functional assessments were used to determine deficits and to evaluate recovery after trauma (576). Three different functional assessments were employed in this project; motor function was assessed using ledged beam test and hanging wire test while behavioural function was determined by open field test. About two weeks prior to commencing behavioural testing, mice habituated for at least two hours each day in the test-specific behaviour room. Likewise, on every day of testing, mice were habituated for 30 minutes in the test-specific behaviour room prior to the start of the experiment. I was blinded to the experimental groups all through the period of testing and analysis.

5.2.3.1 Ledged Beam Task

The ledged beam test is an established and refined non-invasive test that assesses motor function after trauma. It reveals behavioural and motor deficits in rodents as it reduces the need

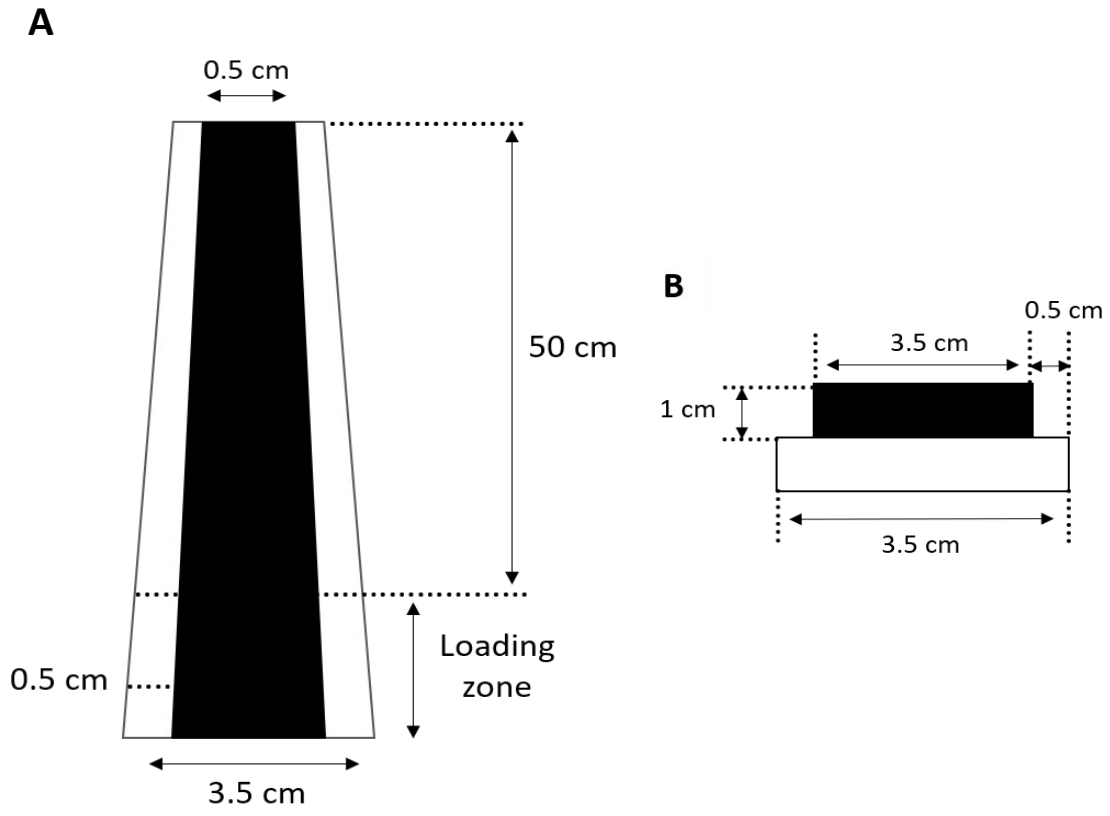


Figure 5.2: Dimensions of the ledged beam apparatus.

*Mice motor function was assessed with ledged beam; top view **(A)** and rear view **(B)**. The ledged beam apparatus was placed at an angle of 30° and foot faults were counted as steps taken on the underlying ledge (clear ledge) or when the lower ledge is touched by the mice following a slip from the central beam surface (black surface).*

for rapidly learning compensatory motor behaviours to prevent falling. The beam, which was adapted from previous assessment in rats (584, 585) is made from plexiglass and consists of a 1 m long central surface that tapers in width from 3.5cm to 0.5cm over a length of 50cm (Figure 5.2A) with underhanging ledges running through the entire length of the beam (323). The underhanging ledges are 0.5 cm wide and 1cm below the surface of the central beam (Figure 5.2B). To determine hemiparesis of the fore and hind limbs, mice were videotaped traversing the beam, placed at an incline of 30°, from the widest to the narrowest end. Foot faults were counted as steps taken on the underlying ledge or when the lower ledge is touched by the mice following a slip from the central beam surface and these were quantified through a blinded analysis of the videotape recordings for the fore and hind limbs contralateral to the side of the brain injury. Each mouse underwent three consecutive trials per session and total foot faults were counted and averaged per trial for statistical analysis. Mice were tested three times in the first week after trauma starting at one day post-TBI or sham operation and thereafter once weekly for three weeks. The apparatus is cleaned with 70% ethanol between each mouse.

5.2.3.2 Hanging wire task.

The hanging wire test (also referred to as the wire hanging or wire grip test in some cases) assesses grip/limb strength, muscle function, endurance and balance (586). The standard linear wire hang apparatus used for this test consists of a metal wire which ran across two plastic poles. The metal wire was suspended 35cm above a padded surface that provided a cushion for the fallen animal to avoid any more injury (Figure 5.3A). Mice were individually tested by placing them on their forelimbs in the centre of the wire for 180 seconds (Figure 5.3B). They could later hang on with their hindlimbs and tail, (Figure 5.3C) however animals were discouraged from balancing on the wire (Figure 5.3D) as it was considered an inappropriate behaviour. Each session was video recorded for future quantification of the total number of falls per session. Mice were tested three times in the first week after trauma and once weekly thereafter for three weeks starting at one day post-TBI or sham operation.

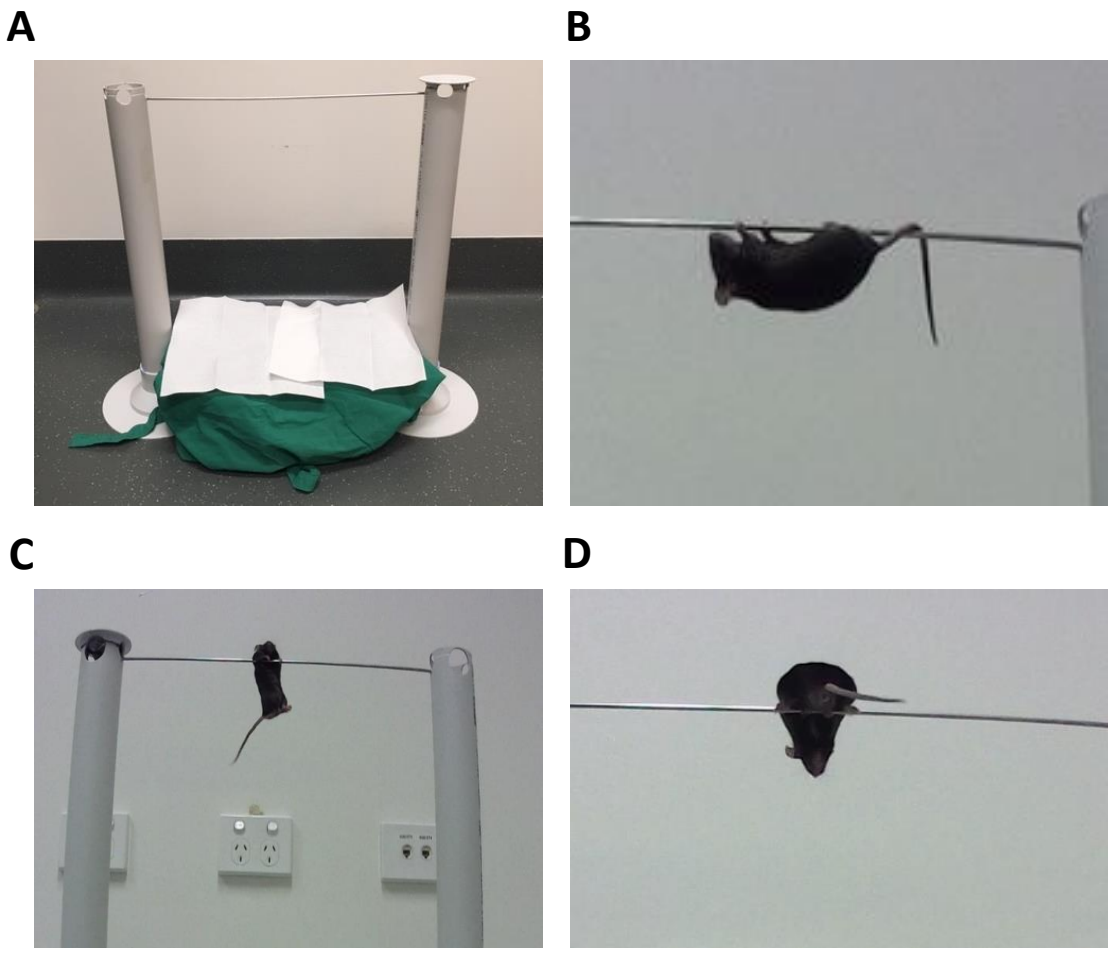


Figure 5.3: The standard linear hanging wire apparatus and explanatory images of testing

A representative image of the hanging wire apparatus setup ***(A)****. The metal wire was placed across two poles and was suspended 35cm above a padded surface. The correct starting position of this test was placing mice on their forelimbs in the centre of the wire* ***(B).*** *Mice could hang on with their hindlimbs and tail afterwards* ***(C)*** *but were not allowed to balance on the wire* ***(D).***

5.2.3.3 Open field Test

Open field test was used to evaluate hyperactivity and anxiety after trauma or sham operation. Mice were individually placed in the centre of an opaque white 30 cm by 30 cm arena and illumination was provided from sodium lights positioned overhead. Mice movements were tracked by an overhead camera connected to the computer with an EthoVision XT software for 10 minutes each. The total distance (cm) and speed travelled (cm/s) as well as the time spent in the inner and outer zone were assessed on days 2, 8, 15, 22 and 29 post-TBI. Nose point, centre point and tail point data were all acquired but only centre point data was analysed

as it reflected the movement of mice in the arena best. The size of the inner area of the arena was defined as approximately 203 cm^2 (13.7 cm x 14.8 cm).

All behaviour tests and the timing of each test are detailed below in Figure 5.4.

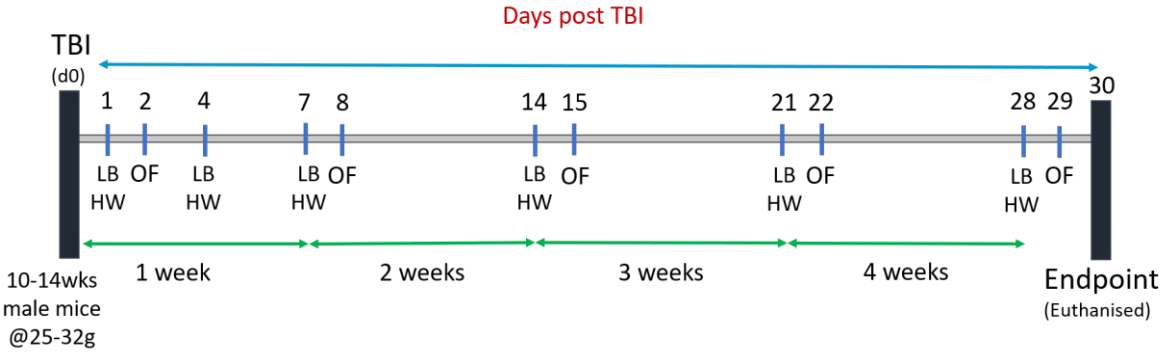


Figure 5.4: Detailed timeline of all behavioural experimental tests

Three behavioural tests, ledged beam (LB), hanging wire (HW) and open field (OF) were performed over a period of 4 weeks starting from day 1 post-TBI to day 29 post TBI. LB and HW tests were conducted on days 1, 4 and 7 in the first week after injury and thereafter once every week until mice were euthanised. OF test on the other hand was carried out once a week beginning from the second day after trauma to day 29 post-TBI.

5.2.3.4 Body weight loss

Animal body weights were measured at day 0, 1, 4, 7, 14, 15, 21, 22, 28 and 29 after TBI and the degree of weight loss was expressed as weight loss ratio [($W_{0\ h} - W_{day\ 1,\ 4,\ 7,\ 14,\ 15,\ 21,\ 22,\ 28\ or\ 29}$) / $W_{0\ h}$ x 100%]. This was performed as part of monitoring the animal's welfare post-surgery and any animal with a weight loss greater than 15% of its original weight was euthanised.

5.2.4 Statistical analyses

Data were presented as mean ± standard deviation (SD) for each treatment group. Group comparisons were made with repeated measures analysis of variance (ANOVA), and Tukey post-hoc analyses with $p<0.05$ considered as statistically significant. All calculations were performed with GraphPad prism (version 9) software.

5.5 Results

5.5.1 Assessment of wellbeing and body weight loss after trauma

To demonstrate that the TBI procedure employed in this study did not impart significantly on animal welfare, mice were monitored at 1, 2, 4 and 7 days after injury using a welfare scoring system and grimace scale. The welfare scoring system assesses general condition, posture, spontaneous behaviour, breathing and movement, while the grimace scale scores mice based on orbital tightening, ear position, whisker change, nose, and cheek bulge. Overall, mice welfare scores were normal in all the parameters checked over the period of 7 days post-TBI. Of the 75 mice that were assessed, two mice demonstrated poor welfare score after trauma. Specifically, one mouse displayed piloerection, significant change in breathing rate, slowed gait and reduced level of behaviour within the first week after trauma. To minimise pain and distress, the mouse was culled on the 8^{th} day after trauma. Upon autopsy, it was discovered that there was a tumour in the thoracic region of the animal which was most likely the reason for its poor welfare and not from the induction of TBI. Meanwhile, the poor welfare score of the second mouse was as a result of the mouse having developed an anal prolapse and it was culled on day 21 after trauma. The poor welfare outcome demonstrated by this mouse was not as a result of trauma as the mouse's general condition, posture, breathing, and movement were normal at 7 days post-TBI.

Furthermore, following grimace scale assessments after trauma, over 80% of the animals scored zero in all the parameters by the 4^{th} day after injury such that by day 7 post-TBI, virtually all mice had attained an apparent pain-free state with a score of zero on all parameters.

Body weight loss, another measure of welfare and wellbeing, was carried out throughout the entire study period of 4 weeks. Mice body weights were measured at days 0, 1, 2, 4, 7, 8, 14, 15, 21, 22, 28, and 29 after trauma. Our results showed that irrespective of treatment, there was no effect of trauma or drug on the amount of weight that was lost and no mouse lost more than 15% of body weight as the average body weight loss was 5% and the most body weight loss observed was 7% in the untreated injured group at day 8 (see table in appendix B).

5.5.2 Evaluation of motor function outcomes after trauma

To assess sensorimotor functional outcomes and the effect of drug treatment (Dp2, Dp4, enoxaparin and the combined therapy of Dp2 and Dp4 (Dp2+Dp4)) after trauma, ledged beam and hanging wire tasks were performed on days 1, 4, 7, 14, 21 and 28 post-TBI. Of the total mice used for this study, the data from eight mice were excluded from the behavioural analyses. Two mice were culled (described above) while the other six mice were excluded from the analysis due to missing experimental values for day 4 post-TBI as behavioural testing was not performed at this timepoint in the last cohort of experimental animals due to unavailability of the behavioural room. TBI causes functional deficits up to 7 days after injury on the ledged beam.

On the ledged beam, foot faults indicate functional deficit. In the first week after trauma, it was observed that the frequency of contralateral hindlimb foot faults made by the injured mice appeared higher than the uninjured mice and this became more evident in the narrowest part of the beam, whereas the uninjured sham controls were observed to traverse the beam in a more upright position without much difficulty.

Analysis of the data showed that there was a significant difference in the number of foot faults made by the contralateral hindlimb between the different experimental groups (Figure 5.5A, two-way repeated measures ANOVA, effect of treatment, $P = 0.0013$), and that the number of errors decreased with time (two-way repeated measures ANOVA, effect of time, $P < 0.0001$), with a significant interaction between treatment and time (two-way repeated measures ANOVA, $P = 0.0038$). Generally, on days 1 and 4, the TBI groups had significantly more foot faults than the shams operated mice (refer to Figure 5.5A for Tukey post-hoc values); On day 7, the number of foot faults in the groups treated with enoxaparin, Dp2 and Dp2+Dp4 were no longer different to shams, while the saline and Dp4-treated TBI groups remained elevated. From day 14, all TBI groups returned to sham levels. Additionally, there were no significant differences in the number of foot faults made by TBI mice of the different treatment groups at all the time points investigated.

In contrast to the contralateral hindlimb, the contralateral forelimb of the injured mice was observed to make fewer foot slips. The analyses of the ledged beam data revealed that there was no significant difference in the number of foot faults by the contralateral forelimb between the different treatment groups post-injury (Figure 5.5B, two-way repeated measures ANOVA, effect of treatment, $P = 0.5765$). However, there was a significant difference in the number of foot faults errors by the contralateral forelimb over time between the experimental groups (Figure 5.5B, two-way repeated measures ANOVA, effect of time, $P < 0.0001$ and

effect of interaction between treatment and time, $P < 0.0001$). Overall, there were substantially more foot fault errors between the experimental groups on day 1 compared to the other timepoints (Figure 5.5A, Tukey post-hoc, $P < 0.05$).

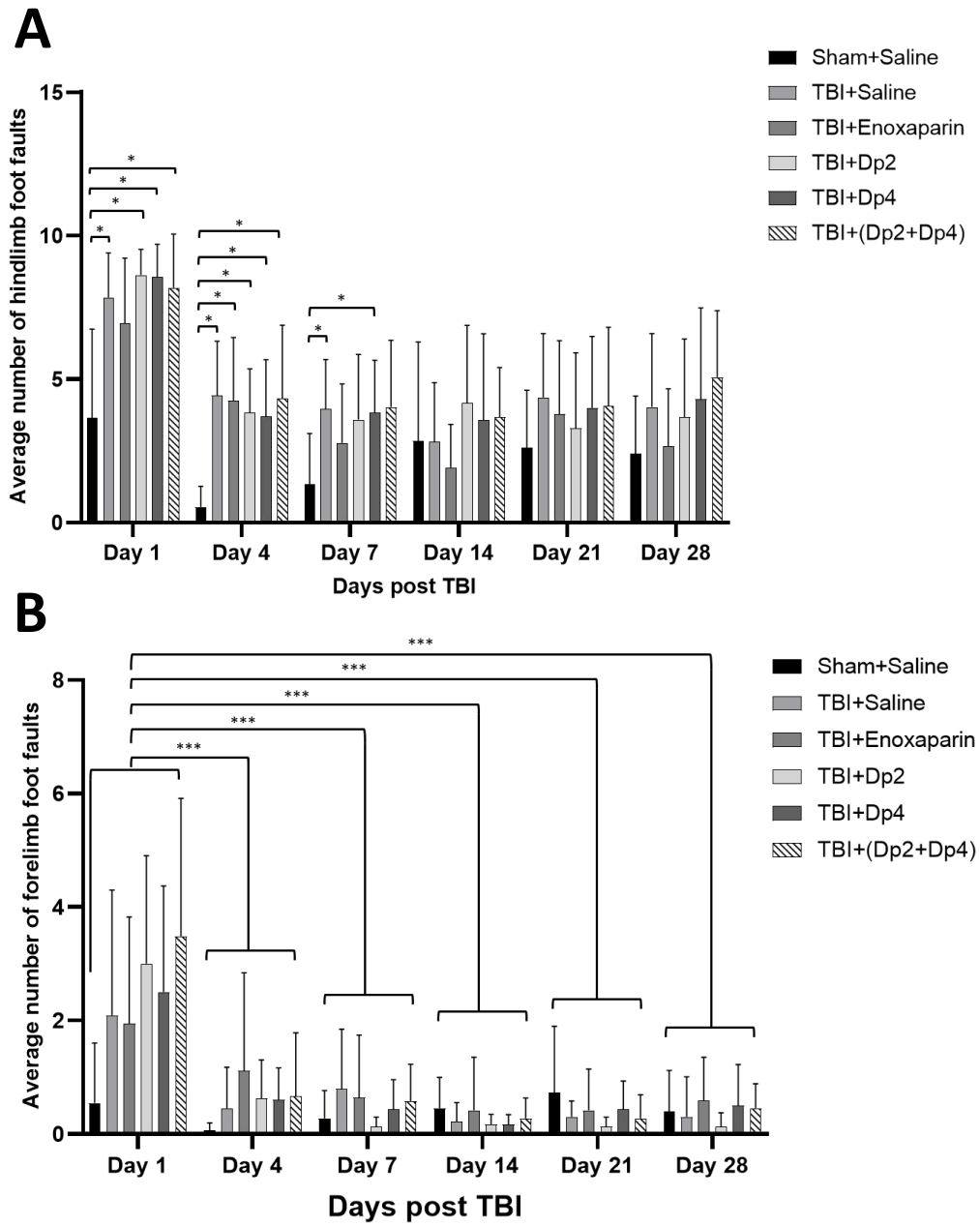


Figure 5.5: Evaluation of contralateral hindlimb and forelimb's ledged beam performance at 4 weeks after trauma.

*Contralateral hindlimb **(A)** and forelimb **(B)** performance was assessed on the ledged beam following TBI sham surgery by calculating the average number of foot faults. Foot faults made by the contralateral hindlimb of the TBI mice were significantly greater compared to the uninjured sham controls and this was most evident within the first week precisely at days 1, 4, and 7 post-injury (effect of treatment, Tukey post hoc, $*P < 0.05$). By day 14 all TBI injured groups had returned to sham levels and this remained constant across the other timepoints (Tukey post-hoc, $P > 0.05$). Furthermore, the number of foot fault errors by the contralateral forelimb was not significantly different between the experimental groups. However, the interaction between treatment and time was significant. Altogether, there were more foot fault errors by the contralateral forelimb at day 1 compared to the other timepoints thus indicating, decreased foot fault errors over time across all experimental groups (Tukey post hoc, $^{***}P < 0.0001$). Data is expressed as mean ± SD; n = 8-12.*

5.5.3 TBI did not lead to motor function deficit detectable by the hanging wire test.

To evaluate muscular strength and motor function, hanging wire test was performed on the same days as the ledged beam test over a period of 4 weeks after trauma and the results show that there was no significant difference in the average number of falls between the experimental groups (Figure 5.6, two-way repeated measures ANOVA, effect of treatment, $P = 0.4177$). However, there was significant effect of time, irrespective of treatment (Figure 5.6, two-way repeated measures ANOVA, effect of time, $P < 0.0001$ and effect of interaction, $P = 0.6167$) with the number of falls decreasing from day 7 post-surgery (refer to Figure 5.6 for Tukey post-hoc p-values).

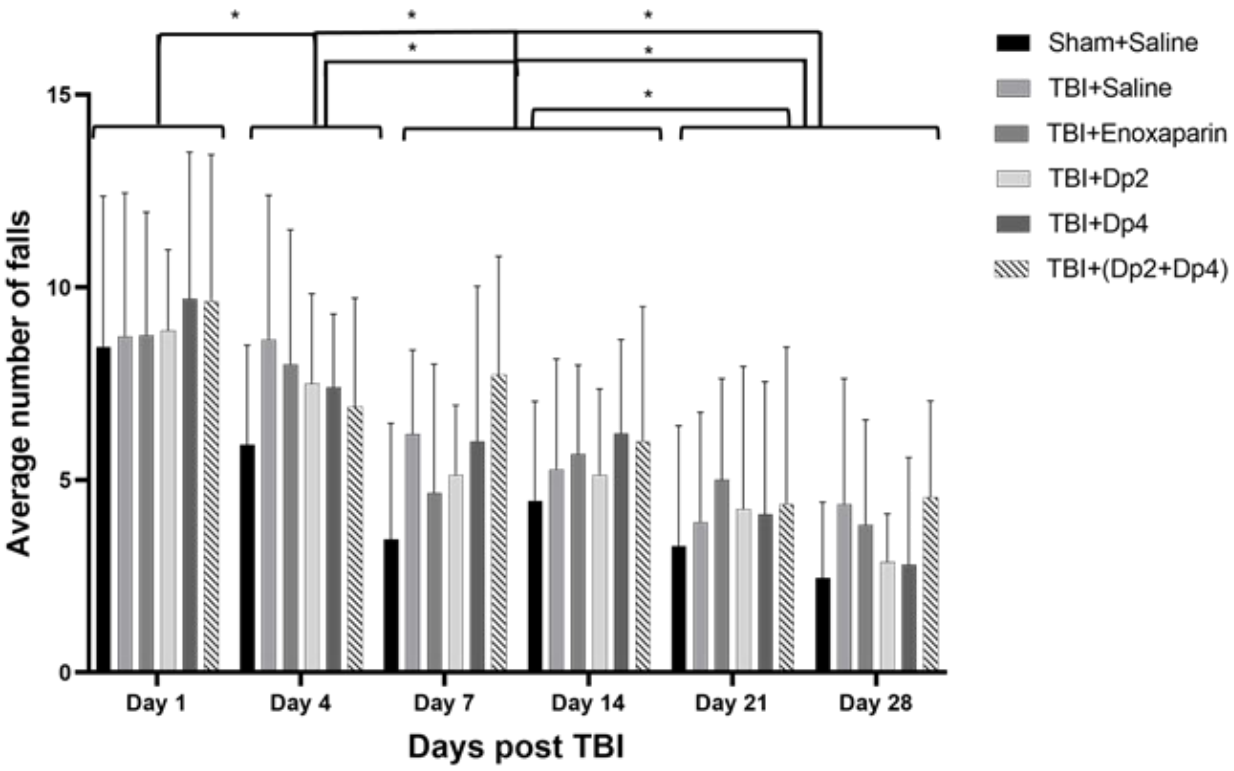


Figure 5.6: Motor function performance improved over time on the hanging wire at 4 weeks after trauma.

*Mice muscular strength and motor function performance was evaluated on the hanging wire at days 1, 4, 7, 14, 21 and 28 after trauma. Whilst there was no significant difference in the average number of falls between all groups (effect of treatment, two-way repeated measures ANOVA, P = 0.4177), over time there was significant difference in the average number of falls across all experimental groups. falls were (effect of time, Tukey post hoc, *P < 0.05). Data is expressed as mean ± SD; n = 8 -12.*

5.5.4 Trauma did not induce increased locomotor activity (hyperactivity) in the open field test.

To evaluate hyperactivity after trauma, mice locomotor activity was assessed in the open field arena on days 2, 8, 15, and 22 post-TBI or sham surgery for a duration of 10 minutes. Total distance travelled (cm) and mean speed (cm/s) were recorded. TBI with or without drug treatment, did not affect total distance travelled and speed in the arena over a period of 4 weeks after injury compared to the sham operated mice (Figure 5.7A and B; two-way repeated measures ANOVA, effect of treatment, $P = 0.1989$, 0.1909 respectively, effect of interaction between treatment and time, $P = 0.0661$, 0.0647 respectively). However, there was an overall significant effect of time in both total distance travelled and speed over a period of 4 weeks post-injury (two-way repeated measures ANOVA, effect of time, $P < 0.0001$, $P < 0.0001$, respectively). For both total distance and speed, day 2 was different to day 22, and day 8 was different to both days 15 and 22 (refer to Figure 5.7 for Tukey post-hoc p-values).

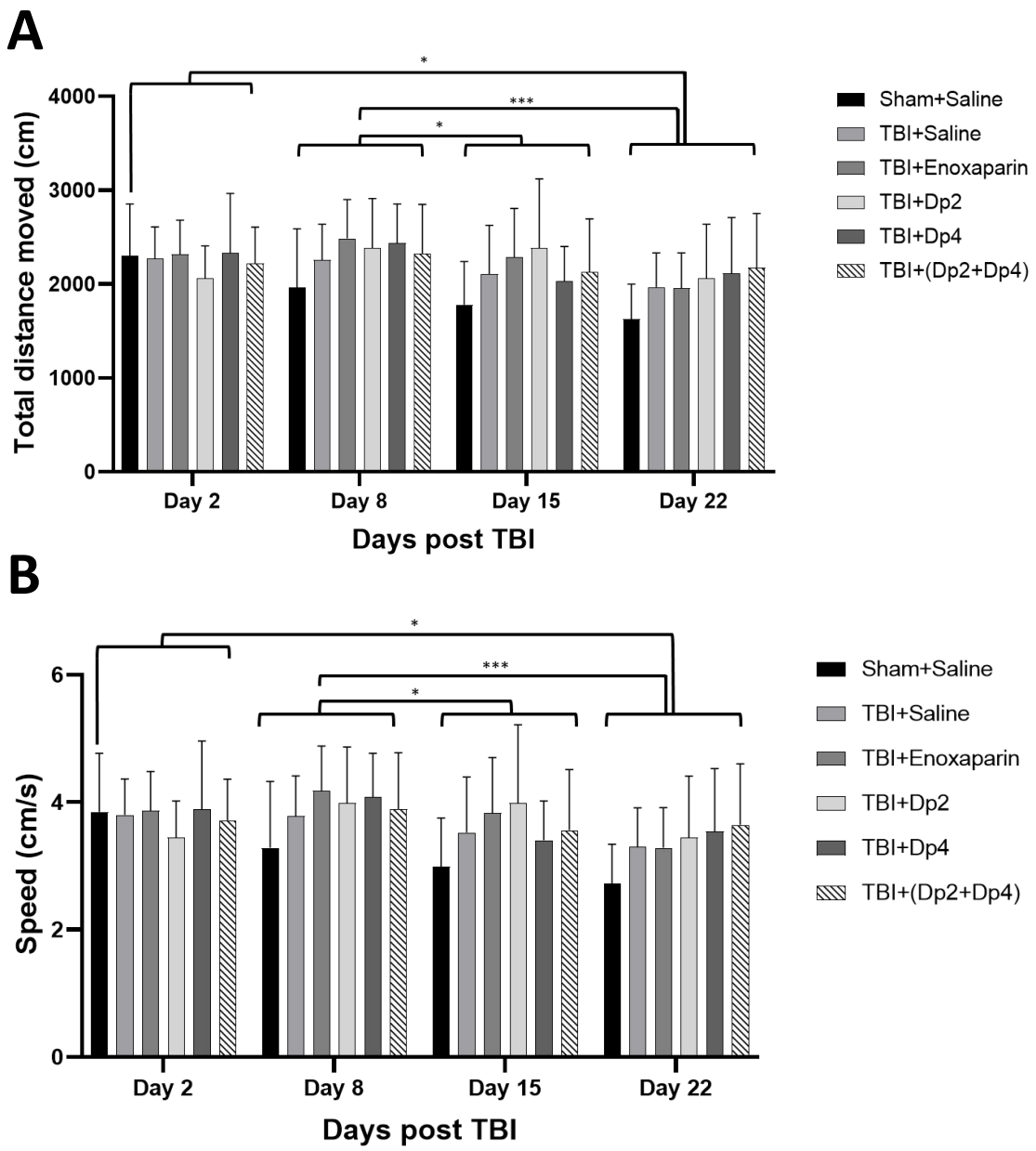


Figure 5.7: Locomotor activity did not change in the open-field test 4 weeks post TBI.

Locomotor activity in mice was assessed in the open-field test at days 2, 8, 15, and 22 post TBI to determine hyperactivity. Total distance travelled in cm ***(A)*** *and speed in cm/s* ***(B)*** *were acquired with Ethovision XT software. There was no change in both total distance moved and speed in the arena of all the experimental groups. However, a significant difference in the total distance travelled as well as speed was demonstrated over time across all groups. For both total distance and speed, day 2 was different to day 22, and day 8 was different to both days 15 and 22 (Tukey post-hoc, *p < 0.05, **P < 0.0001). All data is expressed as mean ± SD; n = 10-13.*

5.5.5 No increase in anxiety after trauma in the open field arena

Having seen that there was no significant change in total distance travelled and speed of mice in the arena after trauma, the relative amount of time spent in the centre and outer zones of the arena was investigated post-sham or injury as a measure of anxiety. In general, mice across all experimental groups spent a lesser amount of time in the centre than in the outer zones. Furthermore, there was no difference in the time spent in the outer zone (defined as areas excluding the centre) and in the inner zone (defined as the centre only) across all experimental groups over the period of 4 weeks post-TBI (Figure 5.8A; effect of treatment and time for outer zone, two-way repeated measures ANOVA, $P = 0.7215$, 0.1542 respectively; Figure 5.8B; for inner zone, two-way repeated measures ANOVA, $P = 0.6804$, 0.2827 respectively). There was also no significant interaction between treatment and the time spent in the outer or inner zones of all the experimental groups (two-way repeated measures ANOVA, $P = 0.4316$, 0.4628 respectively).

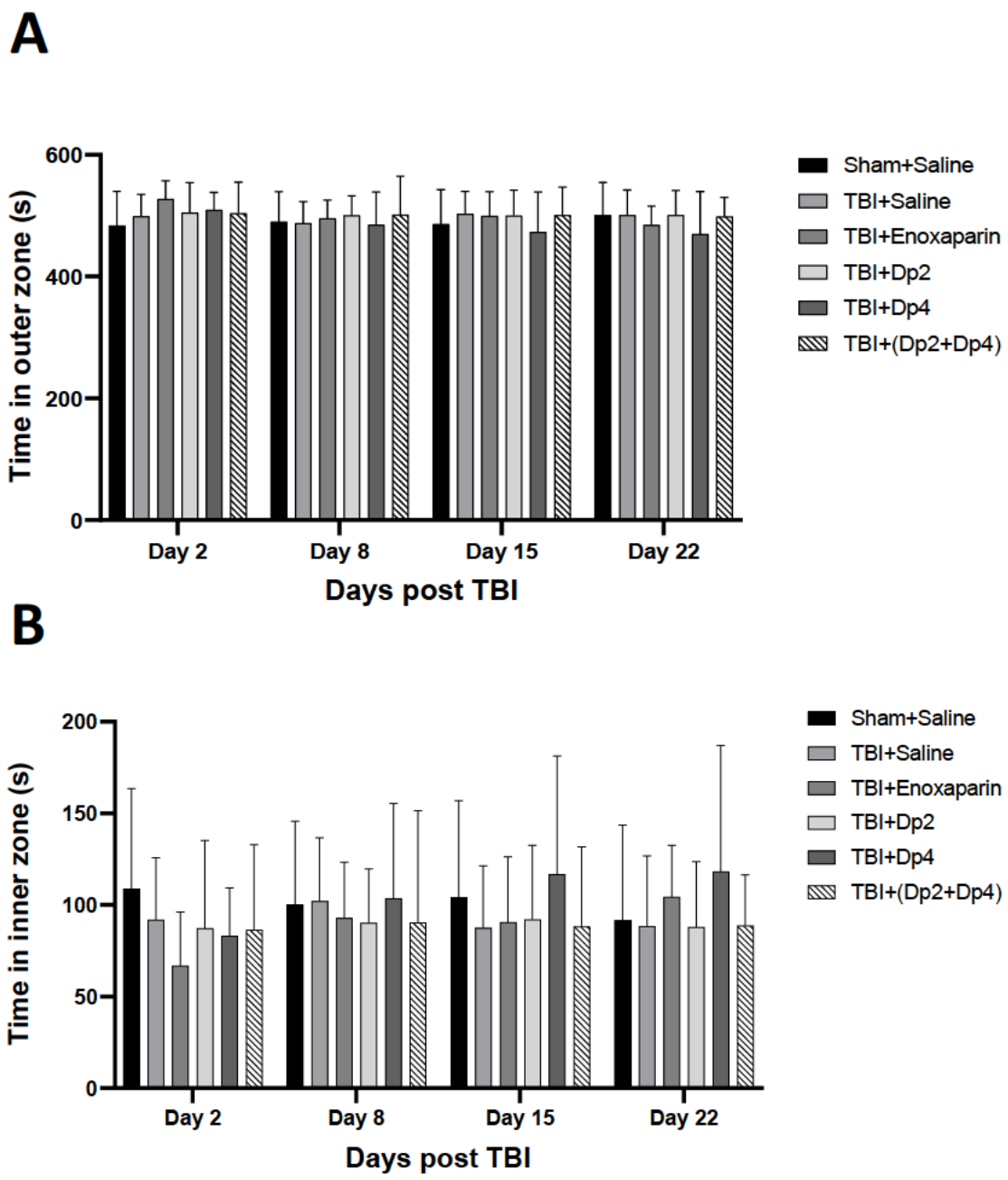


Figure 5.8: Mice spent less time in centre.

Overall, mice across all experimental groups spent less time in the inner zone compared to the outer zone of the arena over 4 weeks post injury. No significant differences were recorded in the time spent within the outer zone ***(A)*** *and the inner zone* ***(B)*** *of all groups over the period specified earlier, also implying that there was no effect of treatment (effect of time and treatment for outer zone, two-way repeated measures ANOVA, P = 0.1542, 0.7215 respectively; for inner zone, two-way repeated measures ANOVA, P = 0.2827, 0.6804 respectively). All data is expressed as mean ± SD; n = 10-13.*

5.6 Discussion

Several studies have examined and established the presence of motor dysfunction and anxiety-like behaviour as long-term outcomes following trauma (564, 574, 587-592). This present study therefore aimed to determine whether treatment with enoxaparin and its fractions Dp2 and Dp4, as well as the combination of Dp2 and Dp4, will reduce motor function impairment and anxiety in a CCI model of TBI over a 4-week period. Our results show that there was no effect of treatment across all the experimental groups after trauma.

Three different tests were employed in this study to assess mouse behaviour after a CCI-induced injury and only one test, the ledged beam task, could show motor deficit in our TBI model. A battery of behavioural test measures appropriate for each deficit is required in order to avoid the misinterpretation of results especially when assessing the therapeutic effect of an intervention/drug in experimental animals (574). Only in this way will the effect of therapeutic intervention be precisely evaluated in experimental animals and the development towards improving functional recovery in TBI-injured patients.

Therefore, in this study, we were interested in assessing hyperactivity, anxiety-like behaviour and motor function deficits in the hindlimb and forelimb after trauma. We chose to use the open field test to assess hyperactivity and anxiety-like behaviour based on other studies in the literature that have successfully utilised this test to demonstrate significance in both of these outcomes following TBI (580, 593). While the ledged beam test has been proven to be a more sensitive test to detect deficits in the hindlimb, it is not a sensitive test for the forelimb (322). Hence, the hanging wire test was introduced for the first time in our lab to assess muscular strength in the forelimb post-injury.

Specifically, my results showed that mice, subjected to TBI demonstrated poor performance on the ledged beam task for up to 7 days post-injury and this motor impairment was not attenuated by treatment. This finding validated our CCI model of focal injury, in that the motor impairment displayed after trauma was consistent with previous studies that observed motor function deficits after trauma on the ledged beam (322, 323, 594). These studies also established that the ledged beam task is a sensitive tool for assessing motor dysfunction in trauma models.

Meanwhile, on the other hand, the finding that enoxaparin did not alleviate the functional deficits exhibited on the ledged beam after trauma was surprising, as it was contrary to earlier studies in which enoxaparin improved motor, cognitive and neurologic function after trauma (386, 388, 391). Similarly, unfractionated heparin, the parent drug of enoxaparin, also

improved cognitive and neurologic function after trauma (365, 396). One major possible explanation that could be attributed to the difference in the findings between these studies and our study could be variability in experimental designs like animal species/strain. For instance, while the injury model and drug dose used in our study is comparable to that of Li et al., 2015 (388), they used a CD1 mouse strain whereas, in our study, we used a C57Bl6 mice.

Furthermore, in this study, a hanging wire task and the well-recognised open field test were also performed to assess neuromuscular function and anxiety-like behaviour after trauma. Unlike the ledged beam, the hanging wire test did not detect motor dysfunction in the trauma mice. Our result supports a study that assessed behaviour in a mouse model of ischaemia where, similar to my observations, they determined that the C57Bl/6 mice were not suited for the hanging wire test. These authors reported that the C57Bl/6 mice tend to let go of the wire or display inappropriate behaviour such as balancing on the wire or intentionally jumping off the wire, especially the uninjured mice, thereby making it difficult to determine true motor function impairment (586, 595, 596). In general, the hanging wire test is a simple, non-invasive and inexpensive sensorimotor test that evaluates motor function and coordination (595, 596) and this test has been successfully used in models of CNS disorders with mice strains other than the C57Bl/6 mouse strain, including a 129/SV mouse model of ischaemia (586), *mdx* or mdx^{5Cv} mouse model of Duchenne muscular dystrophy (DMD) (595) and SOD1G93A mouse model of amyotrophic lateral sclerosis (597).

Collectively, our findings suggest that the ledged beam task is a more suitable tool for assessing motor function deficits following CCI-induced TBI in a C57Bl/6 mouse strain compared to the hanging wire test. The sensitivity of the ledged beam task also provides an efficient platform for testing potential treatments for TBI. For example, treatment with minocycline, an anti-inflammatory drug, improved motor performance on the ledged beam task in a mice CHI model of TBI (322, 323), thus further confirming the suitability of ledged beam as a motor function test after trauma.

Additionally, in my study, TBI did not induce increased locomotor activity (hyperactivity) as evident in the velocity and total distance travelled by the injured mice compared to the uninjured controls in the open field test. However, a decrease in both total distance travelled, and velocity was demonstrated over time in all groups including sham-operated controls. This finding is consistent with prior studies which indicated no significant differences in the velocity and distance travelled between injured animals and uninjured controls across different models of TBI at both early and later timepoints (568, 598-600). For instance, the study by Washington and colleagues (601) reported no difference in the total

distance travelled between the injured mice and sham-operated control at day 21 post-CCI induced injury. Tweedie and colleagues (602) also reported similar observations in a weight drop model of mild TBI at an earlier timepoint of 72 hours (3-days) post-injury. However, hyperactivity following experimental TBI in rodents has been demonstrated in other previous studies (566, 580, 593, 603). For example, Kimbler and colleagues (604) reported increased locomotor activity, evidenced by the total number of squares entered by CCI injured mice compared to uninjured sham controls at 3 days post-injury. Similarly, in a weight drop mouse model of TBI, increased locomotor activity was observed at 10 days post-TBI between injured mice compared to untreated controls (605). The study by Tucker and colleagues (603) also further confirms that severe CCI-induced mice exhibited significant hyperactivity as indicated by the increased distance travelled within the arena compared to the sham-operated animals at days 1, 10 and 20. Interestingly, while there are discrepancies in the outcomes amongst the above-mentioned studies, it is important to note that these inconsistencies could be due to different methodologies employed by different research groups including injury severity, sex and model of TBI. Each of these factors could contribute to varying disease pathologies and responses. For example, the study by Tucker and colleagues (603) shows how injury severity and sex could produce different outcomes after trauma. Therefore, perhaps the injury model we have utilised for this study was not severe enough to show noticeable behavioural changes in the open field.

Moreover, the decrease in locomotor activity as indicated by the total distance travelled and speed in the arena over time in the present study could potentially be due to aging, as this study went on for a period of 30 days post-TBI and it is a well-known fact that the young of any species including mice have a higher motor activity compared to the more mature adult ones. For instance, a steady decrease in total distance was observed in the open field arena across four different mice age group starting at 2-3 months and ending at 8-12 months. The least distance travelled was observed in the 8-12 months age group while the greatest distance travelled was by the 2-3 months age group animals (606). While this does not exactly fit into the age of our animals which was approximately 3-4 months at the start of our behaviour assessments and 4-5 months at the end, it is an important point to note when designing functional experiments.

Notwithstanding, having no differences in locomotor ambulatory activities does not necessarily mean that the animals will have no anxiety-like behaviour in the open field (607). Previous studies have found that TBI-induced mice displayed anxiety-like behaviours such as avoiding the inner area of the arena and a decrease in exploratory activities despite having no

differences in locomotor activity when compared to the uninjured animals up to 6months after injury (599, 608). So, it is important not to draw up conclusion on mice anxiety behaviour with one task or variable (locomotor activity) as the effects of TBI on anxiety-like behaviours are greatly dependent on time and task of assessment (568).

The open field task was also used in this study to assess anxiety-like behaviour, since this disorder has been demonstrated to occur in a range of experimental TBI models. For example, following lateral fluid percussion injury (LFPI), rats spent less time exploring the centre of the open field compared to sham controls despite not showing a significant difference in the total distance travelled at 1-, 3- and 6-months post-injury (608). Similar behavioural responses in which less time was spent in the centre of the arena by the injured mice compared to uninjured controls were also observed at 1, 10 and 20 days post-TBI by Tucker and colleagues, using a CCI mouse model (603). However, in our study, there was no difference in the amount of time spent in the centre of the arena between the sham and TBI mice, so an effect of drug on anxiety could not be assessed. This could reflect that all of the mice were stressed or that our model of TBI did not induce anxiety. One possible reason could be that the test didn't work. It is also possible that the duration (10 minutes) of testing in the open field in our study was not long enough to show any possible anxiety-like behaviour after trauma in our model of TBI since many other studies use open field assessment for a period of 20 minutes (603) or 30 minutes (609).

The negative results found in my studies are in contrast to a number of studies in the literature that have reported beneficial effects of enoxaparin following experimental TBI. With increased research identifying benefits of heparin and enoxaparin to distinct from their non-anticoagulant properties both in the brain and in the periphery (355, 356, 362, 366, 368, 375, 381, 384, 389), several studies have sought to separate and investigate the anti-coagulant fragments from the non-anticoagulant ones, or have chemically modified the heparin molecule such that the anticoagulant activity is significantly reduced and poses no risk of exacerbating haemorrhage (53), a common pathophysiology associated with TBI (54). These studies have successfully demonstrated the potential anti-inflammatory effect after trauma, which ultimately improved outcomes even in neurological disorders. For example, O-desulfated heparin (ODSH) demonstrated significant improvement in motor function in a rat ischaemic model (610) as well as promoted neurological and cognitive recovery after trauma on the Morris water maze (553) suggesting that chemically modified heparin with anti-inflammatory properties can ameliorate functional impairment after TBI.

In view of these observations, we had hypothesized that the non-anticoagulant fractions of enoxaparin, Dp2, Dp4 and the combination of both drugs as a therapy would reduce neuroinflammation and ameliorate behavioural dysfunction associated with TBI. Our findings from the previous two chapters did not show attenuated neuroinflammation and in this study, repeated subcutaneous injections of Dp2, Dp4 and the combination of both drugs did not also ameliorate the motor function deficits induced by trauma. While the administered dose of enoxaparin and mode of delivery was comparable to previous studies that have showed an effect of enoxaparin after trauma (386, 388), the time to first drug administration post-TBI was 1 hour in our study as against 2 hours post-TBI in those studies. It is possible that at least 2 hours is required for subcutaneously injected enoxaparin in rodent models of TBI to become effective. In fact, this was demonstrated in the study by Wahl et al., 2000 (386), where no effect of enoxaparin was exhibited following its time to first administration at 15 minutes post-TBI. Whereas in the same study, a significant effect of the drug on investigated outcomes including motor function was displayed following the time to first drug administration post-TBI at 2 hours. So, perhaps, changing the time to first drug delivery in our study, could make a huge difference in our overall drug effects. Also, perhaps, the use of a different mode of delivery, the inclusion of various kinds of suitable behavioural assessments like the rotarod task and a possible behavioural testing at more chronic timepoints might be pertinent (611).

5.6.1 Justification of Behavioural Tests

Numerous behavioural tests have been established that measure a range of different cognitive, social, and motor impairments in TBI models (574). In this study, an open field test was employed to assess hyperactivity and anxiety-like behaviour, while ledged beam and hanging wire tasks were used to detect motor function deficits in the hindlimb and forelimb after trauma. While the ledged beam test has been proven to be a sensitive test to detect deficits in the hindlimb, it is not a sensitive test for the forelimb (322), and therefore the hanging wire test was introduced for the first time in our lab to assess muscular strength in the forelimb post-injury.

The open field test was also performed in our lab for the first time in this study, and we followed the protocol of Craig and colleagues, (593), since it showed a significant increase in total distance travelled in the arena following a CCI-induced injury similar to ours. Unlike in our study, where there was no behavioural dysfunction on the open field post-injury, hyperactivity and anxiety-like behaviour have been successfully detected following TBI by other studies in the literature (580, 593). Alternative tests that could be used to assess anxiety-like behaviour after trauma include elevated plus maze test, light-dark exploration test, gait

analysis, novelty-induced hypophagia and the social interaction test (568, 598, 612, 613). Each of these tests have been successfully used to show increase in anxiety-like behaviours across different rodent models of TBI. On the contrary, some studies have shown a decrease in anxiety-like behaviour or no difference in anxiety following TBI (603, 614). For instance, decreased anxiety-like behaviour was demonstrated in IL-1RI null mice following a novelty-induced hypophagia test (615).

Cognitive function would also have been interesting to evaluate in my study, since memory loss can be a sensitive indicator of hippocampal dysfunction. Cognitive tests such as Morris water maze or the Y-maze have been used successfully to detect memory deficits in rodent models of TBI (616, 617).

In my study, with the ledged beam task a significant deficit in hind-limb motor function was detected in all groups of TBI mice compared to sham controls, while no effect of treatment with enoxaparin, Dp2 and/or Dp4 was detected. While I have interpreted this data to mean that drug treatment did not improve motor function following TBI, it is also important to consider that the ledged beam task may not be sensitive enough to detect subtle improvements. Therefore, when designing behavioural tasks for future TBI studies, it would be important to perform an injury curve analysis to validate that the selected behaviour test shows a relationship between the extent of the deficit and the degree of injury (i.e. it shows a greater functional deficit for more severe injuries and a lesser deficit for milder injuries and no deficit in sham-operated controls). This would confirm that the test is sufficiently sensitive to detect an improvement resulting from drug treatment. The robustness of behavioural tests could also be improved by first exploring the time course of deficits post-injury to establish the optimal time points for testing the effect of drugs (386, 553).

5.6.2 Study Limitations

An unexpected limitation of this study was the exclusion of data from one cohort of mice that had a missing timepoint for the behavioural task data, due to issues with availability of the mouse behavioural suite. The power of my statistical analysis could be improved by utilising a more sophisticated software package, such as R, in order to incorporate the existing data from those mice I excluded; however, based on the current group means, variability, and P-values, including a few extra mice in the analysis is not likely to reveal any significant differences.

5.6.3 Conclusion

While enoxaparin and its fractions, Dp2 and Dp4, and the combination of both Dp2 and Dp4 did not attenuate motor dysfunction as shown on the ledged beam, alter locomotor activity or anxiety-like behaviour in the open field arena after trauma, it could be that the methodologies used in this study need to be further optimised as it was the first time assessing these drugs on functional outcomes in the brain after trauma. In this regard, methodological considerations and limitations of these experiments will be discussed in detail in Chapter 7.

CHAPTER 6

SYSTEMATIC REVIEW AND META-ANALYSIS

CHAPTER 6: MANUSCRIPT IN PREPARATION

At the start of 2020, I was to embark on a few new cell culture experiments for my last experimental chapter. Unfortunately, due to COVID-19 shutdown, it was no longer possible. So, I took this opportunity to perform the first Systematic Review and Meta-analysis on the use of heparins after TBI and this is now presented in the following chapter.

A systematic review and meta-analysis on the therapeutic efficacy of heparin and Low Molecular Weight Heparins (LMWHs) following experimental Traumatic Brain Injury in animal studies.

Mimieveshiofuo Aiyede, Xin Yi Lim, David W. Howells and Nicole Bye.

Publication

Chapter 6 is a manuscript in preparation and the potential title of the publication is:

A systematic review and meta-analysis on the therapeutic efficacy of heparin and Low Molecular Weight Heparins (LMWHs) following experimental Traumatic Brain Injury in animal studies.

The following individuals and institutions contributed to the preparation of this work undertaken as part of this thesis.

Candidate: Mimieveshiofuo Aiyede[1]

Author 1: Xin Yi Lim[1,2]

Author 2: David W. Howells[3]

Author 3: Nicole Bye[1]

[1]School of Pharmacy and Pharmacology, University of Tasmania (UTAS). [2]Current affiliation: Herbal Medicine Research Centre, Institute for Medical Research, Ministry of Health, Malaysia. [3]Neuroscience and Brain Plasticity Group, Division of Medicine, UTAS.

Author contributions:

Conceived and designed the review: Candidate, Author 2, Author 3

Extraction of data: Candidate, Author 1, Author 3

Analysed the data: Candidate

Wrote the manuscript: Candidate

Edited manuscript: Candidate, Author 2, Author 3

Prior to publication, A/Prof. Nuri Guven[1] and Dr. Rahul Patel[1] will be invited to join as authors and contribute to the discussion and final editing of the manuscript, due to their expertise in the field. Also, PhD candidate Allanna Russell[3] will be invited to join as an author, to acknowledge her helpful discussions regarding statistical techniques of meta-analysis.

6.1 Introduction

Traumatic brain injury (TBI) is one of the leading causes of disability and death among the active population of less than 45 years. It is caused by an external blow or impact to the head, and it is associated with incidents such as falls, motor accidents, bullet injury, industrial accidents or football collision. With the increasing global incidence of TBI, it has been estimated that 69 million people worldwide will suffer from TBI yearly (2, 5, 618). TBI triggers a cascade of events that are classified as primary and secondary injury mechanisms. The primary injury mechanisms, which include disruption of the blood-brain barrier, axonal shearing, tissue destruction and haemorrhage, result from the initial impact at the time of trauma (59, 60, 619), while the secondary injury occurs within minutes to days and is characterised by a number of molecular, metabolic and cellular processes that lead to neuroinflammation, ischemia, apoptosis, oxidative stress, lipid degradation (59, 60, 620, 621) and brain oedema (622) that may eventually result in additional brain dysfunction and chronic neurodegeneration (3, 112). There is currently no effective treatment available for the ongoing brain damage / long-term disability associated with TBI despite many promising pre-clinical studies that have screened and identified potential therapeutics over the years (621). Moreover, drug development for TBI continues to face significant challenges due to the presentation of very diverse and varying pathophysiologies amongst TBI patients (385, 621, 623).

One group of drugs that is of interest as a potential pharmacotherapy for TBI is heparin (including modified heparin like 2,3-O-desulphated heparin, ODSH) and low molecular weight heparin (LMWHs). Heparin and LMWHs are well known for their use as anti-coagulants. They belong to the family of glycosaminoglycans (GAGs) which have a complex chemical structure consisting of recurring monomeric disaccharides units of uronic acid and D-glucosamine residues linked by 1,4 glycosidic bonds. While the average molecular weight of heparin is 15kDa, LMWHs lie in the 4-6kDa range (347, 355, 624). The highly negatively charged parent heparin, has many unwanted effects like drug-induced adverse effects, low bioavailability, and an unpredictable dose response following its administration because it interacts with a wide range of proteins (624, 625). Meanwhile, in addition to their lower molecular size, LMWHs exhibit low affinity for plasma proteins and are hence a preferred option for clinical use than heparin as they have a better safety profile, more predictable pharmacokinetic properties, and efficacy, with reduced risk of bleeding (385, 626). For the purpose of this review, the term ‘heparin’ will also refer to modified heparins such as ODSH and heparin oligosaccharides. Enoxaparin and dalteparin are the two most used LMWHs, therefore, emphasis will be placed on these two LMWHs in this review.

Heparin, and enoxaparin which is one of the most commonly used LMWH, have shown promising neuroprotective effects in a range of different TBI models (385). Following TBI, both enoxaparin and low doses of heparin enhanced neurologic function by decreasing brain contusion (386, 387), cerebral oedema, inflammation, microvascular permeability, recruitment and accumulation of leukocytes (365, 388) as well as by reducing infarct size (389) in TBI related ischaemia. It was suggested that enoxaparin brought about these effects by blocking the signalling of high mobility group box 1 (HMGB1) protein (391), however, more studies need to be done to confirm this. Additionally, the early administration of heparin reduced polymorphonuclear neutrophil (PMN) accumulation and led to prolong cognitive recovery after trauma (365, 396). Nagata and colleagues showed that the non-anticoagulant heparin ODSH reduced oedema, leucocyte recruitment and microvascular permeability at 48 hours post-TBI (553). Dalteparin, which is also a LMWH was reported to reduce apoptosis, brain edema and oxidative stress in a weight drop model of TBI (627). Furthermore, there was a significant decrease in the expression of inflammatory mediators such as COX-2, hippocampal thiobarbituric acid-reactive substances (TBARS), reactive gliosis and oxidized protein levels in a rat lateral fluid percussion brain injury (LFPI) model (390) following treatment by enoxaparin and heparin. Reduced infarct volume and cell apoptosis was also demonstrated in the cortex of mice cold-induced model of TBI (366). Overall, it is believed that most of the beneficial effects demonstrated by heparin and LMWHs in this context are associated with their non-anticoagulant properties (385, 389, 610, 628, 629) but the exact mechanism behind these effects remains unclear. Importantly, despite apparent benefit of heparin and LMWHs in TBI, these drugs are not used clinically, at least in the first 24 hours after trauma, because of the associated risk of bleeding (421).

We have been unable to find evidence that the therapeutic effect of heparin and LMWHs for the treatment of TBI have been studied by rigorous systematic review. Therefore, this systematic review and meta-analysis aims to critically evaluate and summarize the literature on the potential neuroprotective effects of using heparin and LMWHs (enoxaparin and dalteparin) as treatment for TBI. This study will compare the treatment outcomes following the administration of heparin / LMWHs or modified heparin against control group in animal models of TBI. This will provide useful information for future research protocols and potentially a guide for clinicians.

6.1.1 Study questions

The review wishes to answer the following questions:

- Do heparin (including modified heparins) and LMWHs (enoxaparin and dalteparin) have protective effects following experimental TBI in animal models?
- What dose and route of administration will produce the greatest effect following the administration of heparin and LMWHs in animal models of TBI?
- What is the relative efficacy of heparin vs LMWHs in animal models of TBI?
- Does treatment with heparin and LMWHs increase bleeding in TBI animal models?

6.2 Methods

A comprehensive systematic review of both PubMed and Web of Science was conducted based on the current guidelines of Preferred Reporting Items for Systematic reviews and Meta-Analyses for Protocols (PRISMA-P) (630) (Appendix C) and has been published at PROSPERO (https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42020205574).

The inclusion / exclusion criteria and search strategy are detailed below.

6.2.1 Inclusion criteria

For this review and meta-analysis, studies were included based on the following inclusion criteria:

- Peer reviewed primary research articles describing use of heparin (including modified heparins) and/or LMWHs for the potential treatment of TBI in any animal model of TBI, including TBI transgenic models.
- Randomised control, quasi-randomised and non-randomised studies with control groups that investigated the potential neuroprotective effects of heparin, enoxaparin and/or dalteparin in an animal model of TBI compared to vehicle treated TBI control groups.
- Studies that have clearly stated the number of animals in each experimental group.
- Studies where heparin and/or LMWHs were administered at any dose, route, frequency, duration, and time before and after trauma induction.
- Studies with at least one of the following outcome measures: assessment of motor, behavioural or cognitive function, quantification of neuropathological features of TBI, such as lesion volume, blood brain barrier permeability, brain oedema, neuroinflammation, haemorrhage as well as the recruitment of immune cells from the blood.
- Studies where either one or more of these statistical measures: mean / median value, 95% confidence interval and variance (standard error of the mean and standard deviation) were considered.

6.2.2 Exclusion criteria

The exclusion criteria for this review include:

- Studies that are not primary research such as review papers.

- Studies that do not clearly define the model of traumatic brain injury.
- Studies involving other drugs as co-treatment in addition to heparin or LMWH.
- Articles whose full text is not obtainable.
- Studies published in languages other than English.

Two authors (MA and XYL) independently screened the titles and/or abstracts to identify publications meeting our inclusion eligibility criteria and differences were resolved in cases of no consensus by discussion with a third reviewer.

6.2.3 Sources of information

Information was sourced from the following databases:

- Electronic databases: PubMed and Web of Science.
- Relevant articles were manually identified from the reference list of included articles.

6.2.4 Search Strategy

A concept table containing keywords was developed by a quick search through the literature. Then, together with a librarian and Systematic Review Centre for Laboratory Animal Experimentation's (SYRCLE) step by step guide (631), the authors created a detailed search strategy. Comprehensive synonyms and alternate words/terms were generated (Appendix D) for each of the main key words (Heparin/LMWHs, Traumatic Brain injury and animal model) to search titles and abstracts in the databases mentioned above. More alternate terms were added from PubMed MeSH terms as searches were performed and from the references of the relevant articles that was included in the study. The advanced search tools of PubMed and Web of Science were employed to search for both titles and abstracts. The Boolean operators "OR, AND, NOT or AND NOT" were used to combine or exclude keywords in the searches for this review. Duplicate references were removed using the EndNote citation management software (EndNote version X9 Thomson Reuters, New York) once the search was completed. All databases were searched up to October 2020. Detailed information on the search strategy can be found in appendix E.

6.2.5 Data Extraction

The studies were exported to Rayyan QCRI (Qatar Computing Research Institute) software following the removal of duplicates. Here, two independent investigators screened titles and abstracts with a third reviewer resolving any differences in the case of no consensus. All articles approved following the initial title and abstract screening underwent a further full-

text screening step by two independent reviewers and disagreements between the independent reviewers were resolved by a third reviewer. Data was extracted from the manuscripts text or by extrapolating numerical values from graphs from eligible studies. Data were extracted and treated as independent experiments when a single study reported more than one experiment and as recommended by the Cochrane collaboration, the groups were combined to generate a single pair-wise comparison against the control group (632). Authors were contacted directly for further clarification or more information in cases of missing data.

The following information were collected from the full text:

- The title, name of authors, journal, and publication date of articles
- Type of study – randomised, quasi-randomised or non-randomised control studies.
- The animal details including age, sex, species, weight, and strain of animal.
- The TBI model type, severity of injury, anaesthetic agent, duration of anaesthesia, craniotomy / craniectomy / closed cranium, injury location, peak pressure wave / height of weight drop / depth of impactor and impactor velocity.
- All pathological and behavioural outcomes measured to assess injury and treatment.
- The methods used for determining these outcome measures. For instance, motor function test, behavioural assessments, change in lesion size, cognitive function test, histology of inflammatory mediators and cytokine measurement.
- The drug used, mode of administration, dose, and duration of administration.

6.2.6 Quality Assessment

The risk of bias (RoB) of the included studies was assessed with SYRCLE's RoB tool (633) (Appendix F) and the quality assessment of all studies were evaluated according to a modified checklist from the Collaborative Approach to Meta-Analysis and Review of Animal Data from Experimental Studies (CAMARADES) as previously described with slight modification (634, 635). The checklist comprised the following 10 criteria:

1. peer review publication.
2. Random group allocation.
3. Blinded induction of injury.
4. Blinded assessment of outcome measures.
5. Use of an anaesthetics/analgesic agent without marked neuroprotective activity.
6. Statement of compliance with animal welfare regulatory requirements.
7. Statement of potential conflicts of interest.
8. Use of appropriate animal model.

9. Calculation of sample size.
10. Explanation of excluded animals.

One point was given to indicate the presence of each criterion.

6.2.7 Data Analysis

In this review, a meta-analysis and a narrative synthesis were both employed. The effect of heparin and LMWHs on brain oedema, leukocyte rolling, microvascular permeability, haemorrhage/haemorrhagic contusion, neurologic function and body weight loss was estimated by calculating the standardized mean difference (SMD - mean difference between groups divided by the standard deviation of outcome effect) for each comparison and combining these values in a weighted mean difference meta-analysis using a random-effects model (636). This method was chosen because it permits aggregation of data from studies measuring the same outcome in different ways (637, 638). For instance, evaluating neurologic function using different neurological assessment tools. Results are presented as overall SMD and its 95% confidence intervals (CIs).

The presence of heterogeneity was assessed using the I^2 statistic, the percentage of the variability in effect estimates that is due to heterogeneity rather than sampling error (chance) (632). Higher values indicate a greater degree of heterogeneity.

A priori examination of which experimental factors contribute to outcome heterogeneity was performed primarily by subgroup analysis. Subgroup analysis for drug types and their doses were conducted only for the brain oedema outcome where data was most abundant. Additionally, funnel plots and Egger's tests were conducted to investigate the presence of small sample effects that might result from problems such as publication bias, Trim and fill analysis was used for additional visualisation.

In this review, the controls were untreated injured animals while the treatment groups received either heparin or enoxaparin at varied times and doses post-injury. For the narrative synthesis, all 26 outcomes that were extracted from the included studies were summarised in tables based on, but not limited to, the drug dosage, drug type and overall efficacy of the different drugs (heparin, ODSH and enoxaparin), with their major findings discussed. Meanwhile, meta-analysis included only the results of the injured brain hemisphere for brain oedema outcome and the results of only the 48-hour timepoint for neurologic function. This timepoint after injury was chosen as we wanted to access acute neurological deficit post-TBI.

All Meta-analyses were performed using Review Manager (RevMan, Version 5.4. The Cochrane Collaboration, 2020) and/or StataSE software (version 16) StataCorp LP, Texas, USA with a significance level set at $P<0.05$. The effect estimates of all the outcomes in the summarised table (Table 6.3) were also performed using Review Manager (RevMan, Version 5.4. The Cochrane Collaboration, 2020). For data represented as forest plots, the central lines and lateral tips of the diamond signifies pooled point estimates and the corresponding 95% CIs, respectively. The solid black vertical line indicates the line of no effect while the red vertical line is the overall effect size line.

6.3 Results

6.3.1 Study Selection

Our search found 323 potentially relevant publications. 55 were replicates because of database overlap and were removed. Of the remaining 268 publications subject to title and abstract screening, 12 were selected for full-text review. One of these was excluded for not meeting our prespecified inclusion criteria. 11 publications (365, 366, 386-388, 390, 396, 514, 553, 639, 640) were included in the review (Figure 6.1). From the included studies, meta-analysis was conducted on the data from seven publications (365, 386-388, 396, 553, 639) for brain oedema, neurologic function, haemorrhage/haemorrhagic contusion, microvascular permeability, leukocyte rolling and body weight (see appendix H for number of comparisons of each outcome).

6.3.2 Study characteristics

The characteristics of the included studies, published between 2000 and 2020, are shown in Table 6.1. Of the 11 studies, seven administered enoxaparin (366, 386-388, 390, 514, 639), two studies administered unfractionated heparin (365, 396) and one study each administered 2,3-O-desulfated heparin (ODSH) (553) and heparin oligosaccharides (640) respectively. All the animals studied were male. The CD-1 mouse strain was the most commonly used animal (365, 388, 396, 553), followed by Sprague-Dawley (386, 387, 639) and Wister (390, 640) rats. one study each used the C57BL/6 (514) and BALB/c (366) mouse strains respectively. Mice ranged in weight 20 to 32g and rats ranged from 100 to 450g. Six studies reported using adult animals but did not specify an exact age (365, 387, 388, 390, 396, 553) four studies reported using mouse models aged between 4 to 12 weeks (366, 514, 639, 640) and 1 study failed to report the age of animals used (386).

Controlled cortical impact was the most frequently used model of TBI (365, 388, 396, 514, 553) while two studies used the weight-drop impact model (387, 639). In most studies the injury induced was severe (365, 387, 388, 396, 514, 553) while two studies reported moderate injury (386, 390) and one study reported a mild injury (639). Two studies failed to report injury severity (366, 640). Drugs were either administered immediately after trauma induction or between 15 minutes and two hours after injury as a single dose (366, 514, 640) or multiple doses (365, 386-388, 390, 396, 553). Subcutaneous injection was the most used mode of drug delivery as reported by six studies (365, 388, 390, 396, 553, 639), two studies used both intravenous and subcutaneous injections (386, 387). Direct administration into the wound

cavity (640), intravenous (514) and intraperitoneal (366) injection were each reported in one study respectively. Drugs, dosing and frequency of administration are detailed in Table 6.2.

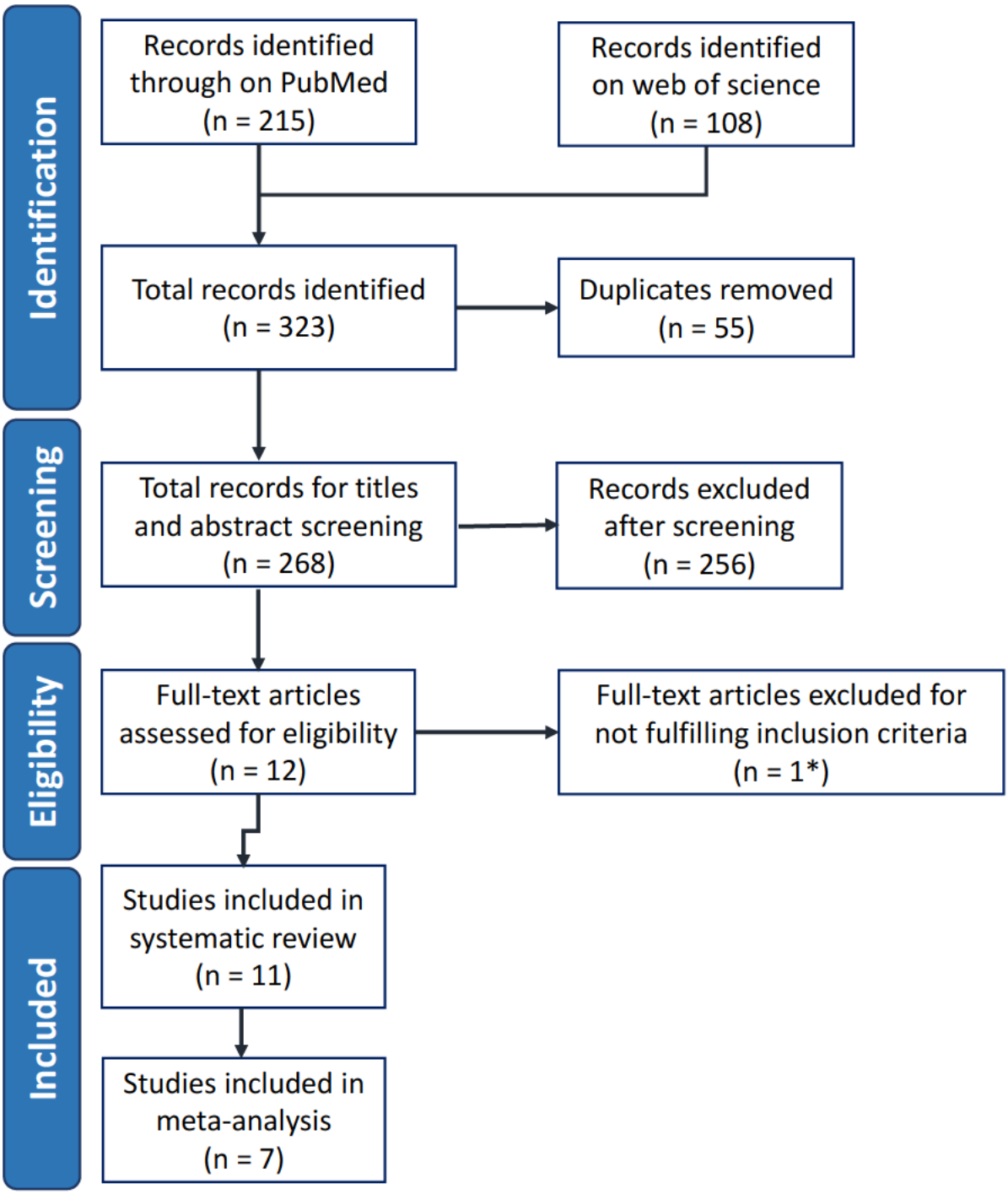


Figure 6.1: Flowchart of study process

*A total of 11 studies were included in this review following the literatures search process and meta-analysis was conducted on seven studies. *One study was excluded during full-text screening because enoxaparin was administered in combination with another drug.*

As detailed in Table 6.3, of the 26 outcomes extracted, brain oedema and neurologic function were the outcomes included in the most number studies (365, 386, 388, 396, 553), followed by leukocyte rolling (365, 388, 553), body weight loss (365, 388, 396), microvascular permeability (365, 388, 553), haemorrhage (387, 388, 639), and apoptosis (366, 387, 390), all with three studies each. Brain infarct volume (366, 386) and leukocyte adhesion (365, 396) were reported by two studies each and the remaining outcomes including oxidative stress (390), cognitive function (386), learning (553), haemoglobin levels (396), motor function (553), Inflammation (387), glial scar formation (640), IL-1β (514), Pro-IL-1β (390), caspase-1 (514), COX expression (390), reactive gliosis (390), lesion surfaces (386), PMN sequestration (396), neurodegeneration (390), total anti-oxidant status and total oxidant status (366), were reported by one study each. The wet/dry weight ratio method was used to assess brain oedema, while the validated, modified Garcia Neurological Test and the Neurological Severity Score were the most frequent methods used to assess neurologic function. The summary of the method of assessment of the other outcome measures used are included in Table 6.3.

Table 6.1: Characteristics of included studies

First author, Year	Animal	Strain	Sex	Age (weeks)	Weight (g)	TBI model	Injury severity	Anaesthetic	Treatment	Mode of administration	QA score
Dhir, 2018 (639)	Rat	Sprague-Dawley	Male	4	100 - 200	Weight drop	Mild	Isoflurane	Enoxaparin	SC injections	5
Hayashi, 2004 (640)	Rat	Wistar	Male	8 - 10	NR	Cryo-cortical injury	NR	Pentobarbital	Heparin oligosaccharides	applied directly to wound cavity after injury	4
Kerr, 2020 (514)	Mouse	C57BL/6	Male	8 - 12	24 - 32	CCI	Severe	Ketamine/Xylazine	Enoxaparin	IV injections	6
Keskin, 2017 (366)	Mouse	BALB/c	Male	8 - 10	20 - 25	Cold induced brain injury	NR	Ketamine hydrochloride / Xylazine hydrochloride	Enoxaparin	IP injections	5
Li, 2015 (388)	Mouse	CD-1	Male	Adult*	25 - 30	CCI	Severe	Ketamine/Xylazine / Acepromazine	Enoxaparin	SC injections	4
Nagata, 2017 (396)	Mouse	CD-1	Male	Adult*	25 - 30	CCI	Severe	Ketamine/Xylazine / Acepromazine	Unfractionated heparin	Repeated SC injections	6
Nagata, 2016 (365)	Mouse	CD-1	Male	Adult*	25 - 30	CCI	Severe	Ketamine/Xylazine / Acepromazine	Unfractionated heparin	Repeated SC injections	4
Nagata, 2018 (553)	Mouse	CD-1	Male	Adult*	25 - 30	CCI	Severe	Ketamine/Xylazine / Acepromazine	O-desulphated heparin	SC injections	5
Sen, 2011 (387)	Rat	Sprague-Dawley	Male	Adult*	200 - 290	Weight drop injury	Severe	Ketamine	Enoxaparin	IV bolus injection+SC injections	4
Wahl, 2000 (386)	Rat	Sprague-Dawley	Male	NR	260 - 300	FPI	Moderate	Halothane	Enoxaparin	IV bolus injection+SC injections	4
Župan, 2011 (390)	Rat	Wistar	Male	Adult*	350 -450	LFPI	Moderate	Isoflurane	Enoxaparin	SC injections	7

Abbreviations: NR-Not reported, CCI - controlled cortical injury, FPI – fluid percussion injury, LFPI - lateral fluid percussion injury, SC - subcutaneous, IV - intravenous, IP - intraperitoneal. *Adult as specified by the authors of the different studies.

Table 6.2: Details of treatment intervention from the included studies

Study	Drug	Time to first drug delivery post-TBI	Treatment dose	Treatment frequency
Dhir, 2018 (639)	Enoxaparin	Unclear	1 mg/kg	Unclear
Hayashi, 2004 (640)	Heparin oligosaccharides	Immediately onto wound cavity	10 mg/ml	Single
Kerr, 2020 (514)	Enoxaparin	30 minutes	1 mg/kg	Single
Keskin, 2017 (366)	Enoxaparin	20 minutes	3 mg/kg, 10 mg/kg	Single
Li, 2015 (388)	Enoxaparin	2 hours	1 mg/kg	Multiple
Nagata, 2017 (396)	Unfractionated heparin	2 hours	75 U/kg, 225 U/kg	Multiple
Nagata, 2016 (365)	Unfractionated heparin	2 hours	75 U/kg, 225 U/kg	Multiple
Nagata, 2018 (553)	ODSH	2 hours	25 mg/kg, 50 mg/kg	Multiple
Sen, 2011 (387)	Enoxaparin	15 minutes	0.5+1 mg/kg	Multiple
Wahl, 2000 (386)	Enoxaparin	1) 15 minutes; 2) 2 hours	1) 0.5+2 mg/kg, 2) 0.5+0.5, 0.5+1, 0.5+2 mg/kg	Multiple
Župan, 2011 (390)	Enoxaparin	1 hour	1 mg/kg	Multiple

Table 6.3: Summary and findings of all outcomes from included studies

Outcomes assessed	**Treatment**	**Vehicle**	**Animal species**	**Model and Timepoints assessed**	**Method of assessment**	**Key laboratory findings post-injury**	**Brain region assessed**	**Effect size % (95% Confidence interval)**
Brain oedema (365, 386, 388, 396)	Enoxaparin, UFH, ODSH	Saline	Mouse, rat	48 and 96 hours post-injury (CCI, FPI)	Calculation of water content	Enoxaparin, high dose ODSH, low and high doses of UFH decreased brain oedema at 48 h. Low dose UFH reduced oedema at 96 h	Cortex, whole brain	0.95 [0.35, 1.55]
Leukocyte rolling (365, 388, 553)	Enoxaparin, UFH, ODSH	Saline	Mouse	48 hours post-TBI (CCI)	Quantification of fluorescently labelled intravascular cells as LEU rolling	Enoxaparin, low and high dose UFH and ODSH reduced LEU rolling	NR	1.93 [1.42, 2.44]
Microvascular permeability (365, 388, 553)	Enoxaparin, UFH, ODSH	Saline	Mouse	48 hours post-TBI (CCI)	Quantified the ratio of FITC-labelled albumin fluorescence	Enoxaparin, both doses of UFH and ODSH decreased albumin leakage	NR	2.23 [1.50, 2.96]
Oxidative stress (390)	Enoxaparin	NR	Rat	48 hours post-TBI (LFPI)	Measured the levels of TBARS, SOD and GSH-Px activity, evaluated immunochemical detection of protein carbonyl groups	Reduced the levels of TBARS and hippocampal oxidized proteins. No effect on hippocampal SOD and GSH-Px activities	Hippocampus	0.11 [-0.38, 0.60]
Body weight loss (365, 388, 396)	Enoxaparin, UFH	Saline	Mouse	24, 48, 72 and 96 hours post-TBI (CCI)	Measured body weight	Enoxaparin reduced body weight loss at 24 h and not at 48 h. Both doses of UFH had no differences on body weight	NR	0.25 [-0.34, 0.85]

Inflammation (387)	Enoxaparin	NR	Rat	48 hours post-TBI (WDI)	Quantified H&E stained mild inflammatory infiltrates	Reduced inflammation	Frontoparietal cortex, hippocampus, corpus callosum	0.61 [-0.29, 1.51]
Glial scar formation (640)	Heparin oligosaccharides	Sterile PBS	Rat	6 - 9 days post-TBI (WDI)	Quantified CS-A and GFAP immunoreactivity	Reduced the formation of glial scar in cortical regions closest to injury. No change in cortical region further away from injury.	Cerebral cortex	0.34 [-0.12, 0.81]
Haemorrhage (387, 388, 639)	Enoxaparin	Saline	Rat, mouse	24, 48 and 72 hours post-TBI (WDI, CCI)	Histologically assessed using H&E staining, measured haemoglobin levels and haemorrhagic contusion.	No differences observed	Frontoparietal cortex, hippocampus, corpus callosum, dorsal surface of the injured hemisphere	0.95 [0.44, 1.46]
IL-1β (514)	Enoxaparin	NR	Mouse	24 hours post-TBI (CCI)	Measured levels of IL-1β using western blot	Reduced expression of IL-1β	Cortex	1.56 [0.42, 2.70]
Neurologic function (365, 386, 388, 396, 553)	Enoxaparin, UFH, ODSH	Saline	Mouse, rat	24, 48, 72 and 96 hours post-TBI, 1-4 weeks post-TBI (CCI, FPI)	A validated modified Neurological Severity Scale (NSS) or Garcia Neurological Test (GNT)	Low dose, but not high dose UFH and enoxaparin improved neurologic function at all timepoints. Both low and high doses of ODSH improved outcome.	NA	-1.11 [-1.75, -0.46]

Brain infarct volume (366, 386)	Enoxaparin	Saline	Mouse, rat	24 hours and 7 days post-TBI (FPI, CIBI)	Histology assessment with H&E and Cresyl Violet staining	Low dose did not reduce infarct volume at 24h post-TBI but reduced lesion volume at 7 days post-TBI. High dose reduced infarct volume at 24 hours post-TBI but did not reduce lesion volume at 7 days after injury.	Throughout the brain	0.65 [0.20, 1.10]
Apoptosis (366, 387, 390)	Enoxaparin	Saline	Mouse, rat	24 and 48 hours post-TBI (WDI, LFPI, CIBI)	Quantified TUNEL positive cells, H&E evaluation of neuronal cell death and assessing the expression of active caspase-3 using western blot	Decreased apoptosis, however, high dose and not low dose decreased outcome in one study	Frontoparietal cortex, hippocampus, corpus callosum	0.62 [0.07, 1.16]
Caspase – 1 (514)	Enoxaparin	NR	Mouse	24 hours post-TBI (LFPI)	Measured levels of caspase-1 using western blot	Reduced the expression of inflammasome protein caspase-1	Cortex	1.12 [0.06, 2.18]
Total anti-oxidant status (TAS) (366)	Enoxaparin	Isotonic saline solution	Mouse	24 hours post-TBI (CIBI)	Measured serum levels of TAS	Low dose had no effect, but high dose increased serum levels	Blood sample	-0.41 [-1.16, 0.34]
Total oxidant status (TOS) (366)	Enoxaparin	Isotonic saline solution	Mouse	24 hours post-TBI (CIBI)	Measured serum levels of TOS	No significant difference observed	Blood sample	-0.60 [-1.36, 0.16]

PMN sequestration (396)	UFH	Saline	Mouse	96 hours post-TBI (CCI)	Counted immunostained positive PMNs cells	Both low and high doses of UFH decreased PMN sequestration	Cortex	1.89 [1.02, 2.75]
Haemoglobin levels (396)	UFH	Saline	Mouse	96 hours post-TBI (CCI)	Measured serum levels of haemoglobin	Low dose had no difference on serum levels. High dose lowered serum haemoglobin levels	Mean arterial blood pressure	0.25 [-0.47, 0.97]
Leukocyte adhesion (365, 553)	UFH, ODSH	Saline	Mouse	48 hours post-TBI (CCI)	Quantification of fluorescently labelled intravascular cells as LEU rolling.	No significant effect	NR	0.82 [0.33, 1.31]
Neurodegeneration (390)	Enoxaparin	NR	Rat	48 hours post-TBI (LFPI)	Quantification of Fluoro-Jade-B stained hippocampal neurons	No significant decrease	Hippocampus	0.09 [-0.55, 0.72]
Motor function (553)	ODSH	Saline	Mouse	14-17 days post-TBI (CCI)	Measured swim velocity on Morris water maze	High dose improved swimming velocity on days 16 and 17	NA	-0.89 [-1.39, -0.38]
Lesion surfaces (386)	Enoxaparin	NR	Rat	7 days post-TBI (FPI)	H&E histological evaluation of coronal sections	Low dose reduced lesion volume. High dose had no effect	NR	0.69 [0.54, 0.84]
Pro IL-1β (390)	Enoxaparin	NR	Rat	48 hours post-TBI (LFPI)	Measured pro IL-1β using western blot	No change in hippocampal pro IL-1β expression	Hippocampus	0.00 [-1.24, 1.24]

COX-2 expression (390)	Enoxaparin	NR	Rat	48 hours post-TBI (LFPI)	Measured COX-2 expression using western blot	Lowered COX-2 expression	Hippocampus	0.99 [-0.37, 2.35]
Reactive gliosis (390)	Enoxaparin	NR	Rat	48 hours post-TBI (LFPI)	Evaluated the area of astrocytes' perikarya and GFAP staining intensity by analysing immunostained GFAP cells	No effect on the area of astrocytes' perikarya. decreased GFAP staining intensity	Hippocampus	0.14 [-0.79, 1.06]
Cognitive function (386)	Enoxaparin*	NR	Rat	2, 6, 13, 20, 27 hours post-TBI (FPI)	Measured latency time on the complex maze at days 2, 6,13, 20 and 27.	Decreased latency time at days 2, 6 and 13 but had no difference on days 20 and 27	NA	0.26 [0.06, 0.45]
Learning (553)	ODSH	Saline	Mouse	14-18 post-TBI (CCI)	Measured Latency to the platform (learning) on Morris water maze	Low dose but not high dose improved learning	NA	-2.13 [-3.28, -0.99]

Abbreviations: UFH - unfractionated heparin, ODSH - 2,3-O-desulfated heparin, TBARS - thiobarbituric acid-reactive substances, SOD - Superoxide dismutase, GSH-Px - glutathione peroxidase, H&E - Haematoxylin and Eosin, PBS - phosphate-buffered saline, CS-A - chondroitin sulfate-A, GFAP – glial fibrillary acidic protein, PMN – Polymorphonuclear, IL-1 – Interleukin-1, COX-2 - Cyclooxygenase-2, CIBI – cold-induced brain injury, CCI – controlled cortical injury, WDI – weight drop injury, FPI – fluid percussion injury, LFPI – lateral fluid percussion injury

*(Only low dose was assessed)

6.3.3 Risk of bias and Quality assessment

The risk of bias (RoB) as shown in Figure 6.2, highlights the possible sources of bias in the included studies. Baseline characteristics were generally well reported (91% of studies) with only one study failing to report these key details (386) . However, most other facets of study reporting suggest the data are at significant risk of bias. No studies described random housing, random outcome assessment or blinding of assessors. 70% of all the included studies had evidence of selective outcome reporting (365, 387, 388, 396, 514, 553, 639, 640) as the sample size planned or analysed was not reported. Only three studies did not have selective outcome reporting as they reported all outcomes and sample sizes evaluated and analysed

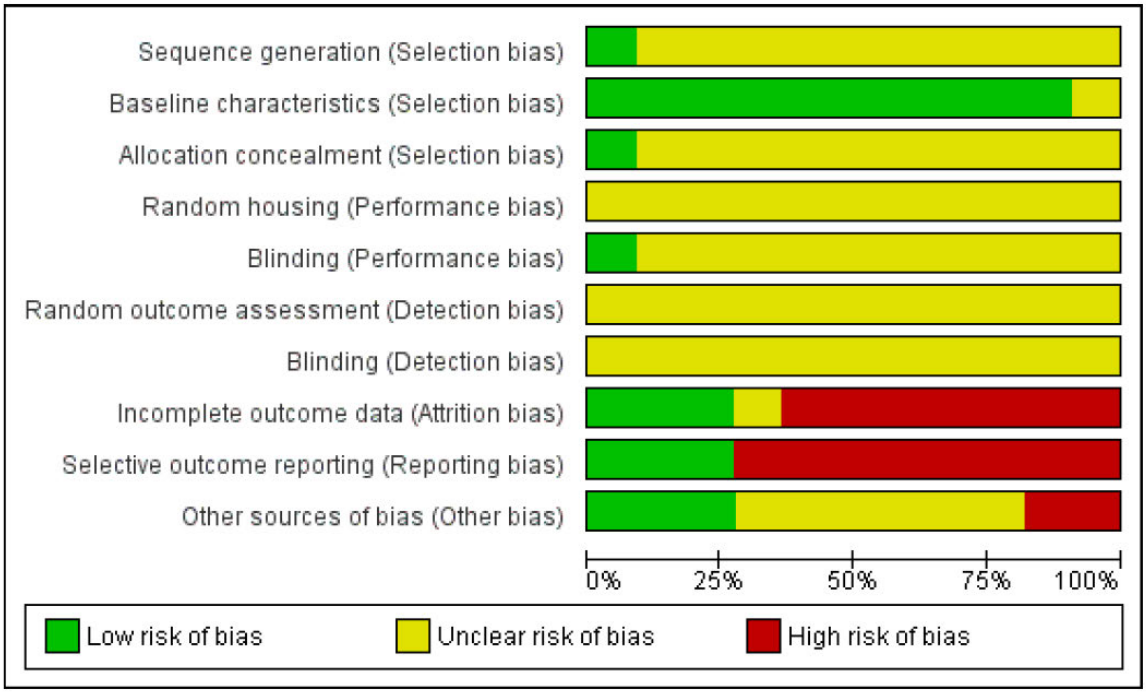


Figure 6.2: Risk of bias graph of included studies evaluated by SYRCLE's RoB tool
Baseline characteristics was the most reported in the included studies (91%) while over 70% of studies demonstrated selective outcome reporting and no study described random housing, random outcome assessment and blinding of assessors (detection bias). Each risk of bias item is presented as percentages.

(366, 386, 390) . In three studies, attrition bias was not evident (366, 386, 390) but was present in seven studies (Over 60%) where the number of sample size did not equate to the final number that was analysed and an explanation was not provided for the missing data (365, 387, 388,

396, 514, 553, 639). One study made no mention of the sample size used or analysed (640). Nine studies reported randomisation into experimental groups, two studies did not report whether randomisation was performed and only one study (about 9%) actually described sequence generation achieved by a double coin toss (396). Of the included studies, only one reported allocation concealment, achieved by using an investigator who was not involved in treatment delivery or analysis (514). The same study was the only one that reported that blinding was achieved by masking treatment bottles with opaque tape (514). No studies indicated whether outcome assessment was blinded.

Other potential source of bias identified included potential conflicts of interest due to sources of funding or materials. While a total of six studies did not report a potential conflict of interest due to sources of funding (365, 366, 386-388, 396), two of the six studies only stated that they had no potential conflict of interest but did not report whether or not it was related to their source of funding (366, 396) whereas the remaining four studies did not report that information at all (365, 386-388). A total of five studies declared their sources of funding (390, 514, 553, 639, 640). Two of the five studies stated that they did not have a conflict of interest (390, 639) , another two studies did appear to have a potential conflict of interest because of their funding and material source (514, 553) whereas one study did not state whether or not if they had a potential a potential conflict of interest as they only reported their source of funding (640).

A modified CAMARADES checklist gave a median quality score of six with scores ranging from four to seven out of 10. In this assessment, by definition, all included studies came from peer-reviewed papers and used the appropriate animal models. Nine out of the 11 included publications (81%) reported compliance with animal welfare regulations and six studies (55%) reported whether a potential conflict of interest existed (366, 390, 396, 514, 553, 639). However, no study reported blinded induction of injury by the investigator or blinded assessment of outcome measures. Random group allocation was reported in nine of the 11 included publications (365, 366, 387, 388, 390, 396, 514, 553, 639). Most studies used ketamine alone or in combination with other agents like Xylazine and Acepromazine for anaesthesia, only three used gaseous anaesthetics (386, 390, 639) and one used pentobarbital (640). Two publications described the sample size calculation (396, 514) and two others provided an explanation for excluded animals (386, 390) . One study didn't have to provide an explanation as there were no excluded animals (366) (Appendix G).

6.3.4 Meta-analysis

Random effects model was used in this study due to the variability of the data, and overall, there was significant heterogeneity between studies investigating drug efficacy on both brain oedema and neurologic function outcomes, which subgroup analyses accounted for.

6.3.4.1 Overall effect of treatment on brain oedema

Five of the publications used in the meta-analysis assessed the effect of treatment on brain oedema (365, 386, 388, 396, 553) and four studies assessed neurologic function (365, 388, 396, 553). The data on therapeutic efficacy of heparin and its derivatives on brain oedema was contained within the 27 experimental comparisons (Appendix H). However, we limited our analysis to data from the ipsilateral cortex, as it best answers our research questions,

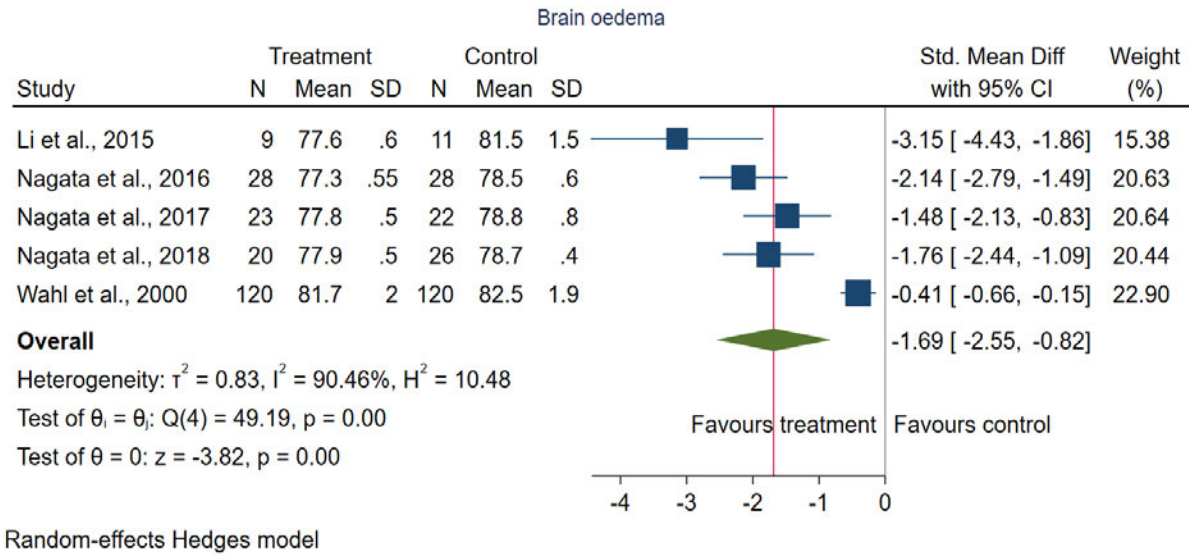


Figure 6.3: Effect of all treatment on brain oedema after trauma

The forest plots represent the pooled analysis of data on the use of heparin and enoxaparin on brain oedema. The overall effect estimate favours treatment compared to untreated control (-1.69 (95% CI: -2.55, -0.82, P < 0.001). However, heterogeneity was significant (I^2 = 90.46%). The square symbols and their error bars (95% CI) are the individual studies contributing to the pooled estimates. The central lines and lateral tips of the diamonds indicates the pooled point estimates and the corresponding 95% CIs, respectively. The solid black vertical line indicates the line of no effect while the red vertical line is the overall effect size line. This annotation is used in all subsequent Forest plots.

the efficacy of treatment on brain oedema was evaluated based on 17 comparisons involving a total of 407 animals. Taken together, drug treatment reduced brain oedema with an overall estimated effect of -1.69 (95% CI: -2.55, -0.82, P < 0.001, Figure 6.3) but with substantial heterogeneity, I^2 of 90.46% among studies (Figure 6.3).

6.3.4.2 Effect of heparin and enoxaparin treatment on brain oedema.

Comparing the two studies that examined the effect of unfractionated heparin (UFH) (365, 396) with the two that examined enoxaparin (386, 388) on brain oedema revealed similar point estimates for both (Test of group difference, brain oedema, P = 0.96, Figure 6.4) but the degree of heterogeneity differed markedly (Enoxaparin I^2 = 94%, UFH I^2 = 34.38%).

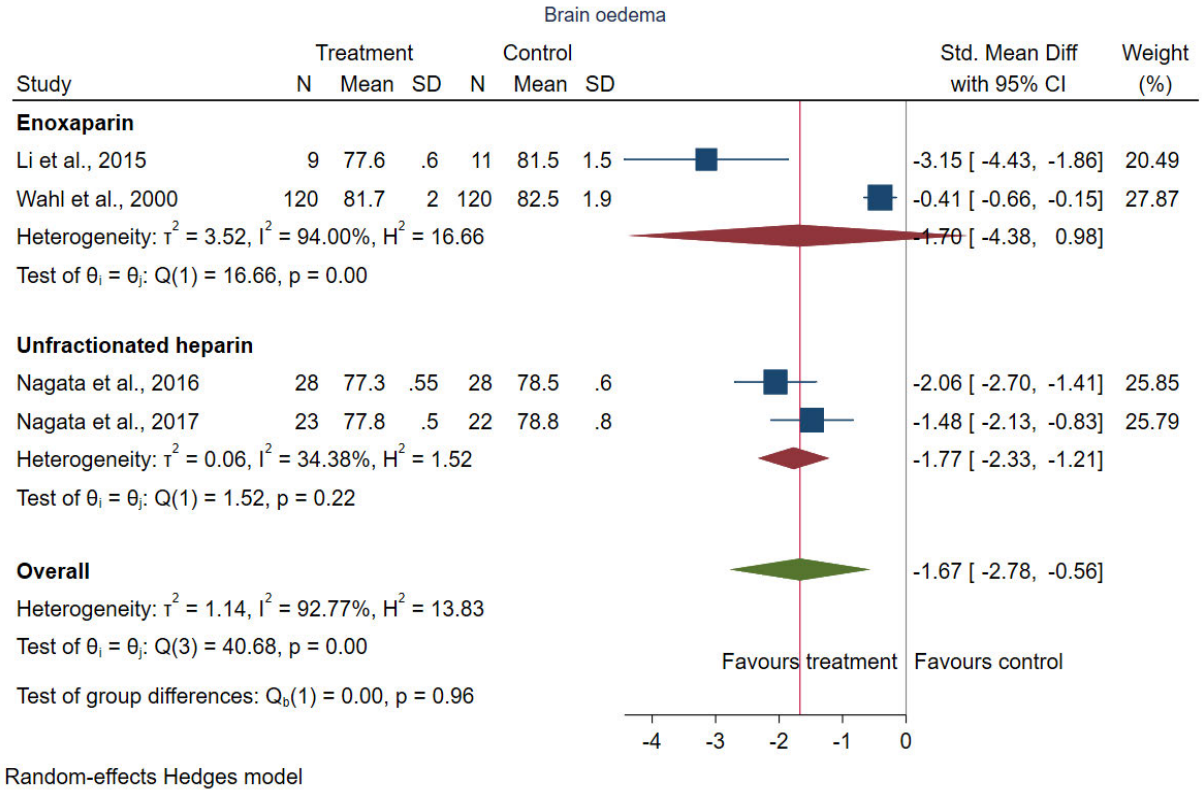


Figure 6.4: No difference in drug subgroup analysis on brain oedema

For enoxaparin and UFH the overall effect favoured treatment compared to the untreated control for brain oedema (-1.67 (95% CI: -2.78, -0.56; P < 0.001). However, there was no difference between the effect estimates for the two drugs although heterogeneity differed substantially (I^2 enoxaparin =93%, I^2 UFH = 34.38%).

6.3.4.3 Effect of different doses of heparin and enoxaparin on brain oedema

Segregating the data for enoxaparin and UFH into low and high dose regimes on brain oedema suggested a trend towards greater effect for low doses (Low dose: -2.40 (95% CI: -3.01, -1.80; High dose: -1.40 (95% CI: -2.42, -0.38), no statistical difference between the doses was detected (Test of group difference, Q_b, 2.76, P = 0.10; Figure 6.5). For all UFH studies, the dose given was recorded as U/kg, so, required conversion to mg/kg for this comparison according to standard dosing guidelines (641). While heterogeneity was not significant in the low dose group (I^2 = 6.55%), it was moderate in the high dose group (I^2 = 60.81%). The study from Wahl et al., 2000 (386) was excluded in this analysis because we could not categorically

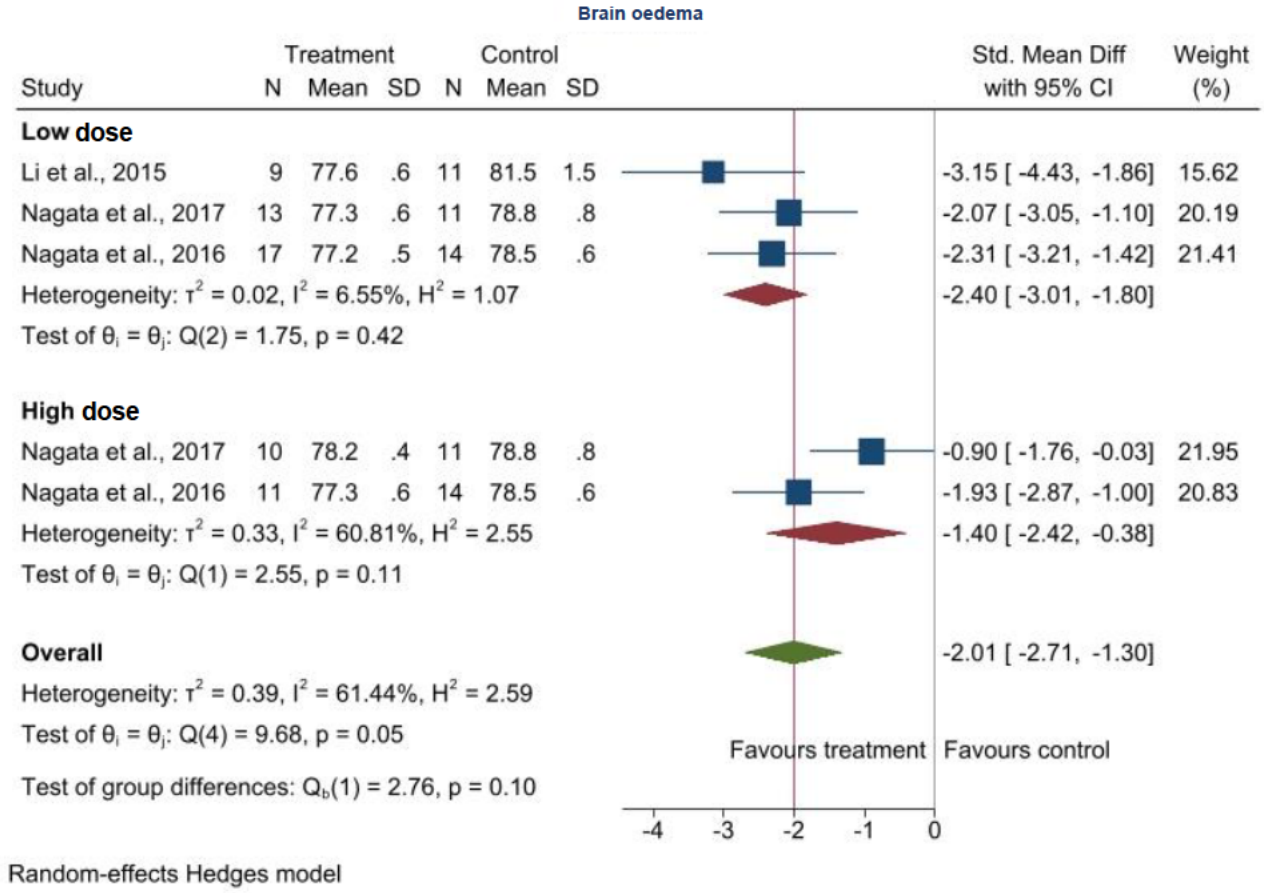


Figure 6.5: Effect of low and high doses on brain oedema after trauma.

The drug doses were grouped into either low or high dose regardless of the mode of administration. Overall, the pooled estimate of both doses favoured treatment compared to control with a substantial heterogeneity (I^2 = 61.44%). There was no significant difference between the effect of low and high dose on brain oedema (P = 0.10). Heterogeneity was moderate in the high dose group (I^2 = 60.81%) and not significant in the low dose group (I^2 = 6.55%).

group the dose administered as either a low or high dose since the authors used a combination of drug doses at various times. For example, they started with an intravenous administration of a low dose a few minutes or hours post-injury and afterwards with a subcutaneous injection of a high dose. Overall, the pooled estimate of both low and high drug doses favoured treatment compared to the untreated controls and the heterogeneity (I^2 = 61.44%) was reduced slightly but remained substantial.

6.3.4.4 Effect of treatment on neurologic function

Neurologic function outcome measured using the validated, modified Garcia Neurological Test and the Neurological Severity Score Severity Score was also evaluated following treatment with heparin and its derivatives in four of the included studies with seven comparisons involving 129 animals. Only the 48-hour timepoint was analysed and overall, the pooled adjusted estimated effect of 1.51 (95% CI: 0.31, 2.72, P = 0.01, Figure 6.6) favoured treatment. There was significant heterogeneity (I^2 = 88.58%).

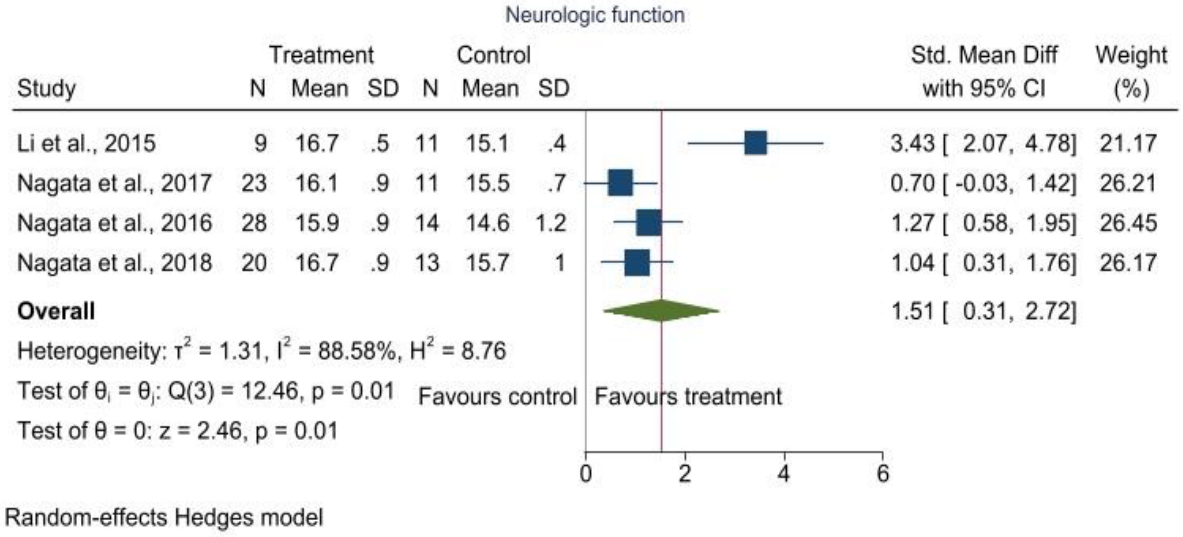


Figure 6.6: Effect of all treatment on neurologic function after trauma

The forest plots represent the pooled analysis of data on the use of heparin and its derivatives on neurologic function. The overall effect estimates favours treatment compared to control (1.51 (95% CI: 0.31, 2.72, P = 0.01). Drug treatment improved neurologic function after trauma. However, heterogeneity was significant (I^2 = 88.58%). The central lines and lateral tips of the diamonds indicates the pooled point estimates and the corresponding 95% CIs, respectively. The solid black vertical line indicates the line of no effect while the red vertical line is the overall effect size line.

6.3.4.5 Effect of treatment on Haemorrhage/haemorrhagic contusion

Only three studies examined the effect of treatment on bleeding using two different methodologies, detection of red cells/clot on haematoxylin and eosin stain (387, 639) and visualisation of haemorrhage on the surface of the brain (388). Together, these studies found that treatment did not increase bleeding (Figure 6.7A).

6.3.4.6 Effect of treatment on inflammation and microvascular permeability

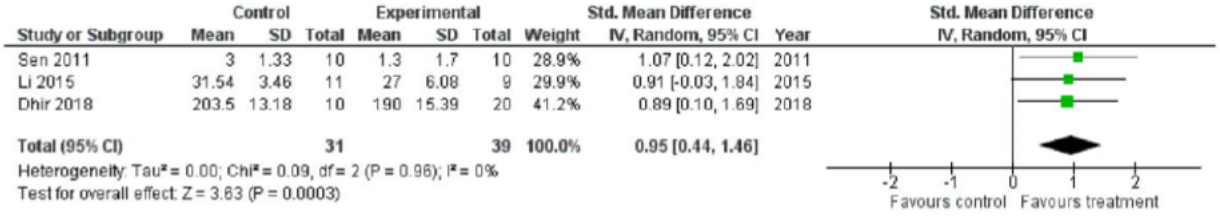
Three studies (365, 388, 553), all in mice, quantified FITC-labelled albumin fluorescence at 48 hours post-TBI. The authors found that enoxaparin, UFH and ODSH, respectively, improved blood brain barrier permeability (Figure 6.7B). The same three studies also reported decreased leukocyte rolling (Figure 6.7C).

6.3.4.7 Effect of treatment on body weight

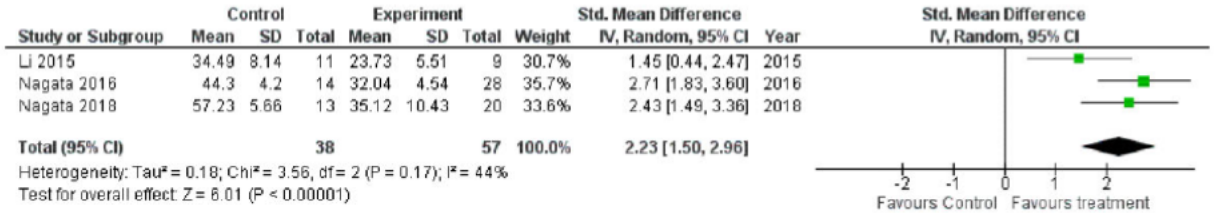
When body weight was examined in the same three studies as above (365, 388, 553), treatment with enoxaparin was associated with reduced body weight loss whilst treatment with either UFH or ODSH was not. Overall, there was no effect of treatment on body weight (Figure 6.7D).

Other variables we had hoped to investigate including the optimal time at which the drug produced the most effect and the best route of administration could not be evaluated due to insufficient data.

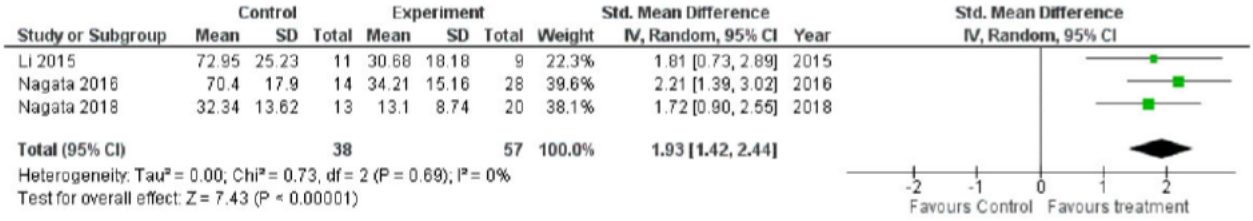
A. Haemorrhage/haemorrhagic contusion



B. Microvascular permeability



C. Leukocyte rolling



D. Body weight loss

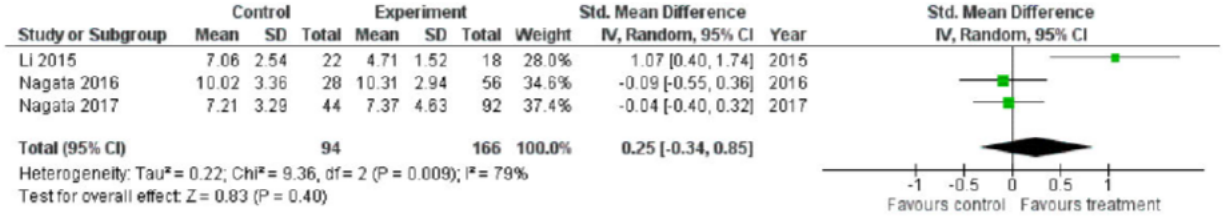


Figure 6.7: Effect of all treatment on haemorrhage, inflammation and vascular permeability.

Leukocyte rolling and microvascular permeability were decreased following treatment with unfractionated heparin, enoxaparin and ODSH. While varying effects of both enoxaparin and unfractionated heparin were observed on body weight loss. Enoxaparin treatment did not increase haemorrhage and treatment.

6.3.4.8 Potential publication bias

Publication bias was assessed using funnel plot on the overall therapeutic effect of treatment on brain edema and neurologic function after trauma. Four studies were evaluated and we found that there was funnel plot asymmetry for both outcomes which was confirmed with egger regression test (brain edema, $z = -4.86$, $P < 0.001$; neurologic function, $z = 3.25$, $P = 0.0012$). Contour-enhanced funnel plot explored funnel plot assymetry at 1%, 5% and 10% (Figure 6.8A and figure 6.8B) for each outcome suggesting the possibility of publication bias.

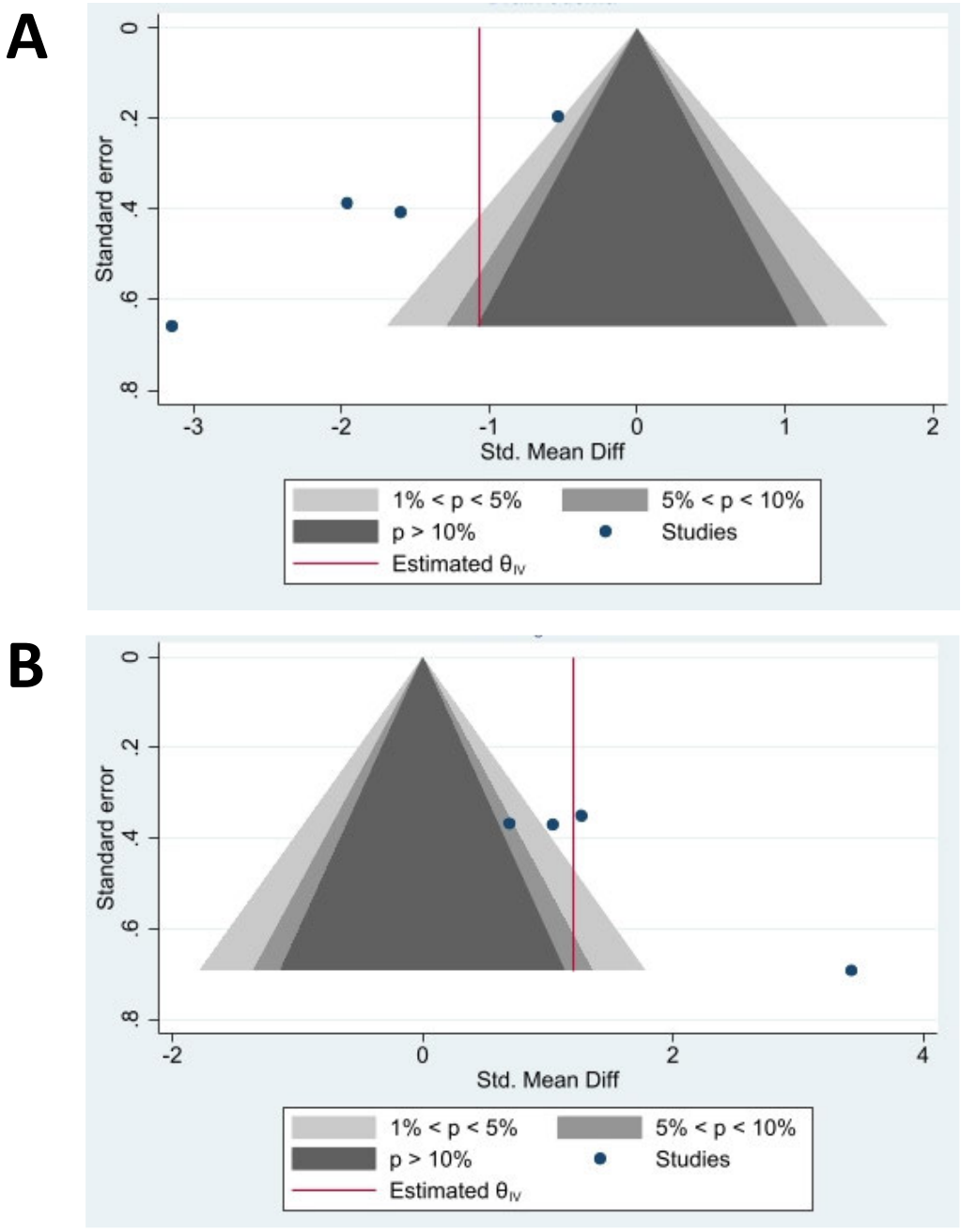


Figure 6.8: Funnel plot asymmetry in the studies evaluated for brain oedema and neurologic function after trauma.

*Contour-enhanced funnel plot assessed at 1%, 5% and 10% show marked asymmetry in reporting of brain oedema (**A**) and neurologic function (**B**) after treatment of TBI with heparins.*

6.4 Discussion

In this review we have evaluated the preclinical data supporting the potential therapeutic use of heparins as treatments for TBI. To our knowledge, this review is the first to systematically collect and analyse all available evidence in the literature regarding the therapeutic efficacy of heparin and low molecular heparins (LMWHs) in animal models of TBI.

There is a significant body of work examining this potential. However, we were surprised at the paucity of data suitable for inclusion in a meta-analysis. Indeed, of the 268 publications that appeared relevant on screening, only 11 met our overall eligibility criteria and only seven contained data suitable for inclusion in meta-analysis. Moreover, within the studies, the injury models and outcome assessments were variable. Not surprising when it is remembered that the presentation of TBI with respect to age, gender, site and nature of injury, severity and pathophysiology are complex and diverse (642).

The paucity of data is in part because of the strict criteria that are applied in order to facilitate valid meta-comparisons across sometimes disparate datasets. For example, many papers were the only reports for a particular outcome and could not sensibly be grouped with others for analysis. Also, we were only able to use data from experiments that provided controlled studies (in this case untreated uninjured animals) where cohort sizes and outcome data and its variability were provided or could be reliably imputed.

This speaks to the need for coordination across the field, particularly with respect to core standardised analyses (643) and better application of current reporting standards such as ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) (644, 645) across the field.

As mentioned above, we were limited in our ability to utilise the analytical power of systematic review (646, 647) by the breadth of different analyses performed and the overall paucity of suitable numeric data, this is an important observation for the field and common in preclinical analyses (648, 649) where exploration of pathobiology is usually combined with evaluation of potential therapeutic efficacy. Nevertheless, these limitations and the meta-analysis itself provide some important additional insights.

Since all the animals used were male (see Table 6.1), we were unable to explore the role of sex on the efficacy of heparins for TBI. This indicates that researchers are ignoring this important facet of human TBI where sex clearly affects outcome (650, 651), the female

hormones oestrogen and progesterone are known to be neuroprotective (277, 652, 653), and published guidelines recommending use of both sexes in studies of TBI (654).

We did find studies that analysed multiple outcomes using multiple model systems as detailed in Tables 6.1-6.3. The main themes were that heparins modify lesion size (366, 386), neurologic function (365, 386, 396, 553), brain oedema (365, 388, 396, 553), BBB dysfunction (365, 388, 553), neuroinflammation (365, 387, 388, 390, 396, 514, 553, 640), a variety of mechanisms of cell death (366, 387, 390, 514), the pathobiology of bleeding in the face of heparin-based drug treatments (387, 388, 396, 639) and a range of other markers of injury such as oxidative stress (390). Here, we first summarise the main findings from the individual papers and report the meta-analysis of components of this data.

Increased lesion volume after TBI, whether due to ischaemic or other secondary changes, is associated with unfavourable outcomes (443, 655). Of the studies included in this review, only two studying the effects of enoxaparin explicitly examined lesion volume. One reported that high dose enoxaparin decreased lesion volume (366), but the other found no effect at high doses but did detect a decrease at low doses (386). These differences might be a consequence of the different models and species used, cold-induced TBI in mice for the former (366), and fluid percussion in rats for the later (386). Unfortunately, there was insufficient data to explore this by meta-analysis.

Poor neurologic function, as a consequence of both the initial lesion and subsequent damaging changes, is perhaps the most critical consequence of TBI (297, 306, 319, 594). The studies reported here that examined enoxaparin, UFH and ODSH all improved neurologic function and enhanced recovery up to 4 weeks post-TBI (365, 386, 396, 553). Motor and cognitive function were also reported to be improved by ODSH (553) and enoxaparin (386).

Brain oedema, which is a common pathology in TBI, could worsen outcomes by increasing intracranial pressure causing secondary ischaemia and reducing cerebral perfusion and tissue oxygenation (101, 656). The studies we assessed reported that enoxaparin, UFH, and ODSH, all decreased brain oedema following experimental TBI (365, 386, 388, 396). The effect of dosing in these studies is unclear. Low dose enoxaparin (388) and both low and high dose UFH (365, 396) but only high dose ODSH (553) were reported to reduce oedema. In related studies in photothrombotic stroke, only higher doses of enoxaparin are reported to improve cerebral oedema (385). These oedema outcomes are discussed further in the meta-analysis section below.

One contributor to brain oedema and worse TBI outcome is damage to the blood brain barrier (BBB), the complex structure that controls the movement of nutrients, proteins and immune cells amongst others in and out of the brain (104, 657, 658). Assessed here as microvascular permeability, enoxaparin, heparin and ODSH all reduced the leakage of BBB function in the CCI model of TBI at 48 hours post-TBI regardless of the drug dose, mode of delivery or drug type (365, 388, 553).

Overall, our meta-analysis suggests that heparin and enoxaparin do appear to reduce brain oedema (Figure 6.3) and improved neurologic function (Figure 6.6) in animal models of TBI. Moreover, it appeared that enoxaparin and UFH provided very similar effects, albeit with considerable differences in experimental heterogeneity which we cannot currently explain (Figure 6.4). Intriguingly, there was a trend suggesting that lower doses (0.75mg/kg minimum, not greater than 1mg/kg) might be more effective than higher doses (≥ 2mg/kg) (Figure 6.5). This observation would be consistent with a randomized double-blinded placebo-controlled trial in humans showing an overall favourable effects following the administration of 0.5mg/kg of enoxaparin in severe TBI (659). However, as an additional note of caution, heterogeneity in the two high dose studies was high despite coming from the same research team and employing very similar methodologies including animal species, mode of drug delivery and dose of drug (365, 396). Conversely, heterogeneity was very low between the low dose studies (365, 388, 396)

The individual studies included in this review did not report an increase in haemorrhage following the administration of enoxaparin after trauma (387, 388, 396, 639), indeed the meta-analysis (Figure 6.7A) would appear to suggest that the treatment improved haemorrhagic outcome suggesting that drugs like enoxaparin may be relatively safe. However, this suggestion has to be weighed against the risk of clinically significant bleeding complications as early use of drugs with anti-coagulant properties (421, 660) might be expected to worsen outcome by increasing intracranial/cerebral haemorrhage which is commonly observed after TBI (62). It should be noted however that, the early administration of enoxaparin in patients with severe TBI did not show significant increase in cerebral haemorrhage in a human clinical trial (659).

Neuroinflammation is a critical secondary injury mechanism that interacts with BBB damage after TBI, contributing to ongoing neuronal damage and death (661, 662). It encompasses infiltration of peripheral immune cells into the brain, increased circulating and

brain concentrations of inflammatory mediators and consequent astrogliosis and microgliosis (127, 196, 511, 513). In related models of subarachnoid haemorrhage, heparin is reported to reduce microglial activation and expression of the inflammatory cytokines, TNF-α and IL-1β (367). Eight of our included studies together investigated the effect of UFH, ODSH and enoxaparin on leukocyte rolling, leukocyte adhesion, PMN sequestration, IL-1β, pro-IL-1β, COX-2 expression, reactive gliosis and glial scar formation following experimental TBI (365, 387, 388, 390, 396, 514, 553, 640). Overall, these inflammatory outcomes were improved by heparin (365, 396, 640), enoxaparin (387, 388, 390, 514) and ODSH (553) regardless of dose. However, there was no effects reported on leukocyte adhesion (365, 553), pro-IL-1β concentration (390) or astrogliosis (390). While most of these parameters provided insufficient data for meta-analysis, three studies which examined both vascular permeability and leukocyte rolling did provide evidence for a consistent beneficial effect (Figure 6.7B and C).

In the studies evaluated here, reduced apoptosis was reported in response to enoxaparin (366, 387, 390) though only one study compared between doses and actually demonstrated that the decrease observed was dose-dependent (366). Similarly, enoxaparin also decreased expression of the necrosis mediator Caspase-1 (514). There was insufficient data to examine this by meta-analysis.

Oxidative stress is one of many other important contributors to the pathophysiology of TBI that were investigated in the articles we reviewed. In TBI, markers of oxidative stress such as TBARS, ROS, NOS are increased in the brain while antioxidant defence enzymes like SOD, GSH are reduced (96, 663, 664). The studies which we report, that examined the effects of enoxaparin on oxidative stress, found decreased concentrations of TBARS and oxidized proteins in the hippocampus in the LFPI rat model of TBI at 48 hours after injury (390). Conversely, there were no changes in hippocampal SOD and GSH-Px activities and while enoxaparin had no effect on TOS levels irrespective of dose, high dose, it did increase TAS levels. (390). It remains to be seen whether anti-oxidant effects are important contributors to the effects of heparins overall.

We had originally pre-planned for the possibility of a range of sub-group analyses (https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42020205574) (e.g. drug type, mode of drug administration, drug dose, sex of animal, animal species, timing, and frequency of treatment) to explore the factors that contributed to experimental heterogeneity. Most proved impractical to analyse effectively and some could not be analysed at all. For

example, many injury models were used but none sufficiently often to allow comparison. Controlled cortical impact (CCI) producing a severe TBI was the most common way to induce TBI (365, 388, 396, 514, 553). Similarly, multiple routes of drug delivery were used (including within studies (386, 387) all with apparent benefit. However, no study provided data indicating that any route of delivery was to be preferred and we found that insufficient data was available for meta-analysis to provide guidance for this important practical question (665, 666). The timing of treatment was also considered important but not possible to analyse.

The potential for bias is another reason for caution. While systematic review and meta-analysis are important tools for finding, aggregating and helping interpret data (667-669), it must be remembered that if the input data is biased, the output data will also be influenced by this bias. While we have endeavoured not to do so, it is also possible for introduction of bias during the process of systematic review and meta-analysis (670-672).

Overall, the studies we were able to find were generally of low quality (median 4 out of 10) based on the modified checklist from CAMARADES (634, 635). Despite having been subjected to peer review, there were notable absences of data reporting, where measures such as blinding had not been taken to help avoid the introduction of bias. These absences are particularly well highlighted by use of the SYRCLE Risk of Bias tool (Figure 6.2). Sadly this finding is consistent with the analyses of the use of these measures across *in vivo* preclinical research (673) where such absences are associated with inflated effect sizes (674, 675). However, it was reassuring to discover that random allocation into groups was very well reported by virtually all the studies included in this review. Funnel plots asymmetry and Egger's regression, albeit based on a small sample size (Figure 6.8), also indicates the possibility of publication bias or some other source of asymmetry in this data (676, 677).

6.4.1 Study Limitations

To our knowledge, this is the first systematic review and meta-analysis investigating the therapeutic effects of heparin and LMWHs in animal models of TBI. However, the study has important limitations, especially with respect to the small sample size available and presence of substantial heterogeneity. Over 50% of the studies included for meta-analysis was from one research group. Analysis of quality metrics depends on presentation of this data in the peer reviewed publications, if it is performed but not reported the studies will score poorly. It should be noted that where this has been formally evaluated, lack of reporting has been associated with overestimation of effect sizes (678). Finally, for brain oedema, we limited our

analysis to only the injured hemisphere as the effect of drug was bigger here compared to the uninjured hemisphere. Similarly, for neurologic function, our analysis was limited to neuroscores acquired at 48-hours post-injury. A more extensive instigation could have been conducted if there had been sufficient data for the other timepoints and brain regions.

6.4.2 Conclusion

Overall, in this review, we found that heparins, including ODSH and enoxaparin, appeared to improve outcome after TBI. With enoxaparin's better bioavailability profile, more predictable dose-response relationship, longer half-life, less anti-IIa (thrombin) activity and half the anti-Xa (prothrombinase) activity than heparin (385, 679), it may have advantages. However, the inferences made above are based on a very limited number of animal studies and moreover four of the seven studies were from a single research group (365, 388, 396, 553). Therefore, this data must be interpreted with caution. Nevertheless, there is a clear need for more studies to evaluate the therapeutic potential of heparins as a treatment for TBI.

CHAPTER 7

GENERAL DISCUSSION

Neuroinflammation is a crucial component of the secondary injury processes that ultimately contribute to the progressive neuronal damage associated with TBI (680). While it is essential for host defence, prolonged and dysregulated neuroinflammation could also lead to unfavourable outcomes (147, 661). Thus, as a therapeutic strategy, drugs targeting the regulation of the neuroinflammatory cascade to promote repair and not to completely impede the process is highly recommended (681).

In this thesis, we aimed to investigate the therapeutic effect of enoxaparin and its non-anticoagulant fragments, Dp2 and Dp4, following experimental TBI. While the potential neuroprotective and anti-inflammatory effects of enoxaparin have been explored across different models of TBI (385), this study is the first instance in which Dp2 and Dp4 have been evaluated for this purpose. As described earlier, Dp2 and Dp4 are disaccharide and tetrasacharide fragments of enoxaparin, respectively, that retain the anti-inflammatory actions but lack the anticoagulant activity of other heparins, and therefore pose no risk of bleeding. We proposed that these properties make these drugs strong candidates for the treatment of TBI. Consequently, we hypothesised that enoxaparin, Dp2 and Dp4 would decrease neuroinflammation and promote recovery following TBI. This was based on previous studies that have identified beneficial effects of enoxaparin - including improving cognitive function, decreased reactive gliosis, decreased activation of the inflammasome, and reduced leukocyte infiltration following TBI, that are distinct from its anticoagulant actions (386, 388, 390, 514). Similar effects were also demonstrated by treatment with the chemically modified heparin ODSH, which has reduced anticoagulant activity (553). However, overall, in this study, treatment did not attenuate neuroinflammation and behavioural impairment in a mouse CCI model of TBI. Hence, leaving many unanswered questions as to why the drugs did not produce expected results despite all the benefits previously demonstrated in the literature.

One possible explanation for our finding could be that the drugs do not interact directly with glial cells to inhibit their activation. While it is well known that LMWHs like enoxaparin are a heterogeneous mixture of oligosaccharides (422), their actual non-anticoagulant mechanisms of action remain unknown (682). However, as mentioned in previous chapters, heparin and LMWHs can inhibit the NF-κB signalling pathway in activated monocytes and endothelial cells (393, 424, 425), and block the signalling of HMGB1 protein (391). These actions could potentially be part of a common pathway, since HMGB1 can activate NF-κB signalling via binding to toll like receptors (TLR) (392, 395). Moreover, NF-κB signalling downstream of TLR4 activation plays an important role in the production of proinflammatory

cytokines by astrocytes and microglia following TBI (393, 395, 424). So, while our supposition that enoxaparin, Dp2 and Dp4 might act to decrease the production of proinflammatory cytokines by inhibiting TLR4 on astrocytes and microglia remains plausible, it has not been supported by our data. It is also important to consider that since Dp2 and Dp4 are two of many fragments of enoxaparin, it is uncertain which binding properties and mechanisms of action of enoxaparin they would retain, if any.

For a better understanding of whether enoxaparin, Dp2 and Dp4 are able to directly inhibit activation of glia and to provide insight into their mechanism of action, I had intended to carry out a series of *in vitro* experiments. These experiments were set to begin as ethics approval had been granted and materials were purchased. Unfortunately, it was not to be as it was one of the many experiments that was affected following the shutdown of the research labs due to the COVID-19 pandemic. The main objective of the cell culture experiment was to directly test the effect of Dp2 and Dp4 on cortical and hippocampal astrocytes and microglia following liposaccharide and/or interferon gamma-induced activation. Overall, this experiment was to address two aims: the first being to determine if activated astrocytes and microglia are dose responsive to Dp2 and/or Dp4 and enoxaparin, while the second aim was to explore whether the mechanism of the drugs was to block the TLR4-NF-κB signalling pathway. The results of this study would help us to determine whether further pursuit of Dp2 and Dp4 as a pharmacotherapy for TBI is worth-while.

A second possible explanation for the failure of the drugs evaluated in this thesis could be that the drugs did not cross the BBB. There were uncertainties as to whether enoxaparin, Dp2 and Dp4 are able to cross the BBB, a semi-permeable protective barrier that strictly regulates substance entry from the blood into the nervous system (104, 657). However, evidence of LMWHs crossing the BBB was demonstrated in an *in vitro* BBB model made up of a co-culture of astrocytes and brain capillary epithelial cells. Here, the authors discovered that depolymerised heparins, including tetrasaccharides and disaccharides, crossed the endothelial cell monolayer barrier more effectively than the full heparin molecule without any observed cell toxicity (683). Another study also demonstrated that depolymerised mixture of heparin oligosaccharides (4-8 dextrose units) could pass through the BBB *in vivo*. Here, C3, a depolymerised heparin derived oligosaccharide dominated mostly by hexa- and octasaccharides with no anti-IIa activity, was administered via intravenous and subcutaneous route in rats and it was discovered that C3 was present in CSF and brain homogenates. Interestingly, the drug crossed the BBB more quickly following intravenous administration

compared to the subcutaneous route. They further went on to demonstrate that a higher intravenous dose of C3 led to a higher concentration of the drug in the brain and also implied that for therapeutic uses, perhaps, to obtain a relatively higher concentration of heparin oligosaccharides in the brain, there has to be a continuous administration of the drug (684). Moreover, with the compromised BBB after trauma, low molecular weight compounds are reported to penetrate into the brain for up to 4 days after injury and larger molecular weight compounds could only penetrate the brain for the first few hours after injury (685) thus, providing a therapeutic window that could be explored for TBI treatments. Although yet to be elucidated, it has been proposed that enoxaparin penetrates the brain through specific glucose transporters like GLUT1, a naturally occurring transporter, readily present on the BBB, whose function is to supply glucose as an energy source to the brain (686). Together these studies suggest that in our experiment, it is likely that enoxaparin, Dp2 and Dp4 were able to cross the BBB, although this was not demonstrated conclusively.

To address this issue directly, it would be valuable to perform a pharmacokinetic analysis of enoxaparin, Dp2 and Dp4. This would provide essential details on the drugs' properties including absorption, bioavailability, distribution, metabolism, and excretion, which could then be used to optimise an effective dosing paradigm (687, 688).

A third possible explanation for the lack of effect of these drugs is that our administration paradigm was not successful. To date, there is no known optimal dose or best mode and timing of drug administration following brain injury for heparins and their derivatives, including enoxaparin. The enoxaparin dose used in rodent studies following experimental TBI model ranges from as low as 0.5 mg/kg to as high as 10 mg/kg which were given subcutaneously (388, 390, 391, 639), intravenously (514), intraperitoneally (366) or by combined routes (386, 387). For instance, the study by Sen et al., 2011 (387) employed the administration of enoxaparin via the combined route in which, they first administered 0.5 mg/kg of enoxaparin intravenously at 15 minutes after TBI as a bolus dose which would likely enable the drug to reach the brain quickly, and then it was followed by an additional 1 mg/kg of drug administered subcutaneously at 30 minutes, 6, 24, and 30 hours after TBI to maintain continuous drug availability to the brain. Comparatively, for the early studies in this thesis, in which the effect of treatment on neuroinflammation was being assessed, the drug regimen involved enoxaparin given either as a continuous SC infusion at a rate of 2.5mg/kg/24hours or as multiple or single daily IP bolus injection of 1 mg/kg. Overall, the daily dose was similar to the study mentioned above. However, our study lacked the early bolus loading dose which

could imply that drug concentration did not get high enough in the brain to have an effect. Also, while no previous enoxaparin study administered drug via continuous SC infusion following trauma, one study did administer enoxaparin through the intraperitoneal route (366). Although this is not a common route for administering heparins and LMWHs clinically, because they are degraded in the first-pass metabolism to lower molecular weight fragments by desulphation and/or depolymerization (679). IP injections was used in our study based on a hypothesis that the short oligosaccharide fragments that we were using wouldn't be further degraded. However, this is yet to be elucidated.

Since I did not see anti-inflammatory effects with the treatment regimen used in my earlier studies, for the final study assessing long-term behavioural outcomes, I changed administration of enoxaparin to 1 mg/kg delivered via repeated SC injections, rather than continuous low-dose infusion, with the frequency of drug administration similar to that used in the study described above by Sen et al., 2011 (387), except that they included an initial 0.5 mg/kg intravenous injection of enoxaparin at 15 minutes post-TBI compared to ours which was given at 30 minutes post-TBI via SC injection. We chose this treatment paradigm because, in as much as we were aiming for a more practicable clinical approximation, another previous study had demonstrated a more efficient result following enoxaparin's administration at a later time of up to 2 hours post-TBI than at 15 minutes (386).

Interestingly, as demonstrated in the study by Keskin et al., 2017 (366), perhaps, employing a much higher dose of drugs could have had a more significant effect on the evaluated TBI pathology in this thesis. For instance, in the Keskin study, the highest dose of 10 mg/kg used produced a greater effect than the lower dose of 3 mg/kg following TBI (366). Further evidence suggest that enoxaparin's beneficial effect in TBI might be dose-related, in that, higher dose enoxaparin reduced infarct volume (386) and high dose of chemically modified heparin, ODSH, reduced brain oedema (553) when compared to their lower dose counterpart. To corroborate this, the C3 study earlier mentioned demonstrated that the higher dose of C3 (10 mg/kg) ultimately led to a significantly higher levels of the drug in the CSF and brain homogenates of rats (684). Meanwhile, in other studies, lower doses of enoxaparin have been reported to produce more neuroprotective effect on the brain following TBI compared to the higher doses. For example, lower doses favoured neurological function recovery and reduced lesion size (386). In fact, some outcomes including body weight loss and haemorrhage were reported to worsen with the administration of high doses of UFH (689, 690). These studies

together show that more work is needed to determine the most effective dose of enoxaparin for the treatment of TBI.

Theoretically, the treatment strategy used in this thesis appeared to be sufficient as it was comparable to previous studies that have shown beneficial effects with enoxaparin However, our study showed no effect of drug on gliosis and motor function impairment following TBI. Perhaps, extending our treatment approach for a few more hours or days could make a difference as we know that neuroinflammation persists for weeks, months and even years after injury (691, 692).

It's important to consider that our treatment strategy could also potentially be verified by examining pathologies other than inflammation that enoxaparin is known to improve after TBI, such as brain oedema (refer to Chapter 6). Since the effect of enoxaparin on glial activation is largely unknown, enoxaparin has not acted as a positive control in this study. Rather, the purpose of including this experimental group in the experiments performed in this thesis was to compare the efficacy of the di- and tetra-saccharide fragments (alone or combined) with efficacy of the parent compound.

Apart from drug doses, severity of the injury is another variable to consider when comparing the negative findings from this study to the beneficial effects seen with heparins in other TBI studies. In my experiments, I used the established CCI model, in which the degree of is severity is determined by the injury induction parameters, including depth and velocity of impact and dwell time (see the review by Siebold and colleagues (693). The parameters used in my studies were the same as those commonly used to generate lesions defined as "moderate-severe injury" based on histological outcomes (603, 694). So, while in this study we used a moderate to severe injury model, a cold induced TBI model was employed in the study by Keskin and colleagues (366). Although, the authors did not report the severity of their injury model, it is worth-noting that the pathological processes involved, if the injury were mild are certainly less damaging than those of moderate to severe injuries, whose neurotoxic environment resulting from the cellular and molecular mechanisms triggered by TBI within the brain could hinder the effective functioning of the drugs (603, 695-697). Therefore, suggesting that the current dose utilised in our various experiments could have been sufficient to produce beneficial effects if the induced injury were milder in this thesis.

Consequently, considering that very little is known about the pharmacokinetics and pharmacodynamics of enoxaparin in the literature except for the only unifying fact that they

seem to have potential anti-inflammatory effects across different neurological diseases, I had to pause, and do a critical evaluation of the literature to determine if heparin and low molecular weight heparins actually do have neuroprotective effects following TBI. Hence, I undertook a systematic review and meta-analysis in which our finding as detailed in chapter 6 demonstrated that heparin and enoxaparin have potential neuroprotective effects following TBI. Although, this conclusion was made from a limited number of included studies. While heparin and enoxaparin appear to be a promising treatment for TBI, more in-depth mechanistic research is necessary to better understand the exact functioning of heparin, enoxaparin and its fragments, Dp2 and Dp4. Until then, there are many unanswered questions including, if enoxaparin and its non-anticoagulant fraction, Dp2 and Dp4 act directly on the brain cells? Could they bring about their effect by indirectly acting on the brain, probably by suppressing T-cells? On the other hand, could they be acting through a different pathology we did not investigate in this thesis, for example, supressing cell apoptosis, as discussed in Chapter 5.

With the increasing incidence of TBI globally, there is a need to design and develop effective therapies for the treatment of this debilitating disorder. An essential component required to achieve this goal is preclinical research. Preclinical studies ultimately provide information on efficacy and safety of drugs or other medical procedures for clinical trials following testing in animals (698). Despite the successes recorded in preclinical TBI studies, no clinical trials have translated into an effective TBI treatment. One major challenge however, for failure to translate TBI preclinical studies is that TBI animal models are a poor representation of the actual TBI pathophysiology occurring in the human brain following injury (699). Therefore, considering that it is highly impossible to fully replicate human presentation of TBI pathologies in rodents, it is important for animal models to be well characterised to suit the exact injury pathology that is to be investigated with specific research questions to be asked accordingly (700). Other factors that could significantly impact the quality of preclinical drug trials include poor experimental design and inadequate funding (435, 701).

An additional important consideration in evaluating the potential of a novel drug based on preclinical trials is the prevalence of publication bias, which arises because negative results are not readily reported (702, 703). Also, publishing negative results could provide relevant information that could contribute significantly to the advancement of scientific research and consequently, prevent possible waste of resources (704). Thus, despite my negative findings, the studies undertaken in this thesis were carefully designed and executed, and my results will

contribute valuable information to the field regarding the potential limitations of the use of heparins in the treatment of TBI.

In conclusion, the search for a TBI treatment has definitely come a long way and has faced its fair share of challenges. There is urgency to identify effective TBI treatments due to its increasing global burden. I am hopeful for the future as several novel therapeutic treatments targeting various aspects of TBI pathophysiology, including the neuroinflammatory cascade, are being explored. However, in the context of the studies carried out in this thesis, future research should focus on determining the glial response to the drugs by a direct application in an *in vitro* medium, and also elucidation of the drugs' mechanisms of action. If the *in vitro* work showed that these drugs can directly inhibit pro-inflammatory glial responses, further *in vivo* studies would be warranted to explore the pharmacokinetics of the drugs and their ability to cross the BBB, before determining the best dosing regimen and route of administration. Also, future work should include female animals to determine whether there is a sex-effect of drug treatment following experimental TBI (705-707), and should also explore potential neuroprotective mechanisms of these drugs. The outcome of these would be an interesting find for the future of TBI therapeutics.

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Appendix

Appendix A: Comparing the quantification of immunofluorescent labelling between imageSURF and thresholding image analysis methods

The thresholding image analysis technique is the most commonly used method of quantifying immunofluorescent labelling. This method could be subjective with a high risk of bias. Therefore, the quantification of immunofluorescent labelling of glial cells in TBI tissue was compared for the first time between the automated newly developed imageSURF tool and the ImageJ thresholding method of image analysis.

GFAP immunostaining in only the injured cortex following IP injection of Dp4High at 3 days post-TBI was evaluated using both image analysis methods and it was revealed that while quantitatively, a similar pattern was observed across the groups in both analyses, the percentage area positive for GFAP staining in the injured cortex was lower in the imageSURF analysis compared to the thresholding method analysis. In addition, from the size of the error bars, there seem to be greater error variability in the data analysed by thresholding as indicated by the large error bars compared to the imageSURF analysis data with smaller error bars. Lastly, there was no significant difference across the various groups following analysis with both imageSURF and thresholding image analysis methods (Figure A1 A and B; one-way ANOVA, $P = 0.5720$, $P = 0.3130$; $n = 4$).

Furthermore, the sensitivity of imageSURF as an analytical tool was validated in chapter 3 when it was shown to effectively differentiate between levels of GFAP in the uninjured hippocampus and cortex. It is known that astrocytes across different regions of the mouse brain are heterogeneous in regard to physiological, molecular, functional and morphological properties (708, 709). Under basal conditions, GFAP expression has been reported to be higher in the hippocampus than in the cortex (710), and likewise, a systematic review evaluating cell densities in the mouse brain revealed that the ratio of astrocytes to neurones was higher in the hippocampus than in the cortex (711). Our data resulting from the quantification of GFAP in the uninjured brain hemisphere using imageSURF supports these earlier studies, with a percentage area immunostaining in the hippocampus of 8.4% compared to 2.1% in the cortex.

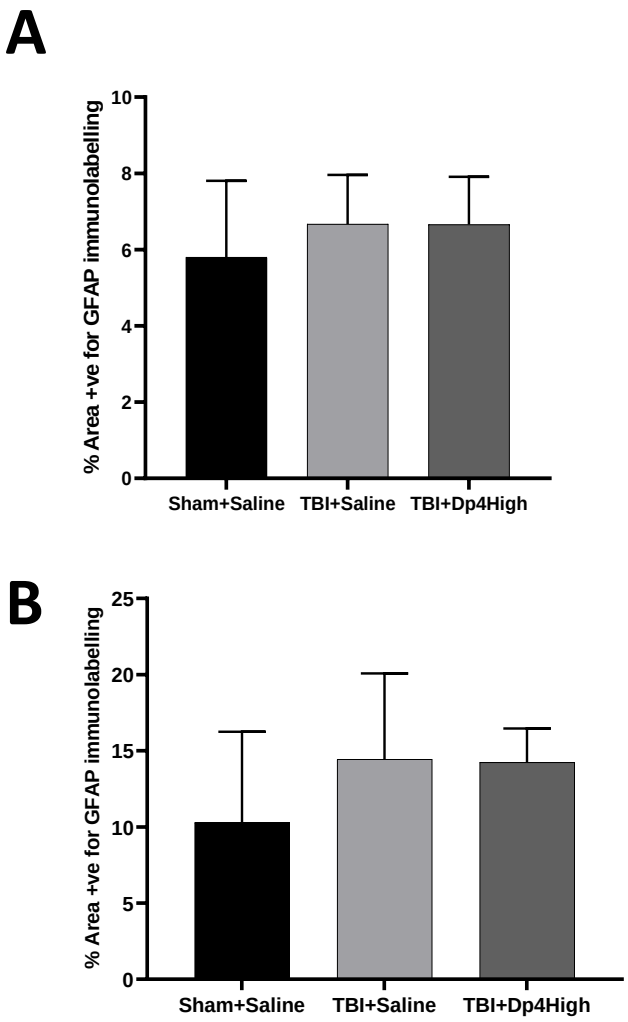

Figure A 1: Comparing between imageSURF and thresholding image quantification methods.

*ImageSURF **(A)**, an automated image segmentation tool and thresholding technique **(B)**, the most used method for quantifying immunofluorescent labelled images were used to assess GFAP immunostaining in the injured cortex at 3 days post-TBI. Overall, following both analyses, a similar pattern was observed across all experimental groups. The percentage area positive for GFAP staining was lower following imageSURF analysis compared to thresholding analysis and there was no change in GFAP positive cells across the groups (one-way ANOVA, P = 0.5720, P = 0.3130; n = 4). All data are expressed as mean ± standard deviation (SD).*

Appendix B: Percentage body weight loss across the experimental groups over a period of 4 weeks post-TBI (mean±SD)

Experimental group	D1	D2	D4	D7	D8	D14	D15	D21	D22	D28	D29
Sham+saline	1.0±2.7	1.6±3.5	5.2±2.3	5.5±3.3	5.5±2.8	5.5±2.8	5.5±3.3	4.7±3.5	5.7±2.6	4.7±2.7	5.5±2.8
TBI+saline	3.1±2.8	3.3±2.2	4.6±2.7	6.5±2.5	7.0±2.4	5.3±3.0	5.0±3.0	4.8±2.8	5.6±2.8	5.3±2.5	5.9±1.8
TBI+Enoxaparin	4.1±3.0	4.5±3.3	4.9±2.3	6.4±2.1	6.8±1.8	4.6±2.6	4.6±2.3	5.5±2.0	5.4±2.4	4.6±2.7	5.4±2.4
TBI+Dp4	3.1±1.9	4.2±2.4	6.5±2.5	5.8±2.4	6.7±2.4	6.0±3.1	6.0±3.5	4.7±3.0	4.8±3.6	4.0±3.9	4.9±3.2
TBI+Dp2	4.0±2.2	4.2±1.6	6.8±1.7	5.9±2.8	6.7±2.3	4.1±4.0	4.5±2.6	4.4±3.8	6.0±3.3	5.8±3.4	4.4±3.1
TBI+Dp2	3.3±2.0	5.7±1.7	7.7±3.0	6.9±2.9	6.6±3.3	6.8±4.3	6.7±5.3	5.9±3.6	6.3±3.6	5.9±3.5	5.9±3.6

Appendix C: PRISMA Checklist (630)

Title: A systematic review and meta-analysis on the therapeutic efficacy of heparin and Low Molecular Weight Heparins (LMWHs) following Traumatic Brain Injury (TBI) in animal studies.

Author list: *Mimieveshiofuo Aiyede, MRes; Xin Yi Lim, MSc; David Howells, PhD; Nuri Guven, PhD; Nicole Bye, PhD.*

Section and Topic	Item #	Checklist item	Location where item is reported
TITLE			
Title	1	Identify the report as a systematic review.	123
ABSTRACT			
Abstract	2	See the PRISMA 2020 for Abstracts checklist.	
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	126
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	128
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	129
Information sources	6	Specify all databases, registers, websites, organisations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	130
Search strategy	7	Present the full search strategies for all databases, registers and websites, including any filters and limits used.	130
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	130
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they worked independently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used in the process.	130
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (e.g. for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	130
	10b	List and define all other variables for which data were sought (e.g. participant and intervention characteristics, funding sources). Describe any assumptions made about any missing or unclear information.	131

Section and Topic	Item #	Checklist item	Location where item is reported
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each study and whether they worked independently, and if applicable, details of automation tools used in the process.	131
Effect measures	12	Specify for each outcome the effect measure(s) (e.g. risk ratio, mean difference) used in the synthesis or presentation of results.	132
Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g. tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5)).	132
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or data conversions.	132
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	132
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe the model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	132
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g. subgroup analysis, meta-regression).	132
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results.	N/A
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	N/A
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	133
RESULTS			
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studies included in the review, ideally using a flow diagram.	134
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded.	134
Study characteristics	17	Cite each included study and present its characteristics.	134
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	144
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimate and its precision (e.g. confidence/credible interval), ideally using structured tables or plots.	139
Results of syntheses	20a	For each synthesis, briefly summarise the characteristics and risk of bias among contributing studies.	137
	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision (e.g. confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	146
	20c	Present results of all investigations of possible causes of heterogeneity among study results.	146
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results.	147-148

Section and Topic	Item #	Checklist item	Location where item is reported
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	152-153
Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	146-152
DISCUSSION			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	154
	23b	Discuss any limitations of the evidence included in the review.	158
	23c	Discuss any limitations of the review processes used.	158
	23d	Discuss implications of the results for practice, policy, and future research.	159
OTHER INFORMATION			
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered.	129
	24b	Indicate where the review protocol can be accessed, or state that a protocol was not prepared.	129
	24c	Describe and explain any amendments to information provided at registration or in the protocol.	N/A
Support	25	Describe sources of financial or non-financial support for the review, and the role of the funders or sponsors in the review.	
Competing interests	26	Declare any competing interests of review authors.	
Availability of data, code and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted from included studies; data used for all analyses; analytic code; any other materials used in the review.	168

Appendix D: List of keywords for database search

	Concept 1	Concept 2	Concept 3
Key concepts	**Heparin/LMWHs**	**Traumatic Brain injury**	**Animal models**
Free text terms (Titles and abstracts)	Unfractionated heparin UFH Low molecular weight heparin LMWH Heparin Enoxaparin Dalteparin ODSH	Traumatic brain injury Experimental traumatic brain injury Head injury TBI Concussion Intracerebral haemorrhage Neuroinflammation Brain injury mild Traumatic Brain Injury mTBI severe Traumatic Brain Injury sTBI moderate TBI moderate Traumatic Brain Injury Controlled Cortical Impact injury CCI	Previously developed animals filter will be employed for this search (712).

		Weight drop Injury WDI Fluid Percussion Injury FPI Cerebral haemorrhage Cerebral hemorrhage Concussion Intracerebral haemorrhage Closed Head Injury Blast injury model	
Medline via Pubmed MeSH **(Titles and abstract)**	Heparin	Craniocerebral trauma	Previously developed animals filter will be employed for this search (712).
Web of Science: Key words **(Titles and Abstract)**	Heparin Low molecular weight heparin LMWH Enoxaparin Dalteparin	Traumatic Brain Injury Craniocerebral trauma Cerebral haemorrhage Concussion Intracerebral haemorrhage	Previously developed animals filter will be employed for this search (712).

		Closed Head Injury Blast injury model	

Appendix E: Search strategy of the electronic databases

Search	Database	Search terms
	Medline via Pubmed	
#1		(((((((Heparin[MeSH Terms]) OR (Heparin[Title/Abstract])) OR (UFH[Title/Abstract])) OR (LMWH[Title/Abstract])) OR (LMWHs[Title/Abstract])) OR (Enoxaparin[Title/Abstract])) OR (Dalteparin[Title/Abstract])) OR (ODSH[Title/Abstract])
#2		((((((((((((((((((((((((Craniocerebral trauma[MeSH Terms]) OR (Craniocerebral trauma[Title/Abstract])) OR (Craniocerebral traumas[Title/Abstract])) OR (Head trauma[Title/Abstract])) OR (Head traumas[Title/Abstract])) OR (Brain trauma[Title/Abstract])) OR (Brain traumas[Title/Abstract])) OR (Cranial Injury[Title/Abstract])) OR (Cranial injuries[Title/Abstract])) OR (Brain Injury[Title/Abstract])) OR (Brain injuries[Title/Abstract])) OR (Head Injury[Title/Abstract])) OR (Head injuries[Title/Abstract])) OR (Blast Injury[Title/Abstract])) OR (Blast injuries[Title/Abstract])) OR (Traumatic brain injury[Title/Abstract])) OR (TBI[Title/Abstract])) OR (Fluid Percussion Injury[Title/Abstract])) OR (FPI[Title/Abstract])) OR (Controlled Cortical Impact injury[Title/Abstract])) OR (CCI[Title/Abstract])) OR (Weight drop Injury[Title/Abstract])) OR (WDI[Title/Abstract])) OR (Concussion[Title/Abstract])) OR (Concussions[Title/Abstract])
#3		(((((((((Animal experimentation[MeSH Terms]) OR (invertebrates[Title/Abstract])) OR (models, animal[MeSH Terms])) OR (chordata[MeSH Terms])) OR

		(chordata, nonvertebrate[MeSH Terms])) OR (vertebrates[MeSH Terms])) OR (amphibians[MeSH Terms])) OR (birds[MeSH Terms])) OR (fishes[MeSH Terms])) OR (reptiles[MeSH Terms])) OR (mammals[MeSH Terms])) OR (primates[MeSH Terms])) OR (artiodactyla[MeSH Terms])) OR (Animals[MeSH Terms])) OR (carnivora[MeSH Terms])) OR (cetacea[MeSH Terms])) OR (chiroptera[MeSH Terms])) OR (elephants[MeSH Terms])) OR (hyraxes[MeSH Terms])) OR (insectivora[MeSH Terms])) OR (lagomorpha[MeSH Terms])) OR (marsupialia[MeSH Terms])) OR (monotremata[MeSH Terms])) OR (perissodactyla[MeSH Terms])) OR (rodentia[MeSH Terms])) OR (scandentia[MeSH Terms])) OR (sirenia[MeSH Terms])) OR (xenarthra[MeSH Terms])) OR (haplorhini[MeSH Terms])) OR (strepsirhini[MeSH Terms])) OR (platyrrhini[MeSH Terms])) OR (tarsii[MeSH Terms])) OR (catarrhini[MeSH Terms])) OR (cercopithecidae[MeSH Terms])) OR (hylobatidae[MeSH Terms])) OR (hominidae[MeSH Terms])) OR (gorilla gorilla[MeSH Terms])) OR (pan paniscus[MeSH Terms])) OR (pan troglodytes[MeSH Terms])) OR (pongo pygmaeus[MeSH Terms])) OR (animals[Title/Abstract])) OR (animal[Title/Abstract])) OR (mice[Title/Abstract])) OR (mus[Title/Abstract])) OR (mouse[Title/Abstract])) OR (murine[Title/Abstract])) OR (woodmouse[Title/Abstract])) OR (rats[Title/Abstract])) OR (rat[Title/Abstract])) OR (murinae[Title/Abstract])) OR (muridae[Title/Abstract])) OR (cottonrat[Title/Abstract])) OR (cottonrats[Title/Abstract])) OR (hamster[Title/Abstract])) OR (hamsters[Title/Abstract])) OR (cricetinae[Title/Abstract])) OR (rodentia[Title/Abstract])) OR (rodent[Title/Abstract])) OR (rodents[Title/Abstract])) OR (pigs[Title/Abstract])) OR (pig[Title/Abstract])) OR (swine[Title/Abstract])) OR (swines[Title/Abstract])) OR (piglets[Title/Abstract])) OR (piglet[Title/Abstract])) OR (boar[Title/Abstract])) OR (boars[Title/Abstract])) OR (sus scrofa[Title/Abstract])) OR (ferrets[Title/Abstract])) OR (ferret[Title/Abstract])) OR (polecat[Title/Abstract])) OR (polecats[Title/Abstract])) OR (mustela putorius[Title/Abstract])) OR (guinea pigs[Title/Abstract])) OR (guinea pig[Title/Abstract])) OR (cavia[Title/Abstract])) OR (callithrix[Title/Abstract])) OR (marmoset[Title/Abstract])) OR (marmosets[Title/Abstract])) OR (cebuella[Title/Abstract])) OR (hapale[Title/Abstract])) OR (octodon[Title/Abstract])) OR (chinchilla[Title/Abstract])) OR (chinchillas[Title/Abstract])) OR (gerbillinae[Title/Abstract])) OR (gerbil[Title/Abstract])) OR (gerbils[Title/Abstract])) OR (jird[Title/Abstract])) OR (jirds[Title/Abstract])) OR (merione[Title/Abstract])) OR (meriones[Title/Abstract])) OR (rabbits[Title/Abstract])) OR (rabbit[Title/Abstract])) OR (hares[Title/Abstract])) OR (hare[Title/Abstract])) OR (diptera[Title/Abstract])) OR (flies[Title/Abstract])) OR (fly[Title/Abstract])) OR (dipteral[Title/Abstract])) OR (drosphila[Title/Abstract])) OR (drosophilidae[Title/Abstract])) OR (cats[Title/Abstract])) OR (cat[Title/Abstract])) OR (carus[Title/Abstract])) OR (felis[Title/Abstract])) OR (nematoda[Title/Abstract])) OR (nematode[Title/Abstract])) OR (nematodes[Title/Abstract])) OR (sipunculida[Title/Abstract])) OR (Dogs[Title/Abstract])) OR (dog[Title/Abstract])) OR (canine[Title/Abstract])) OR (canines[Title/Abstract])) OR (canis[Title/Abstract])) OR (sheep[Title/Abstract])) OR (sheeps[Title/Abstract])) OR (mouflon[Title/Abstract]))

		OR (mouflons[Title/Abstract])) OR (ovis[Title/Abstract])) OR (goats[Title/Abstract])) OR (goat[Title/Abstract])) OR (capra[Title/Abstract])) OR (capras[Title/Abstract])) OR (rupicapra[Title/Abstract])) OR (chamois[Title/Abstract])) OR (haplorhini[Title/Abstract])) OR (monkey[Title/Abstract])) OR (monkeys[Title/Abstract])) OR (anthropoidea[Title/Abstract])) OR (anthropoids[Title/Abstract])) OR (saguinus[Title/Abstract])) OR (tamarin[Title/Abstract])) OR (tamarins[Title/Abstract])) OR (leontopithecus[Title/Abstract])) OR (hominidae[Title/Abstract])) OR (ape[Title/Abstract])) OR (apes[Title/Abstract])) OR (pan[Title/Abstract])) OR (paniscus[Title/Abstract])) OR (pan paniscus[Title/Abstract])) OR (bonobo[Title/Abstract])) OR (bonobos[Title/Abstract])) OR (troglodytes[Title/Abstract])) OR (pan troglodytes[Title/Abstract])) OR (gibbon[Title/Abstract])) OR (gibbons[Title/Abstract])) OR (siamang[Title/Abstract])) OR (siamangs[Title/Abstract])) OR (nomascus[Title/Abstract])) OR (symphalangus[Title/Abstract])) OR (chimpanzee[Title/Abstract])) OR (chimpanzees[Title/Abstract])) OR (prosimians[Title/Abstract])) OR (bush baby[Title/Abstract])) OR (prosimian[Title/Abstract])) OR (bush babies[Title/Abstract])) OR (galagos[Title/Abstract])) OR (pygmaeus[Title/Abstract])) OR (pongo pygmaeus[Title/Abstract])) OR (orangutans[Title/Abstract])) OR (pygmaeus[Title/Abstract])) OR (lemur[Title/Abstract])) OR (lemurs[Title/Abstract])) OR (lemuridae[Title/Abstract])) OR (horse[Title/Abstract])) OR (horses[Title/Abstract])) OR (equus[Title/Abstract])) OR (cow[Title/Abstract])) OR (calf[Title/Abstract])) OR (bull[Title/Abstract])) OR (chicken[Title/Abstract])) OR (chickens[Title/Abstract])) OR (gallus[Title/Abstract])) OR (quail[Title/Abstract])) OR (bird[Title/Abstract])) OR (birds[Title/Abstract])) OR (quails[Title/Abstract])) OR (poultry[Title/Abstract])) OR (poultries[Title/Abstract])) OR (fowl[Title/Abstract])) OR (fowls[Title/Abstract])) OR (reptile[Title/Abstract])) OR (reptilia[Title/Abstract])) OR (reptiles[Title/Abstract])) OR (snakes[Title/Abstract])) OR (snake[Title/Abstract])) OR (lizard[Title/Abstract])) OR (lizards[Title/Abstract])) OR (alligator[Title/Abstract])) OR (alligators[Title/Abstract])) OR (crocodile[Title/Abstract])) OR (crocodiles[Title/Abstract])) OR (turtle[Title/Abstract])) OR (turtles[Title/Abstract])) OR (amphibian[Title/Abstract])) OR (amphibians[Title/Abstract])) OR (amphibia[Title/Abstract])) OR (frog[Title/Abstract])) OR (frogs[Title/Abstract])) OR (bombina[Title/Abstract])) OR (salientia[Title/Abstract])) OR (toad[Title/Abstract])) OR (toads[Title/Abstract])) OR (epidalea calamita[Title/Abstract])) OR (salamander[Title/Abstract])) OR (salamanders[Title/Abstract])) OR (eel[Title/Abstract])) OR (eels[Title/Abstract])) OR (fish[Title/Abstract])) OR (fishes[Title/Abstract])) OR (pisces[Title/Abstract])) OR (catfish[Title/Abstract])) OR (catfishes[Title/Abstract])) OR (siluriformes[Title/Abstract])) OR (arius[Title/Abstract])) OR (heteropneustes[Title/Abstract])) OR (sheatfish[Title/Abstract])) OR (perch[Title/Abstract])) OR (perches[Title/Abstract])) OR (percidae[Title/Abstract])) OR (perca[Title/Abstract])) OR (trout[Title/Abstract])) OR (trouts[Title/Abstract])) OR (char[Title/Abstract])) OR (chars[Title/Abstract]))

		OR (salvelinus[Title/Abstract])) OR (fathead minnow[Title/Abstract])) OR (minnow[Title/Abstract])) OR (cyprinidae[Title/Abstract])) OR (carps[Title/Abstract])) OR (carp[Title/Abstract])) OR (zebrafish[Title/Abstract])) OR (zebrafishes[Title/Abstract])) OR (goldfish[Title/Abstract])) OR (goldfishes[Title/Abstract])) OR (guppy[Title/Abstract])) OR (guppies[Title/Abstract])) OR (chub[Title/Abstract])) OR (chubs[Title/Abstract])) OR (tinca[Title/Abstract])) OR (barbels[Title/Abstract])) OR (barbus[Title/Abstract])) OR (pimephales[Title/Abstract])) OR (promelas[Title/Abstract])) OR (poecilia reticulata[Title/Abstract])) OR (mullet[Title/Abstract])) OR (mullets[Title/Abstract])) OR (seahorse[Title/Abstract])) OR (seahorses[Title/Abstract])) OR (mugil curema[Title/Abstract])) OR (atlantic cod[Title/Abstract])) OR (shark[Title/Abstract])) OR (sharks[Title/Abstract])) OR (catshark[Title/Abstract])) OR (anguilla[Title/Abstract])) OR (salmonid[Title/Abstract])) OR (salmonids[Title/Abstract])) OR (whitefish[Title/Abstract])) OR (whitefishes[Title/Abstract])) OR (salmon[Title/Abstract])) OR (salmons[Title/Abstract])) OR (sole[Title/Abstract])) OR (solea[Title/Abstract])) OR (sea lamprey[Title/Abstract])) OR (lamprey[Title/Abstract])) OR (lampreys[Title/Abstract])) OR (pumpkinseed[Title/Abstract])) OR (sunfish[Title/Abstract])) OR (sunfishes[Title/Abstract])) OR (tilapia[Title/Abstract])) OR (tilapias[Title/Abstract])) OR (turbot[Title/Abstract])) OR (turbots[Title/Abstract])) OR (flatfish[Title/Abstract])) OR (flatfishes[Title/Abstract])) OR (sciuridae[Title/Abstract])) OR (squirrel[Title/Abstract])) OR (squirrels[Title/Abstract])) OR (chipmunk[Title/Abstract])) OR (chipmunks[Title/Abstract])) OR (suslik[Title/Abstract])) OR (susliks[Title/Abstract])) OR (vole[Title/Abstract])) OR (voles[Title/Abstract])) OR (lemming[Title/Abstract])) OR (lemmings[Title/Abstract])) OR (muskrat[Title/Abstract])) OR (muskrats[Title/Abstract])) OR (lemmus[Title/Abstract])) OR (otter[Title/Abstract])) OR (otters[Title/Abstract])) OR (marten[Title/Abstract])) OR (martens[Title/Abstract])) OR (martes[Title/Abstract])) OR (weasel[Title/Abstract])) OR (badger[Title/Abstract])) OR (badgers[Title/Abstract])) OR (ermine[Title/Abstract])) OR (mink[Title/Abstract])) OR (minks[Title/Abstract])) OR (sable[Title/Abstract])) OR (sables[Title/Abstract])) OR (gulo[Title/Abstract])) OR (gulos[Title/Abstract])) OR (wolverine[Title/Abstract])) OR (wolverines[Title/Abstract])) OR (mustela[Title/Abstract])) OR (llama[Title/Abstract])) OR (llamas[Title/Abstract])) OR (alpaca[Title/Abstract])) OR (alpacas[Title/Abstract])) OR (camelid[Title/Abstract])) OR (camelids[Title/Abstract])) OR (guanaco[Title/Abstract])) OR (guanacos[Title/Abstract])) OR (chiroptera[Title/Abstract])) OR (chiropteras[Title/Abstract])) OR (bat[Title/Abstract])) OR (bats[Title/Abstract])) OR (fox[Title/Abstract])) OR (foxes[Title/Abstract])) OR (iguana[Title/Abstract])) OR (iguanas[Title/Abstract])) OR (xenopus laevis[Title/Abstract])) OR (parakeet[Title/Abstract])) OR (parakeets[Title/Abstract])) OR (parrot[Title/Abstract])) OR (parrots[Title/Abstract])) OR (donkey[Title/Abstract])) OR (donkeys[Title/Abstract])) OR (mule[Title/Abstract])) OR (mules[Title/Abstract])) OR (zebra[Title/Abstract]))

		OR (zebras[Title/Abstract])) OR (shrew[Title/Abstract])) OR (shrews[Title/Abstract])) OR (bison[Title/Abstract])) OR (bisons[Title/Abstract])) OR (buffalo[Title/Abstract])) OR (buffaloes[Title/Abstract])) OR (deer[Title/Abstract])) OR (deers[Title/Abstract])) OR (bear[Title/Abstract])) OR (bears[Title/Abstract])) OR (panda[Title/Abstract])) OR (pandas[Title/Abstract])) OR (wild hog[Title/Abstract])) OR (wild boar[Title/Abstract])) OR (fitchew[Title/Abstract])) OR (fitch[Title/Abstract])) OR (beaver[Title/Abstract])) OR (beavers[Title/Abstract])) OR (jerboa[Title/Abstract])) OR (jerboas[Title/Abstract])) OR (capybara[Title/Abstract])) OR (capybaras[Title/Abstract])
#4		#1 AND #2 AND #3
#5		#4 NOT (patient[Title/Abstract])) NOT (patients[Title/Abstract])
	Web of Science	
#1		TS=(Heparin OR UFH OR LMWH OR LMWHs OR Enoxaparin OR Dalteparin OR ODSH)
#2		TS=("Craniocerebral trauma" OR "Craniocerebral traumas" OR "Head trauma" OR "Head traumas" OR "Brain trauma" OR "Brain traumas" OR "Cranial Injury" OR "Cranial injuries" OR "Brain Injury" OR "Brain injuries" OR "Head Injury" OR "Head injuries" OR "Blast Injury" OR "Blast injuries" OR "Traumatic brain injury" OR TBI OR "Fluid Percussion Injury" OR FPI OR "Controlled Cortical Impact injury" OR CCI OR "Weight drop Injury" WDI OR Concussion OR Concussions)

#3		TS=("animal experimentation" OR "models, animal" OR invertebrates OR Animals OR "animal population groups" OR chordata OR "chordata, nonvertebrate" OR vertebrates OR amphibians OR birds OR fishes OR reptiles OR mammals OR primates OR artiodactyla OR carnivora OR cetacea OR chiroptera OR elephants OR hyraxes OR insectivora OR lagomorpha OR marsupialia OR monotremata OR perissodactyla OR rodentia OR scandentia OR sirenia OR xenarthra OR haplorhini OR strepsirhini OR platyrrhini OR tarsii OR catarrhini OR cercopithecidae OR hylobatidae OR hominidae OR "gorilla gorilla" OR "pan paniscus" OR "pan troglodytes" OR "pongo pygmaeus" OR animal OR mice OR mus OR mouse OR murine OR woodmouse OR rats OR rat OR murinae OR Muridae OR cottonrat OR cottonrats OR hamster OR hamsters OR cricetinae OR rodentia OR rodent OR rodents OR pigs OR pig OR swine OR swines OR piglets OR piglet OR boar OR boars OR "sus scrofa" OR ferrets OR ferret OR polecat OR polecats OR "mustela putorius" OR "guinea pigs" OR "guinea pig" OR cavia OR Callithrix OR marmoset OR marmosets OR cebuella OR hapale OR octodon OR chinchilla OR chinchillas OR gerbillinae OR gerbil OR gerbils OR jird OR jirds OR merione OR meriones OR rabbits OR rabbit OR hares OR hare OR diptera OR flies OR fly OR dipteral OR drosphila OR drosophilidae OR cats OR cat OR carus OR felis OR nematoda OR nematode OR nematode OR nematodes OR sipunculida OR dogs OR dog OR canine OR canines OR canis OR sheep OR sheeps OR mouflon OR mouflons OR ovis OR goats OR goat OR capra OR capras OR rupicapra OR chamois OR haplorhini OR monkey OR monkeys OR Anthropoidea OR anthropoids OR saguinus OR tamarin OR tamarins OR leontopithecus OR Hominidae OR ape OR apes OR pan OR paniscus OR "pan paniscus" OR bonobo OR bonobos OR troglodytes OR "pan troglodytes" OR gibbon OR gibbons OR siamang OR siamangs OR nomascus OR symphalangus OR chimpanzee OR chimpanzees OR prosimians OR "bush baby" OR prosimian OR bush babies OR galagos OR galago OR pongidae OR gorilla OR gorillas OR pongo OR pygmaeus OR "pongo pygmaeus" OR orangutans OR pygmaeus OR lemur OR lemurs OR lemuridae OR horse OR horses OR pongo OR equus OR cow OR calf OR bull OR chicken OR chickens OR gallus OR quail OR bird OR birds OR quails OR poultry OR poultries OR fowl OR fowls OR reptile OR reptilia OR reptiles OR snakes OR snake OR lizard OR lizards OR alligator OR alligators OR crocodile OR crocodiles OR turtle OR turtles OR amphibian OR amphibians OR amphibia OR frog OR frogs OR bombina OR salientia OR toad OR toads OR "epidalea calamita" OR salamander OR salamanders OR eel OR eels OR fish OR fishes OR pisces OR catfish OR catfishes OR siluriformes OR arius OR heteropneustes OR sheatfish OR perch OR perches OR percidae OR perca OR trout OR trouts OR char OR chars OR Salvelinus OR "fathead minnow" OR minnow OR cyprinidae OR carps OR carp OR zebrafish OR zebrafishes OR goldfish OR goldfishes OR guppy OR guppies OR chub OR chubs OR tinca OR barbels OR barbus OR pimephales OR promelas OR "poecilia reticulata" OR mullet OR mullets OR seahorse OR seahorses OR mugil curema OR atlantic cod OR shark OR sharks OR catshark OR Anguilla OR salmonid OR salmonids OR whitefish OR whitefishes OR salmon OR salmons OR sole OR solea OR "sea lamprey" OR lamprey OR lampreys OR pumpkinseed OR sunfish OR sunfishes OR tilapia OR tilapias OR turbot OR turbots OR flatfish OR flatfishes OR Sciuridae OR squirrel OR squirrels OR chipmunk OR chipmunks OR suslik OR susliks OR vole OR voles OR lemming OR lemmings OR muskrat OR muskrats OR lemmus OR otter OR otters OR marten OR martens OR martes OR weasel OR badger OR badgers OR ermine OR mink OR minks OR sable OR sables OR gulo OR gulos OR wolverine OR wolverines OR minks OR Mustela OR llama OR llamas OR alpaca OR alpacas OR camelid OR camelids OR guanaco OR guanacos OR chiroptera OR chiropteras OR bat OR bats OR fox OR foxes OR iguana OR iguanas OR “xenopus laevis” OR parakeet OR parakeets OR parrot OR parrots

		OR donkey OR donkeys OR mule OR mules OR zebra OR zebras OR shrew OR shrews OR bison OR bisons OR buffalo OR buffaloes OR deer OR deers OR bear OR bears OR panda OR pandas OR "wild hog" OR "wild boar" OR fitchew OR fitch OR beaver OR beavers OR jerboa OR jerboas OR capybara OR capybaras)
#4		#1 AND #2 AND #3

Appendix F: SYRCLE's risk of bias tool for animal studies (633)

Item	Type of bias	Domain	Description of domain	Review authors judgment
1	Selection bias	Sequence generation	Describe the methods used, if any, to generate the allocation sequence in sufficient detail to allow an assessment whether it should produce comparable groups.	Was the allocation sequence adequately generated and applied? (*)
2	Selection bias	Baseline characteristics	Describe all the possible prognostic factors or animal characteristics, if any, that are compared in order to judge whether or not intervention and control groups were similar at the start of the experiment.	Were the groups similar at baseline or were they adjusted for confounders in the analysis?
3	Selection bias	Allocation concealment	Describe the method used to conceal the allocation sequence in sufficient detail to determine whether intervention allocations could have been foreseen before or during enrolment.	Was the allocation adequately concealed? (*)
4	Performance bias	Random housing	Describe all measures used, if any, to house the animals randomly within the animal room.	Were the animals randomly housed during the experiment?
5	Performance bias	Blinding	Describe all measures used, if any, to blind trial caregivers and researchers from knowing which intervention each animal received. Provide any information relating to whether the intended blinding was effective.	Were the caregivers and/or investigators blinded from knowledge which intervention each animal received during the experiment?
6	Detection bias	Random outcome assessment	Describe whether or not animals were selected at random for outcome assessment, and which methods to select the animals, if any, were used.	Were animals selected at random for outcome assessment?
7	Detection bias	Blinding	Describe all measures used, if any, to blind outcome assessors from knowing which intervention each animal received. Provide any information relating to whether the intended blinding was effective.	Was the outcome assessor blinded?
8	Attrition bias	Incomplete outcome data	Describe the completeness of outcome data for each main outcome, including attrition and exclusions from the analysis. State whether attrition and exclusions were reported, the numbers in each intervention group (compared with total randomized animals), reasons for attrition or exclusions, and any re-inclusions in analyses for the review.	Were incomplete outcome data adequately addressed? (*)
9	Reporting bias	Selective outcome reporting	State how selective outcome reporting was examined and what was found.	Are reports of the study free of selective outcome reporting? (*)
10	Other	Other sources of bias	State any important concerns about bias not covered by other domains in the tool.	Was the study apparently free of other problems that could result in high risk of bias? (*)

Appendix G: Quality assessment of studies using a modified checklist from the Collaborative Approach to Meta-Analysis and Review of Animal Data from Experimental Studies (CAMARADES) (634)

First author, Year	Peer review publication (via Ulrichs Web)	Random group allocation	Blinded induction of injury	Blinded assessment of outcome measures	Use of an alternative anaesthetics/analgesic agent without marked neuroprotective activity (ketamine)	Statement of compliance with animal welfare regulatory requirements	Statement of potential conflicts of interest	Use of appropriate animal models	Calculation of sample size	Explanation of excluded animals	Score	Quality
Dhir, 2018 (639)	1	1	0	0	1	0	1	1	0	0	5	Fair
Hayashi, 2004 (640)	1	0	0	0	1	1	0	1	0	0	4	Fair
Kerr, 2020 (514)	1	1	0	0	0	1	1	1	1	0	6	Good
Keskin, 2017 (366)	1	1	0	0	0	1	1	1	0	0	5	Fair
Li, 2015 (388)	1	1	0	0	0	1	0	1	0	0	4	Fair
Nagata, 2017 (396)	1	1	0	0	0	1	1	1	1	0	6	Good

Nagata, 2016 (365)	1	1	0	0	0	1	0	1	0	0	4	Fair
Nagata, 2018 (553)	1	1	0	0	0	1	1	1	0	0	5	Fair
Sen, 2011 (387)	1	1	0	0	0	1	0	1	0	0	4	Fair
Wahl, 2000 (386)	1	0	0	0	1	0	0	1	0	1	4	Fair
Župan, 2011 (390)	1	1	0	0	1	1	1	1	0	1	7	Good

Appendix H: Study characteristics of all outcome measures extracted.

Outcomes measure	No of study	Enoxaparin	Heparin	ODSH	Heparin oligosaccharides	#separate authors	No. of overall comparisons	Meta-analysis
Brain edema(365, 386, 388, 396)	5	2	2	1	NA	3	27	Yes
Leukocyte rolling (365, 388, 553)	3	1	1	1	NA	2	5	No
Microvascular permeability (365, 388, 553)	3	1	1	1	NA	2	5	No
Oxidative stress (390)	1	1	NA	NA	NA	1	5	No
Body weight loss (365, 388, 396)	3	1	2	NA	NA	2	14	No
Inflammation (387)	1	1	NA	NA	NA	1	1	No
Glial scar formation (640)	1	NA	NA	NA	1	1	3	No
Haemorrhage (387, 388, 639)	3	3	NA	NA	NA	3	4	No
IL-1β (514)	1	1	NA	NA	NA	1	2	No

Neurologic function (365, 386, 388, 396, 553)	5	2	2	1	NA	3	26	Yes
Brain infarct volume (366, 386)	2	2	NA	NA	NA	2	4	No
Apoptosis (366, 387, 390)	3	3	NA	NA	NA	3	5	No
Caspase – 1 (514)	1	1	NA	NA	NA	1	2	No
Total anti-oxidant status (TAS) (366)	1	1	NA	NA	NA	1	2	No
Total oxidant status (TOS) (366)	1	1	NA	NA	NA	1	2	No
PMN sequestration (396)	1	NA	1	NA	NA	1	2	No
Haemoglobin levels (396)	1	NA	1	NA	NA	1	2	No
Leukocyte adhesion (365, 553)	2	NA	2	NA	NA	1	6	No
Neurodegeneration (390)	1	1	NA	NA	NA	1	3	No
Motor function (553)	1	NA	NA	1	NA	1	8	No
Lesion surfaces (386)	1	1	NA	NA	NA	1	28	No
Pro IL-1β (390)	1	1	NA	NA	NA	1	1	No

COX-2 expression (390)	1	1	NA	NA	NA	1	1	No
Reactive gliosis (390)	1	1	NA	NA	NA	1	3	No
Cognitive function (386)	1	1	NA	NA	NA	1	10	No
Learning (553)	1	NA	NA	1	NA	1	2	No

Appendix I: Ethics Approvals

Appendix I1: Ethics Approval for Dp4 studies

UNIVERSITY of TASMANIA

Animal Ethics Committee
ETHICS APPROVAL PERMIT

Office of Research Services
Phone : 03 62267283
Fax: 03 62267148
animal.ethics@utas.edu.au

To: Dr Nicole Bye

From: Jude Vienna-Hallam

Date: 08 February 2017

Project: A0016233 - Investigating the therapeutic efficacy of Dp4 following experimental traumatic brain injury

Approved on: 08 February 2017

Approval expires: 08 February 2020

1st Annual Report due: 08 February 2018

Please read this permit carefully as **approval may be withdrawn** for non-compliance with the conditions stated below.

The Animal Ethics Committee has approved the above project and a copy of the initial application document is attached. The approval is subject to the review and AEC approval of an annual report which is due before the approval anniversary. **Please note the due date in your diary.**

As the Responsible Investigator, you MUST ensure that:

1. All aspects of the work conform to the requirements of the current edition of the *Australian code of practice for the care and use of animals for scientific purposes* 8th edition 2013

2. The project is conducted in accordance with the provisions of the Tasmanian Veterinary Surgeons Act 1987 and Veterinary Surgeons Regulations 2012. If the project involves a veterinary service or other animal service, it is **your responsibility** to contact the University Veterinarian to discuss the legal requirements of competency assessment.

3. The University Veterinarian and the Animal Ethics Committee are promptly notified of any unexpected event which was not considered in the initial application and impacts on the welfare of any animal directly or indirectly involved in the project.

4. You contact the University Veterinarian to advise when and where your experiments will be conducted. Sufficient notice needs to be given so that an inspection can be easily arranged.

5. In the event of any unexpected death, you contact the University Veterinarian to perform an autopsy.

6. A full record is maintained of all animals used in this project. If at any stage you anticipate the need to use additional animals this must be communicated to the committee before use. Using additional animals without AEC approval is a breach of your ethics permit.

7. That all investigators attend Ethics training sessions every three years. Contact the Executive Officer Animal Ethics for the next available session.

The project is approved for a maximum of 3 years. If the project is to continue past the expiry date, a new initial application will need to be submitted.

If the investigation necessitates a Parks & Wildlife permit or other permits, you are required to send copies to animal.ethics@utas.edu.au before commencing work.

Ethics Officer
Animal Ethics Committee

Appendix I2: Ethics approval for Dp2 study

Research Integrity and Ethics Unit

Animal Ethics Committee - Ethics Approval Permit

To: Nicole Bye

Date: 26/03/2020

Project: 18665

Approved On: 24/3/2020

Approval Expires: 24/03/2023

1st Annual Report Due:24/03/2021

Please read this permit carefully as **approval may be withdrawn for non-compliance** with the conditions stated.

The Animal Ethics Committee has approved the above project. This approval is subject to the review and AEC approval of an annual report. **Please note the due report date in your calendar.**

As the Responsible Investigator, you *must* ensure:

1. All aspects of the work conform to the requirements of the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes 8th Edition 2013*
2. The project is conducted in accordance with the provisions of the Tasmanian Veterinary Surgeons Act 1987 and the Veterinary Surgeons Regulations 2012. If the project involves a veterinary service or other animal service, it is **your responsibility** to contact the University Veterinarian to discuss the legal requirements of competency assessment.
3. It is the responsibility of institutions and researchers to be aware of and conduct research in accordance with both general and specific legal requirements, wherever relevant.
4. The University Veterinarian and the Animal Ethics Committee are promptly notified of any unexpected event which was not considered in the initial application and impacts on the welfare of any animal directly or indirectly involved in the project.
5. You contact the University Veterinarian to advise when and where your experiments will be conducted. Sufficient notice needs to be given so that an inspection can be easily arranged.
6. In the event of any unexpected death, you contact the University Veterinarian to perform an autopsy.
7. A full record is maintained of all animals used in this project. If at any stage you anticipate the need to use additional animals this must be communicated to the committee before use. Using additional animals without AEC approval is a breach of your ethics permit.
8. That all investigators complete the MyLo Animal Ethics Online Training module every three years.

Project approval is granted for <u>three</u> years.

If the project necessitates a Parks & Wildlife permit, or other permits, you are required to send copies to the Animal Ethics Team before commencing work.

University of Tasmania - Animal Ethics Committee

Project ID:	18665
Project Title:	Investigating the therapeutic efficacy of Dp2 and Dp4 following experimental traumatic brain injury (A0018665)
Responsible Investigator:	Nicole Bye
Person Responsible for day-to-day care:	
Emergency Contact:	