ACTIVATION OF HEME OXYGENASE-2 TO IMPROVE OUTCOME AFTER TRAUMATIC BRAIN INJURY

by

WALLACE LEE

A thesis submitted to the Graduate Program in Pharmacology and Toxicology in the Department of Biomedical and Molecular Sciences in conformity with the requirements for the degree of Master of Science

Queen’s University

Kingston, Ontario, Canada

June 2014

Copyright © Wallace Lee, 2014
Abstract

Traumatic brain injury (TBI) is an injury of the brain most often caused by blunt force trauma to the head and typically characterized by an increase in reactive oxygen species (ROS), inflammation, and hemorrhaging. Heme oxygenase (HO) catalyzes the breakdown of heme into carbon monoxide (CO), biliverdin which is further reduced to bilirubin, and ferrous iron. There are two active isoforms: HO-1 which is inducible and found predominantly in liver and spleen tissue; and HO-2 which is constitutive and found predominantly in the brain and testis. The metabolites of heme possess cytoprotective properties that can limit damage resulting from TBI. Our laboratory has found a selective HO-2 activator known as menadione (MD) that has been found to increase HO-2 activity by 4-fold while not affecting HO-1 in vitro. Given the higher amounts of HO-2 found in the brain and the cytoprotective properties of heme metabolites, we postulate that activation of HO-2 using menadione would mitigate further damage after TBI. The rat controlled cortical impact (CCI) model was used to simulate TBI with spontaneous locomotor activity (SLA), spontaneous alternation behaviour (SAB), and beam balance (BB) as the behavioural tasks to assess cognitive and motor function. A dose-response study (25, 50, 100, 200 µmol/kg) was performed to ascertain the effect of MD treatment on injured animals comparing to uninjured controls and injured animals treated with the vehicle (saline). We found that BB performance improved to control levels after MD treatment at 25 µmol/kg and 50 µmol/kg whereas animals treated with saline did not improve. SLA and SAB performance did not improve after treatment with MD. The findings suggest that HO-2 activation may be a viable method in mitigating further injury after TBI.
Acknowledgements

Completing a masters is...difficult. Yes, that is definitely the word I would use to describe it. Difficult with a side order of tumultuous, sprinkle in a few spoonfuls of exhaustion and you have a masters! I kid, I kid, there are many positive aspects to taking on a project of this size, the discovery, the fact that you will be the sole possessor of one piece of knowledge (for the time being), the wonderful people you meet, and the growth you experience. But don’t get me wrong, there are many challenges, the ones you foresee and plan for, and also the ones that come running out of left field and kick you repeatedly while you are down and calling for help.

Luckily for me, when in trouble I always had people around me who would offer assistance or tell me to keep going towards the light (the light here being a metaphor for project completion). I would like to thank my supervisor, Dr. Kanji Nakatsu for offering me an opportunity to study an area that I am very passionate about, and for being an inspiration in personal fitness. Also, I would like to thank Drs. Dragic Vukomanovic, Mona Rahman, Andrew Winterborn, James Brien, Tom Massey, and Yohan D’Souza, Brian McLaughlin, and Maaike Hum. Whenever I had doubts, someone would always offer to push those doubts aside, whether it be Kanji and floor hockey, Dragie and his poems, Mona and her adventures with her sons, Yohan and his personal experiences, Brian and baseball, or Maaike and her sarcastic and upbeat nature, someone was always there. Lastly, I would like to thank my family for providing unwavering support and patience as I figure out what I would like to do with my life.
Table of Contents

Abstract.......................................................................................................................... ii  
Acknowledgement.......................................................................................................... iii  
Table of Contents .......................................................................................................... iv  
List of Figures................................................................................................................ vii  
List of Tables ................................................................................................................ x  
List of Abbreviations ..................................................................................................... xi  

Chapter 1 – Introduction .............................................................................................. 1  
A.1. Traumatic Brain Injury .......................................................................................... 1  
A.2. Heme Oxygenase and Cytoprotection .................................................................. 11  
A.3. Traumatic Brain Injury in Relation to Heme Oxygenase ....................................... 15  
A.4. Selecting the Models and Approaches for TBI ...................................................... 25  
A.5. Hypothesis and Objectives ..................................................................................... 31  

Chapter 2 – Materials and Methods .......................................................................... 32  
B.1. Animals ................................................................................................................... 32  
B.2. In vitro Experiments ............................................................................................... 32  
   B.2.1. Microsomal Fraction Preparation .................................................................... 32  
   B.2.2. Carbon Monoxide Assay for HO Activity ...................................................... 35  
B.3. In vivo Experiments ................................................................................................. 36  
   B.3.1. Stereotaxic Surgery ......................................................................................... 36  
   B.3.2. Drug Preparation and Treatments .................................................................... 39  
   B.3.3. Experimental Groups ...................................................................................... 40
B.3.4. Behavioural Studies

B.4. Statistical Analysis

Chapter 3 – Results

C.1. In vitro Experiments

C.1.1. Menadione Selectivity for HO-2 Activation

C.2. In vivo Experiments

C.2.1. Establishment of TBI Model: Craniectomy

C.2.1.1. Craniectomy and Animal Weight

C.2.1.2. Craniectomy and SLA Performance

C.2.1.3. Craniectomy and SAB Performance

C.2.1.4. Craniectomy and BB Performance

C.2.2. Establishment of TBI Model: CCI Speed and Depth

C.2.2.1. Optimization: SLA Performance

C.2.2.2. Optimization: SAB Performance

C.2.2.3. Optimization: BB Performance

C.2.3. Menadione Effects in Absence of TBI

C.2.3.1. SLA Performance

C.2.3.2. SAB Performance

C.2.3.3. BB Performance

C.2.4. MD Dose-Response Study

C.2.4.1. Dose-Response Overview: SLA

C.2.4.2. Dose-Response Overview: SAB

C.2.4.3. Dose-Response Overview: BB
Chapter 4 – Discussion ...............................................................................................................71

D.1. HO-2 Activation by MD ..................................................................................................71
D.2. Cranietomy and MD in Absence of Injury ....................................................................72
D.3. Validity of the TBI Model ..............................................................................................72
D.4. Behavioural Tasks .........................................................................................................74
D.5. MD Dose-Response ......................................................................................................76

Chapter 5 – Conclusion .......................................................................................................81

References ...........................................................................................................................82
List of Figures

Figure 1: Traumatic brain injury reactive oxygen species pathway ........................................8
Figure 2: Traumatic brain injury inflammation pathway .........................................................9
Figure 3: Heme catalysis by heme oxygenase pathway ...........................................................12
Figure 4: Various functions of heme metabolites .................................................................12
Figure 5: Anti-inflammatory functions of heme metabolites and HO-2 activity .....................20
Figure 6: Comparison of heme and zinc protoporphyrin structures .....................................20
Figure 7: Structure of menadione .........................................................................................22
Figure 8: Comparison of vitamin K family ............................................................................22
Figure 9: Comparison of menadione and menadione sodium bisulphite structures ..............24
Figure 10: Traumatic brain injury induction methods ............................................................28
Figure 11: Picture of controlled cortical impact setup .........................................................28
Figure 12: Diagram of craniectomy with landmarks ..............................................................38
Figure 13: Picture of spontaneous locomotor activity open-field container .........................43
Figure 14: Picture of Y-maze ...............................................................................................43
Figure 15: Effect of menadione on HO-1 and HO-2 activity via CO production ....................47
Figure 16A: Effect of craniectomy on animal weight (absolute) ........................................50
Figure 16B: Effect of craniectomy on animal weight (normalized to pre-injury) .................50
Figure 17A: Effect of craniectomy on spontaneous locomotor activity performance (absolute) ....51
Figure 17B: Effect of craniectomy on spontaneous locomotor activity performance (normalized to pre-injury) .................................................................51
Figure 18A: Effect of craniectomy on spontaneous alternation behaviour performance (absolute) ........................................................................................................52
Figure 18B: Effect of craniectomy on spontaneous alternation behaviour performance (normalized to pre-injury) ........................................................................52
Figure 19A: Effect of craniectomy on beam balance performance (absolute) ........................................53
Figure 19B: Effect of craniectomy on beam balance performance (normalized to pre-injury) ....53
Figure 20A: CCI optimization – spontaneous locomotor activity (absolute) .................................56
Figure 20B: CCI optimization – spontaneous locomotor activity (normalized to pre-injury) .....56
Figure 21A: CCI optimization – spontaneous alternation behaviour (absolute) ...........................57
Figure 21B: CCI optimization – spontaneous alternation behaviour (normalized to pre-injury) .57
Figure 22A: CCI optimization – beam balance (absolute) ..........................................................58
Figure 22B: CCI optimization – beam balance (normalized to pre-injury) .....................................58
Figure 23A: Effect of menadione in absence of injury – spontaneous locomotor activity (absolute) .................................................................................................................................................60
Figure 23B: Effect of menadione in absence of injury – spontaneous locomotor activity (normalized to pre-injury) .................................................................................................................................................60
Figure 24A: Effect of menadione in absence of injury – spontaneous alternation behaviour (absolute) ....................................................................................................................................................61
Figure 24B: Effect of menadione in absence of injury – spontaneous alternation behaviour (normalized to pre-injury) ....................................................................................................................................................61
Figure 25A: Effect of menadione in absence of injury – beam balance (absolute) .................62
Figure 25B: Effect of menadione in absence of injury – beam balance (normalized to pre-injury) .................................................................................................................................62
Figure 26A: Effect of various doses of menadione on spontaneous locomotor activity performance post-injury (absolute) ........................................................................................................66
Figure 26B: Effect of various doses of menadione on spontaneous locomotor activity performance post-injury (normalized to pre-injury) ........................................................................................................66
Figure 27A: Effect of various doses of menadione on spontaneous alternation behaviour performance post-injury (absolute) ........................................................................................................67
Figure 27B: Effect of various doses of menadione on spontaneous alternation behaviour performance post-injury (normalized to pre-injury) ........................................................................................................67
Figure 28A: Effect of various doses of menadione on beam balance performance post-injury (absolute)........................................................................................................................................68

Figure 28B: Effect of various doses of menadione on beam balance performance post-injury (normalized to pre-injury).......................................................................................................................................68

Figure 29A: Effect of 25 µmol/kg of menadione on beam balance performance (normalized to pre-injury) ................................................................................................................................................69

Figure 29B: Comparison of various doses of menadione on beam balance performance on Day 8 (normalized to pre-injury)........................................................................................................................................69

Figure 30A: Effect of 25 µmol/kg of menadione on spontaneous locomotor activity performance (normalized to pre-injury).........................................................................................................................................70

Figure 30B: Effect of 25 µmol/kg of menadione on spontaneous alternation behaviour performance (normalized to pre-injury)........................................................................................................................................70
List of Tables

Table 1: Head injury hospital admissions in Canada organized by age groups...............................4
Table 2: Non-exhaustive list of current metalloporphyrin HO-2 inhibitors .................................19
Table 3: Most common animal models for traumatic brain injury ..............................................26
Table 4: Behavioural tasks and faculties tested ............................................................................30
Table 5: Biuret method conditions for determination of protein concentration .............................34
Table 6: Menadione treatment and behavioural task regimen .........................................................41
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>BB</td>
<td>beam balance</td>
</tr>
<tr>
<td>BINT</td>
<td>blast-induced neurotrauma</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BVR</td>
<td>biliverdin reductase</td>
</tr>
<tr>
<td>CCI</td>
<td>controlled cortical impact</td>
</tr>
<tr>
<td>cGMP</td>
<td>guanosine 3',5'-cyclic monophosphate</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CO</td>
<td>carbon monoxide</td>
</tr>
<tr>
<td>DAI</td>
<td>diffuse axonal injury</td>
</tr>
<tr>
<td>dH₂O</td>
<td>distilled water</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>EAA</td>
<td>excitatory amino acid</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>H₂O</td>
<td>water</td>
</tr>
<tr>
<td>HO</td>
<td>heme oxygenase</td>
</tr>
<tr>
<td>HO-1</td>
<td>heme oxygenase-1</td>
</tr>
<tr>
<td>HO-2</td>
<td>heme oxygenase-2</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram</td>
</tr>
<tr>
<td>LFP</td>
<td>lateral fluid percussion</td>
</tr>
<tr>
<td>m</td>
<td>milli</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>m/s</td>
<td>meters per second</td>
</tr>
<tr>
<td>MD</td>
<td>menadione</td>
</tr>
<tr>
<td>MHA</td>
<td>methemalbumin</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>MP</td>
<td>metalloporphyrin</td>
</tr>
<tr>
<td>MSB</td>
<td>menadione sodium bisulphite</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>MWM</td>
<td>Morris water maze</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate (reduced)</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RR</td>
<td>rotarod</td>
</tr>
<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>SAB</td>
<td>spontaneous alternation behaviour</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>sGC</td>
<td>soluble guanylate cyclase</td>
</tr>
<tr>
<td>SLA</td>
<td>spontaneous locomotor activity</td>
</tr>
<tr>
<td>TBI</td>
<td>traumatic brain injury</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumour necrosis factor-α</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume</td>
</tr>
<tr>
<td>µ</td>
<td>micro</td>
</tr>
</tbody>
</table>
A.1. Traumatic Brain Injury

Traumatic brain injury (TBI) is a rather peculiar ailment in that it finds itself in a unique position, overlooked by the general public, yet it affects a very large population. Unlike other maladies, TBI is most often discussed in the context of sports, yet sports are estimated to only account for 3-10% of all reported cases (Echemendia et al, 2001). The rudimentary cause of TBI is blunt force trauma to the head, resulting in damage to the brain (Zink, 1996). This is an overly simplistic view that is most applicable to the sporting world and also in TBI cases resulting from recreational activities, but it is important to keep in mind that TBI can also result from a multitude of events, not restricted only to blunt force trauma. TBI can be separated into two broad categories: open-skull TBI where the skull is penetrated by an object, and closed-skull TBI where the skull is not penetrated (Saatman et al, 2008). Open-skull TBI, the less common form of TBI, can also be caused by a multitude of events, but the one consistent factor is that the skull is penetrated by a foreign object (Saatman et al, 2008). The foreign objects can vary greatly, ranging from household items to bullets and shrapnel, but due to the mortality rates of the resulting injury, treatments for open-skull TBI are not well studied (Griffin et al, 2012). The much more common closed-skull TBI may be caused by blunt force trauma to the head and also by rapid acceleration and deceleration as in the case of vehicular accidents, which can also result in rotational injury whereby the brainstem is damaged (Faul et al, 2010). TBI is also prevalent in military settings, often referred to as Blast-Induced Neurotrauma (BINT), whereby shockwaves from explosions result in tissue damage including the brain (Hoge et al, 2008). Soldiers who experience BINT often get discharged with dizziness, amnesia, and nausea, ailments that fall
under the broad spectrum of post-concussion syndrome (Cernak et al, 2010). Moving beyond the military component of TBI, a similar symptomology affects the civilian population. Due to the nature of TBI symptoms, cases are grossly underreported and even when reported, treatment options are limited to relieving symptoms rather than directly treating the injury. Until recently, the topic of TBI has been a socially unacceptable topic even in the professional sporting world as it is not necessarily an injury with a physical manifestation of symptoms. Within the past decade, there has been an increasing number of athletes falling victim to the effects of years of mistreated or even untreated TBI (Gilchrist et al, 2007). Coinciding with these increased incidences, despite increased safety regulations, were multi-million dollar lawsuits filed by former professional athletes against their respective major sports leagues. Beyond the economic risk of TBI, player health is a major problem as many former athletes who have died at young ages with substantial brain damage revealed during autopsy. A study by Omalu et al (2005) found that chronic mild TBI from football is a contributing factor in post-traumatic encephalopathy, resulting in various symptoms including memory loss, inattention, and executive dysfunction. As previously mentioned, sporting injuries only account for 3-10% of all reported TBI cases. The vast majority of reported TBI cases are a result of common mishaps, vehicular accidents, and military events (Faul et al, 2010; Okie, 2005) indicating that it can affect not only a large population, but also affects extremely diverse groups. A recent Canadian study found that falls were the leading cause of TBI, accounting for 45% of TBI hospital admissions in Canada with vehicular accidents being the second leading cause (CIHI, 2006).

Although TBI is indiscriminant of age and gender, the ratio of males to females affected ranges from 3:2 up to an astonishing 7:3, while affecting individuals belonging to the age groups of 0-19, and 60+ at a slightly higher percentage as opposed to the age groups of 20-39 and 50-59
(Table 1; Faul et al, 2010; CIHI, 2006). The slight age variance can be partially explained by increased supervision and caution for younger children and older individuals whereas most cases of TBI go unreported.
Table 1. Head injury hospital admissions in Canada organized by age groups. 2003-2004. Adapted from CIHI (2006), LOS represents length of stay.

<table>
<thead>
<tr>
<th></th>
<th>0-19 years</th>
<th>20-39 years</th>
<th>40-59 years</th>
<th>60+ years</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number admitted</td>
<td>4,966</td>
<td>3,637</td>
<td>3,306</td>
<td>4,902</td>
<td>16,811</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3,318 (67%)</td>
<td>2,870 (79%)</td>
<td>2,403 (73%)</td>
<td>2,772 (57%)</td>
<td>11,363 (68%)</td>
<td></td>
</tr>
<tr>
<td>Average age (years)</td>
<td>10</td>
<td>29</td>
<td>49</td>
<td>76</td>
<td>41</td>
</tr>
<tr>
<td>Average LOS in hospital (days)</td>
<td>5</td>
<td>11</td>
<td>13</td>
<td>15</td>
<td>11</td>
</tr>
<tr>
<td>Deaths in hospital</td>
<td>120 (9%)</td>
<td>217 (16%)</td>
<td>222 (16%)</td>
<td>809 (59%)</td>
<td>1,368</td>
</tr>
</tbody>
</table>
In the USA, Zink (1996) found TBI to be the leading cause of death for individuals under the age of 45, surpassing vehicular accidents, assaults, and various diseases. This is startling considering that the under-45 working population constitutes 55-60% of the entire labour force (Lee et al, 2008), not to mention the under-16 future working population. In 2003, TBI cases resulted in over 1.9 million days in hospital in Canada alone (CIHI, 2006). TBI is the rare case that affects an entire population while also posing a great financial risk and possessing the ability to disrupt an otherwise healthy economy.

Patients suffering from TBI can display anywhere from no outward symptoms, to coma and death depending on the severity of the injury (Imajo et al, 1984). That being said, the pathophysiology of TBI also varies amongst patients but there are several features that are common to the majority of patients. After the initial insult, the brain undergoes several changes. The most common is the appearance of a cerebral contusion surrounding the area of impact, caused by the rupturing of small blood vessels in the cerebral cortex (Okubo et al, 2013). Cerebral contusions can heal depending on severity and prolonged contusions can lead to neuronal death. Diffuse axonal injury (DAI) is another prominent sign of TBI, often caused by shearing forces from rapid deceleration, most commonly associated with motor vehicle accidents (Hilton et al, 1995). The result of DAI is often a tearing of neural tracts resulting in disrupted membrane permeability and action potential propagation (Johnson et al, 2012). DAI carries with it lasting effects as the damage caused is often irreparable due to the nature of the tissue, and the fact that it is often not at the site of the initial insult, contrasting cerebral contusions (Adams et al, 1989). In addition to DAI, another notable characteristic of TBI is hemorrhaging, bleeding caused by the rupture of larger vessels, and also edema, an increase in fluid permeation to allow immune cells to reach injured area. Hemorrhaging is a troubling issue with blood loss starving
neurons of needed nutrients, resulting in cell death. A second more sinister negative effect of hemorrhaging is the increase in reactive oxygen species (ROS). The TBI ROS pathway is a positive feedback loop (Figure 1). After TBI, there is an increase in excitatory amino acids (EAAs), resulting in an increase in intracellular Ca\(^{2+}\) concentration, decreasing protein phosphorylation and function. The altered enzyme function results in ROS formation via the uncoupling of the potent oxidant heme from hemoglobin, and the production of nitric oxide (NO) from nitric oxide synthase (NOS). In the presence of oxidative stress, NO is converted to peroxynitrite, further contributing to the ROS population. The increased presence of ROS leads to lipid peroxidation, membrane dysfunction, and finally the release of more EAAs, completing the positive feedback cycle (Zink, 1996).

Beyond increased ROS formation damaging cells, another aspect of TBI is inflammation. Inflammation is typically the body's way of fighting infection, to prevent the growth and spread of a foreign body while also causing harm to non-infected areas. This dual nature of inflammation is important in the case neuroinflammation as the damage caused to neurons can be permanent (Morganti-Kossmann et al, 2001). Neuroinflammation following TBI if left alone can persist for years, resulting in irreversible white matter degeneration and also decreased brain plasticity (Johnson et al, 2013; Clausen 2009). Recently Johnson et al (2013) found that persistent neuroinflammation caused by TBI and greatly increased the likelihood of Alzheimer's disease later in life. The pathophysiology of neuroinflammation is interconnected with hemorrhaging and the ROS pathway described earlier. After initial insult resulting in TBI, hemorrhaging and hemolysis results in an increase in the extra-cellular concentration of the pro-oxidant heme, further contributing to the increase in ROS from altered enzyme function (Figure 2). ROS increases the expression of cell adhesion molecules that allow leukocyte adhesion to
occur, while also increasing the pro-inflammatory and pro-apoptotic factor tumour necrosis factor-α (TNF-α).
Figure 1. **Traumatic brain injury reactive oxygen species pathway.** Adapted from Zink (1996). Excitatory amino acids (EAAs), nitric oxide (NO), nitric oxide synthase (NOS), reactive oxygen species (ROS).

Figure 2. **Traumatic brain injury inflammation pathway.** TBI inflammation pathway showing relevance to ROS and hemorrhaging. Reactive oxygen species (ROS), intercellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), tumour necrosis factor-α (TNF-α).
The different aspects of TBI injury are closely linked together, requiring attention and care from all aspects to ensure the patient receives the best treatment possible to improve recovery. This leads to the next issue with regards to TBI. As alluded to previously, despite the importance and the impact TBI has on a population, it is not an injury that receives the proportionate attention from the public and this is partially due to two facts: TBI cases are underreported, and secondly because there are very limited treatment options available. Most current treatment protocols are designed to manage symptoms that a patient may experience, and to educate patients on the best approach to certain tasks in order to minimize the appearance of symptoms (Lombard et al, 2005). Although symptom management does indeed help a patient cope and possibly reach a healthier state, mitigating further injury offers better chances for recovery. There are currently very limited pharmacological agents being used to treat TBI beyond those used to alleviate nausea and other symptoms. There are several facets as to why there is a lack of consensus about drug treatment for TBI. Firstly, TBI is a complex injury that relies heavily on secondary injury mechanisms that are still being elucidated (McIntosh, 1993). Furthermore, there have been numerous inconsistencies when attempting to translate laboratory findings and animals models to human TBI cases suffered in the real world (Kabadi and Faden, 2014). Lastly, although TBI manifests in specific signs when tissue is studied, this is not readily apparent with patients, instead relying more on subjective symptoms (Saatman et al, 2008). Physical therapy is often recommended for those suffering from persistent motor impairments, with cognitive programs to assist with amnesia and learning impairments (Alexander, 1995). There has recently been a shift in focus from merely symptom management to treating the cause of the symptoms although such interventions are still in the experimental phase. As more information on the specific pathways associated with TBI are being elucidated, pharmacological
treatments can play a larger role in recovery. Faden et al (1989) attempted to target the ROS pathway and counteract the increase in excitatory amino acids by utilizing an N-methyl-D-aspartate (NMDA) receptor antagonist. There was limited success with this treatment but more importantly, it showed the progression from symptom management to injury mitigation. As the injury pathways of TBI become more well studied, newer ideas can be implemented, opening the door to pharmacological intervention. Another method to limit damage after TBI is to use hypothermia immediately post-injury. The rationale is that hypothermia will slow down the rate of metabolism and also decrease the presence of leukocytes, limiting harmful cytokines from being released (Beilin et al, 1998). While mitigating further injury after the initial insult, the use of hypothermia also limits recovery, in essence placing the injured area into a static state. Dietrich et al (1994) applied this theory to rats injured under experimental conditions and found that hypothermia after mild TBI significantly reduced neuronal cell death when compared to injured rats exposed to normothermic conditions. Using similar conditions, cooling human patients to an internal temperature of 32°C to 33°C for 24 hours immediately after TBI resulted in faster neurological recovery compared to individuals not exposed to mild hypothermia (Marion et al, 1997). Utilizing hypothermia to slow metabolism has been increasingly used as pharmacological interventions continue to be studied. The main caveats that arise with using hypothermia as a treatment option is that it is most effective only if the patient can be placed into a hypothermic state very shortly after TBI, and secondly the resources needed are not always readily available, and when inducing the hypothermic state via surface cooling, more time is needed (Clifton et al, 1991). An ideal first line treatment for TBI requires the injured area to be directly affected, either by promoting healing or by mitigating injury, requires fast onset of action, and lastly needs to be able to be implemented with limited assistance and resources.
(Williamson et al, 1996). Pharmacological intervention would appear to be an option although such treatment does not exist currently despite being an area with increased focus.

A.2. Heme Oxygenase and Cytoprotection

Heme oxygenase (HO) is an important stress-inducible enzyme that catalyzes the breakdown of the pro-oxidant heme into carbon monoxide (CO), biliverdin, and Fe$^{2+}$ (Chen et al, 2000) (Figure 3).

Due to its role in breaking down the pro-oxidant heme, HO displays numerous cytoprotective properties in various tissues. Besides actively removing a pro-oxidant, the catalysis also yields a potent antioxidant in biliverdin which is quickly reduced to bilirubin via biliverdin reductase (Baranano et al, 2002), CO a small gaseous molecule that acts as a messenger and also imparts its own cytoprotective properties via various pathways, and Fe$^{2+}$ that becomes sequestered by the protein ferritin (Chen et al, 2000) (Figure 4).
Figure 3. Heme catalysis by heme oxygenase pathway. Heme oxygenase catalyzing breakdown of heme into biliverdin, CO, and Fe$^{2+}$ (Wu and Wang, 2005). Nicotinamide adenine dinucleotide phosphate/reduced (NADP/H), carbon monoxide (CO), water (H$_2$O).

Figure 4. Various functions of heme metabolites. Metabolites of heme catabolism and their various protective functions, adapted from Jozkowicz et al (2007). Tumour necrosis factor-α (TNF-α), interleukin (IL), interferon-γ (IFNγ), guanosine triphosphate (GTP), guanosine 3′,5′-cyclic phosphate (cGMP).
HO has been studied in numerous capacities due to its widespread expression. Biliverdin and bilirubin thought previously to be only lipid soluble waste byproducts of heme catabolism in fact have beneficial physiological functions under the right conditions. Stocker et al (1987) found that although biliverdin and bilirubin can be cytotoxic at higher concentrations and not excreted, under normal conditions these antioxidants were able to suppress lipid peroxidation better than the gold standard, α-tocopherol. Lipid peroxidation leads to membrane disruptions and instability that can result in cell death. Bilirubin produced by HO was also found to be neuroprotective against oxidative stress in rat neuronal cell cultures (Dore et al, 1999). Choi et al (1996) found that an isoform of HO, heme oxygenase-1 (HO-1), was highly inducible by oxidative stress and provided protection from oxidant-induced lung injury. In addition to anti-oxidative properties, biliverdin and bilirubin were also found to reduce P- and E-selectin expression in vascular beds, providing evidence of anti-inflammatory properties (Vachharajani et al, 2000). The specific pathway for reducing expression of pro-inflammatory genes is by inhibiting the transcription factor nuclear factor-κB (Soares et al, 2003). Moving beyond the beneficial functions of biliverdin and bilirubin, CO also plays a large role in the body.

As alluded to previously, CO is an endogenous cellular messenger similar to NO formed by NOS (Verma et al. 1994), but also provides anti-apoptotic and anti-inflammatory properties amongst others. Like biliverdin and bilirubin, CO in large enough concentrations is poisonous as it binds to hemoglobin with greater affinity than oxygen, crippling gas exchange (Otterbein et al, 2000). Endogenous CO production never reaches levels high enough to interfere with respiration, maintaining the beneficial properties. HO-derived CO has been the focus of numerous studies due to its anti-inflammatory and anti-apoptotic properties. Willis et al (1996) found that HO modulation can provide a new path to treating chronic inflammatory diseases. Sheikh et al
(2011) studied mice genetically modified for colitis, an inflammatory disease of the colon, and found that treatment with CO or induction of HO-1 with cobalt protoporphyrin resulted in improved histological scores, whereas inhibition of HO-1 using tin protoporphyrin reduced scores and generally proved to be detrimental. The anti-inflammatory properties of CO were thought to be exploitable in lung tissue as HO-1 has been found to be highly inducible in lung tissue with lung injuries including asthma, acute complement-dependent lung inflammation, and lung inflammation caused by hyperoxia, endotoxemia, bleomycin, and heavy metals (Otterbein et al, 2000). CO has also been found to be able to reduce diffuse alveolar damage, offering promise to future studies utilizing the activation or induction of HO-1 in lung tissue (Jin et al, 2005). HO-1 when overexpressed in neurons has been found to be able to resist cell death caused by oxidative stress (Chen et al, 2000).

The non-iron HO metabolites, CO and biliverdin/bilirubin, all impart protection to cells without discrimination to type. The topics and studies highlighted thus far have painted a picture where this cytoprotection is beneficial, but there are instances where this indiscriminate nature may also be a disadvantage. HO metabolites being cytoprotective offers unwanted cells such as cancer cells an advantage in addition to their inherent survivability. Jozkowicz et al (2007) described HO-1 as a false friend when spoken in the context of tumours as the protective properties of biliverdin/bilirubin, CO, and even ferritin all function to reduce oxidative stress by scavenging free radicals, inhibiting pro-apoptotic and inflammatory factors, and protecting cells from oxidative stress by sequestering Fe^{2+}. The inducible nature of HO-1 further complicates matters as it is upregulated in quickly proliferating cells such as cancer cells (Hanselmann et al, 2000).
Up to this point, much of the discussion and examples have been based on studies with HO-1. Although all of the previous discussions can be applied to HO in general, an injustice would occur if it is not mentioned that there are two active isoforms of HO expressed in cells, HO-1 as previously described, and HO-2. Both HO-1 and HO-2 are expressed throughout the body although there are certain tissues where one isoform is predominant. HO-1 is the 32-kDa inducible isoform, capable of being induced by stressors such as oxidative conditions, heavy metals, and various cytokines. HO-2 is the 36-kDa constitutive isoform, expressed under homeostatic conditions. As alluded to earlier, although HO-1 and HO-2 are expressed throughout the body, there are certain tissues where one isoform predominates. HO-1 is found at highest concentrations in liver and spleen cells (Schacter, 1978), whereas HO-2 is found at highest concentrations in brain, testes, and endothelial cells (Jozkowicz, 2007).

A.3. Traumatic Brain Injury in Relation to Heme Oxygenase

TBI is an injury that affects the cerebrum and occasionally the brain stem, tissues where HO-2 is the predominant isoform. HO-2 shares many of the same properties and functions as HO-1 including its anti-inflammatory properties. Free heme is toxic and has been found to be a potent inducer of inflammation (Wagener et al, 2001). The pathophysiology of TBI is centered around two pathways, the ROS cycle where injury results in increased ROS formation and cell death, and the inflammation pathway resulting in impaired recovery and also cell death via apoptosis. Dore et al (1999) utilizing rat hippocampal and cortical neuronal cultures performed a study to ascertain whether bilirubin produced by increased HO-2 catalytic activity protects cells from oxidative stress and found that in the presence of bilirubin in nanomolar concentrations, neuronal cell death was decreased compared to a control culture with no increased HO-2.
catalytic activity. The previous study suggests that HO-2 may be a useful target in neurons to reduce oxidative stress and to limit injury and prevent cell death. A follow-up study by Dore et al (2000) further demonstrated the importance of HO-2 in neuroprotection as HO-2⁻/⁻ mice displayed greater apoptotic morphology after an induced ischemic event, with the cultures displaying greater survivability after transfection with a functional HO-2 gene. Despite having a similar role as HO-1 in terms of the anti-inflammatory properties HO-2 possesses, this aspect of HO-2 activity has not yet been exploited. Inflammation is a major issue in TBI, furthering cell injury and death while also preventing recovery. In addition to TBI, neuroinflammation has also been linked to numerous CNS related diseases including Parkinson's disease, where degradation of dopaminergic neurons in the substantia nigra result in disrupted motor functions. Hirsch et al (2009) found that neuroinflammation contributed to increased neurodegeneration, exacerbating the effects of Parkinson's disease. Multiple sclerosis is a neuroimmunological disease characterized by neuroinflammation amongst other pathologies, with mounting evidence that oxidative stress further contributes to the disease (LeVine et al, 2004). HO-2 being highly concentrated in the brain offers a novel target in the fight against CNS diseases and injuries such as TBI. A study performed by Yoneyama-Sarnecky et al (2010) found that HO-2⁻/⁻ mice were less capable of recovering fine motor coordination and mitigating secondary damage after TBI. The metabolites of heme catabolism have been shown to offer neuronal cells protective benefits that have yet to be successfully exploited in vivo. With regards to TBI, an injury characterized and exacerbated by oxidative stress and inflammation, HO-2 activation would appear to be a suitable and novel method for mitigation of further injury (Figure 5).
Increased HO-2 activity would be able to remove the pro-oxidant heme, negating its ability to contribute to the population of ROS formed by the release of excitatory amino acids (Figure 1), while also preventing heme from increasing the expression of the pro-apoptotic and pro-inflammatory factor TNF-α, and the expression of various cell adhesion molecules (Zink, 1996), further reducing inflammation. Increased concentrations of antioxidant biliverdin, which is quickly further reduced to bilirubin via biliverdin reductase (Baranano et al, 2002), scavenges ROS thereby limiting lipid peroxidation and also decreasing the expression of cell adhesion molecules, limiting inflammation. CO plays a large role in the TBI inflammation pathway as it has been found to limit the expression of cell adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and P-selectin amongst others (Marilena et al, 1997). Cell adhesion molecules are important molecules that mediate leukocyte adhesion, prior to the release of inflammatory cytokines (Jozkowicz et al, 2007; Otterbein et al, 2000). CO has also been found to inhibit the expression of pro-inflammatory cytokines including TNF-α, interleukin-1β, and also macrophage inflammatory protein-1β, while simultaneously increasing the expression of the anti-inflammatory cytokine interleukin-10 via the p38 mitogen-activated protein kinase pathway (Otterbein et al, 2000). Limiting expression of TNF-α also has the added benefit of reducing apoptotic cell death, an important factor when considering treatment options for TBI. The metabolites formed by the breakdown of heme by HO-2 in conjunction with the high quantities of HO-2 found in brain tissue is cause for optimism in regards to TBI treatment.

Increasing the activity of HO-2 appears to be a viable and worthwhile option for the treatment of TBI given the beneficial properties of the metabolites of heme and how they coincide with the pathologies of TBI. Thus far, there has been limited research in the area of HO-
2 activation or enzyme activation in general as most research has been focused on means of inhibiting enzyme function, leaving a wide gap in knowledge, especially in pharmacology (Zorn et al, 2010). Finding a chemical compound that can selectively activate HO-2 and not HO-1 can be a challenge. Currently, there exist many non-selective inhibitors of HO-2 including many metalloporphyrins (MPs), but there is limited literature on HO-2 activators (Table 2). MPs share the same general structure and in some cases, an identical structure with heme, but with a different metal cation as opposed to iron (Figure 6).
Table 2. Non-exhaustive list of current metalloporphyrin HO-2 inhibitors. Organized by decreasing potency (Wong et al, 2011; Meffert et al, 1994).

<table>
<thead>
<tr>
<th>Metalloporphyrin non-selective HO-2 inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td>• tin mesoporphyrin</td>
</tr>
<tr>
<td>• chromium mesoporphyrin</td>
</tr>
<tr>
<td>• zinc bis-glycol porphyrin</td>
</tr>
<tr>
<td>• tin protoporphyrin</td>
</tr>
<tr>
<td>• chromium protoporphyrin</td>
</tr>
<tr>
<td>• chromium deuteroporphyrin</td>
</tr>
<tr>
<td>• zinc mesoporphyrin</td>
</tr>
<tr>
<td>• tin deuteroporphyrin</td>
</tr>
</tbody>
</table>
Figure 5. Anti-inflammatory functions of heme metabolites and HO-2 activity. TBI inflammation pathway with markers indicating the beneficial property of each metabolite. Reactive oxygen species (ROS), intercellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), tumour necrosis factor-α (TNF-α), carbon monoxide (CO).

Figure 6. Comparison of heme and zinc protoporphyrin structures. Heme (left) and zinc protoporphyrin (right).
The similarity in structure between heme and MPs allow MPs to act as competitive inhibitors of HO (Wong et al, 2011). The similarity in structure also makes MPs very non-selective not only in terms of HO-1 and HO-2 inhibition, but also inhibition of other enzymes in cells. Zinc protoporphyrin and tin protoporphyrin have been shown to also inhibit NOS and soluble guanylate cyclase (sGC) (Luo et al, 1994; Meffert et al, 1994; Grundemar et al, 1997). The non-selectiveness of MPs allows for in vitro research where variables and the enzymes present can be controlled, but quickly becomes problematic when applied to theories in vivo.

As previously mentioned, molecules that affect HO activity have employed structures similar to its substrate heme, with the majority being nonselective inhibitors. Moving away from structures homologous to heme, a molecule known as menadione (MD; Figure 7) was recently found to selectively activate HO-2 in rat brain microsomal preparations and also recombinant human HO-2 protein, and to increase activity by up to 30-fold compared to control (Vukomanovic et al, 2011).

MD, also known as vitamin K3, is a member of the K vitamin family. A similar study performed by Vukomanovic et al (2014) found that the bulky aliphatic groups of vitamins K1 and K2 resulted in no HO-2 activation, whereas smaller groups at positions -2 and -3 allowed for activation (Figure 8).
Figure 7. Structure of menadione.

Figure 8. Comparison of K vitamin family. Numbers indicate positions.
Vitamins K1 and K2 are naturally occurring forms found in leafy green vegetables and in the colon, produced by gut bacteria, respectively (Hitomi et al 2005). MD (C\textsubscript{11}H\textsubscript{8}O\textsubscript{2}) on the other hand is synthetic, highly lipophilic, and has a molecular weight of 172.18. K vitamins have displayed potential as pharmacological agents against cancer cells with MD being the most well-studied due to its size and inherent redox properties (Okayasu et al, 2001; Vukomanovic et al, 2014). MD has been the focus of numerous studies relating to both rodent and human cancer cells and has displayed beneficial actions under the right conditions. Wu et al (1993) in a study using nasopharyngeal carcinoma cells found that MD was able to induce cell cycle arrest and also inhibit apoptotic cell death. Similar studies performed by Prasad et al (1981), Chlebowski et al (1985), and Okayasu et al (2001) have all demonstrated the anti-cancer properties of MD in human oral tumour cells, human promyelocytic leukemia cells, human gingival fibroblast, and also the cytotoxic nature of this synthetic compound. Single bolus injection of MD at high dose (150 mg/kg) has resulted in cardiac, renal, liver and lung toxicity in rats (Chiou et al, 1997). Since MD is highly lipophilic and insoluble in water, a water-soluble salt is often administered for \textit{in vivo} studies. Menadione sodium bisulphite (MSB; Figure 9) is a popular choice for studies of MD as it has an enzymatic MSB-to-MD conversion half-life of approximately 1.70 min (Hu et al, 1996). Once converted, MD has a half-life of 17 hours in the rat and a high apparent volume of distribution, with no preference for specific organs or tissues regardless of vitamin K deficiency, indicating its sequestration in tissues and organs (Thierry et al, 1969; Hu et al, 1996). These pharmacokinetic properties, coupled with data demonstrating the selectivity of MD for activation of HO-2 and not HO-1 (Vukomanovic et al, 2011), make MD a promising compound to use for the activation of HO-2.
Figure 9. Comparison of menadione and menadione sodium bisulphite structures. Menadione (left) with a water-soluble salt, menadione sodium bisulphite (right).
A.4. Selecting the Models and Approaches for TBI

There are many animal models of TBI, varying between animals and also the method by which the TBI is produced (Table 3). Animals that have been used most often to study the effects of TBI include rat, mouse, and ferret (Cernak, 2005). Methods of TBI induction are also numerous, with impact injuries being the most studied, further broken down to open-skull/penetrating, and closed-skull/non-penetrating (Figure 10). All open-skull models require a craniectomy, a surgical procedure where a small portion of the skull is removed, in order to gain direct access to the brain.

<table>
<thead>
<tr>
<th>Animal</th>
<th>TBI Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>• Open-skull/penetrating</td>
</tr>
<tr>
<td></td>
<td>o Controlled cortical impact (CCI)</td>
</tr>
<tr>
<td></td>
<td>o Lateral fluid percussion (LFP)</td>
</tr>
<tr>
<td></td>
<td>o Guided weight drop</td>
</tr>
<tr>
<td></td>
<td>• Closed-skull/non-penetrating</td>
</tr>
<tr>
<td></td>
<td>o Guided weight drop</td>
</tr>
<tr>
<td>Mouse</td>
<td>• Open-skull/penetrating</td>
</tr>
<tr>
<td></td>
<td>o CCI</td>
</tr>
<tr>
<td></td>
<td>o LFP</td>
</tr>
<tr>
<td></td>
<td>o Guided weight drop</td>
</tr>
<tr>
<td></td>
<td>• Closed-skull/non-penetrating</td>
</tr>
<tr>
<td></td>
<td>o Guided weight drop</td>
</tr>
<tr>
<td>Ferret</td>
<td>• Open-skull/penetrating</td>
</tr>
<tr>
<td></td>
<td>o CCI</td>
</tr>
</tbody>
</table>
Despite the many animal models of TBI available, the most common model is the rat CCI model as it offers great reproducibility, while utilizing a common lab animal that is neither too small nor too large (Dixon et al, 1991; Cernak, 2005). The CCI model utilizes a machine called the impactor which houses an extendable tip of varying diameter, attached to a control box where the operator can select the speed of the impact. The impactor is attached to an actuator arm that allows for the impact depth to be adjusted (Figure 11). The animal and the actuator arm are mounted onto a stereotaxic frame to prevent animal movement, allowing for increased reproducibility. CCI machines can use pneumatic, hydraulic, or electromagnetic mechanisms to control impact speeds.
Figure 10. Traumatic brain injury induction methods. Adapted from Cernak (2005).

Figure 11. Picture of controlled cortical impact setup. 1: Control box. 2: Stereotaxic frame. 3: Impactor. 4: Impact tip. 5: Actuator arm.
LFP is another model that is often used by researchers as it offers similar reproducibility when compared to CCI, albeit at an increased cost (Cernak, 2005). The mechanism LFP uses to induce TBI is similar to CCI in that it requires a craniectomy to be performed, but instead of a solid impact tip contacting the brain, it utilizes a small fluid pulse (Alder et al, 2011). Guided weight drops require a weight of known mass to calculate the speed of impact. A guide tube is used to ensure it follows the right path (Flierl et al, 2009). The main disadvantages of a guided weight drop are two-fold. Firstly the weight remains resting on the site of impact until manually removed, and secondly the reproducibility of this method relies heavily on external conditions (Hallam et al, 2004). All methods listed above produce similar histological results, and behavioural and cognitive deficits in animals when optimal with reproducibility being the main difference. We used the rat CCI model due to the abundant literature and reproducibility.

Behavioural deficits from CCI include motor deterioration and also cognitive deterioration (Dixon et al, 1991; Fujimoto et al, 2004). Tasks that have been used to measure these deficits include the Morris water maze (MWM), spontaneous locomotor activity (SLA), spontaneous alternation behaviour (SAB), rotarod task (RR), and the beam balance task (BB) amongst many others (Table 4).
Table 4. Behavioural tasks and faculties tested.

<table>
<thead>
<tr>
<th>Motor Deficits</th>
<th>Cognitive Deficits</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLA</td>
<td>MWM</td>
</tr>
<tr>
<td>RR</td>
<td>SLA</td>
</tr>
<tr>
<td>BB</td>
<td>SAB</td>
</tr>
<tr>
<td>MWM*</td>
<td></td>
</tr>
</tbody>
</table>

* MWM mainly used to measure cognitive deficits but due to the nature of the task, can also serve as a task measuring motor deficits
MWM has been the gold standard behavioural task to measure deficits following TBI as it allows for measurement of both cognitive and motor deficits, despite being mainly used to assess spatial learning and memory (Morris, 1984; D'Hooge et al., 2001). Francis et al (1995) found that stress induced by placing an animal into a survival situation introduces an alternate variable other than the animal's cognitive abilities. The SLA task tracks an animal's movements to measure motor deficits, but also its exploratory nature (Fibiger et al., 1971). The SAB task measures an animal’s cognitive abilities by utilizing a Y-maze and tracking the number of successful alternations. RR and BB tasks measure motor deficits and require an animal to balance on either a rotating rod or a narrow beam, respectively. For the deficits caused by TBI, SLA, SAB, and BB are appropriate tasks to measure deficits and improvements.

A.5. Hypothesis and Objectives

Given the beneficial properties of heme metabolites upon HO-2 activation, our hypothesis was that treatment of animals with an HO-2 activator such as MD after TBI would mitigate brain injury as measured by behavioural and cognitive tasks. Our objectives were to (a) induce TBI using the rat model of CCI as described by Dixon et al. (1991), (b) refine CCI procedure to ensure our behavioural tasks would be sensitive, (c) establish the actions of MD in the absence of injury, (d) perform a dose-response study of MD comparing performances from animals uninjured and untreated, injured and untreated, and injured and treated.
Chapter 2 – Materials and Methods

B.1. Animals

Adult male Sprague-Dawley rats (275-300 g) were purchased from Charles River Inc. (Montreal, QC, Canada) and housed in a 12 hour reversed light cycle room (1900h-700h) located in the animal care facility at Botterell Hall (Queen's University, Kingston, ON, Canada). Animals had ad libitum access to water and standard Ralston Purina laboratory chow 5001 (Ren’s Feed Supplies, Ltd., Oakville, ON, Canada). Animals were given one week to acclimatize to surroundings prior to respective studies, and were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care with experimental protocols approved by Queen’s University Animal Care Committee.

B.2. In vitro Experiments

B.2.1. Microsomal Fraction Preparation

Methodology for the preparation of microsomal fraction was adapted from Marks et al (1997). Four male Sprague-Dawley rats were anaesthetized with isoflurane (Pharmaceutical Partners of Canada, Inc., Richmond Hill, ON, Canada) and then decapitated. The spleen and brain were harvested from each animal, with all four spleens and four brains being placed into respective pre-weighed 50-mL beakers containing 10 mL of 20 mM phosphate buffer. Once the weight of the respective tissues was established, tissues were placed into separate clean Petri dishes on ice and minced with a razor blade. Homogenates (15% w/v) of each tissue was prepared by pouring the minced spleen or brain tissue with buffer into a 50-mL plastic conical tube, and adding the appropriate volume of 20 mM phosphate buffer. Spleen or brain tissue was
further homogenized using a Sonic Dismembrator 60® at 20 watts for 1 min with the conical tube on ice. Homogenate was then transferred to a 40-mL round-bottom centrifuge tube and centrifuged at 10,000 x g for 20 min at 4°C. Supernatant was then transferred to a 10-mL centrifuge tube and centrifuged at 106,500 x g for 60 min at 4°C. The supernatant was removed and the pellet was resuspended by adding 0.9 mL or 0.6 mL of 100 mM phosphate buffer/20% glycerol solution to spleen or brain pellet, respectively, followed by mixing. The resuspended pellet mixture was transferred to a separate glass homogenizing tube and homogenized by hand using a Potter-Elvehjem tissue grinder with a Teflon pestle. Thereafter, 90-μL or 140-μL aliquots of spleen or brain microsomal fraction homogenate were added to microcentrifuge tubes, which then were frozen at -80°C until analysis. Protein concentration of the microsomal fraction homogenate was determined using the Biuret method, with bovine serum albumin (BSA) as the standard (Table 5).
Table 5. Biuret method conditions for determination of protein concentration.

<table>
<thead>
<tr>
<th>Protein conc. (mg/mL)</th>
<th>BSA 5 mg/mL (µL)</th>
<th>Vehicle (µL)</th>
<th>Biuret solution (µL)</th>
<th>Total volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>0</td>
<td>200</td>
<td>800</td>
<td>1000</td>
</tr>
<tr>
<td>0.2</td>
<td>40</td>
<td>160</td>
<td>800</td>
<td>1000</td>
</tr>
<tr>
<td>0.4</td>
<td>80</td>
<td>120</td>
<td>800</td>
<td>1000</td>
</tr>
<tr>
<td>0.6</td>
<td>120</td>
<td>80</td>
<td>800</td>
<td>1000</td>
</tr>
<tr>
<td>0.8</td>
<td>160</td>
<td>40</td>
<td>800</td>
<td>1000</td>
</tr>
<tr>
<td>1.0</td>
<td>200</td>
<td>0</td>
<td>800</td>
<td>1000</td>
</tr>
</tbody>
</table>
B.2.2. Carbon Monoxide Assay for HO Activity

Individual aliquots of 120 µL reaction mixture containing 100 mM phosphate buffer, 1.5 mM methemalbumin (MHA), and spleen or brain microsomal fraction homogenate were prepared for incubation with MD dissolved in dimethyl sulfoxide (DMSO). Volume of microsomal fraction homogenate added was determined by protein concentration of microsomal preparation determined previously with Biuret method. Final concentrations of reaction mixture components were as follows: 50 µM MHA, 0.5 mg/mL spleen microsome or 1.0 mg/mL brain microsomal fraction homogenate, with phosphate buffer bringing final master mix volume to 120 µL, extrapolated volumes so that there would be 20 samples of each microsomal fraction homogenate. Each 120-µL aliquot was placed into a 1.5-mL amber screw-cap septum vial (Chromatographic Specialties Inc., Brockville, ON, Canada). Aliquots (10 µL) of MD solutions at concentrations of 1.5 mM, 375 µM, 150 µM, 15 µM, 1.5 µM, and 0.15 µM were added to individual samples for final concentrations of 100 µM, 25 µM, 10 µM, 1.0 µM, 0.1 µM, and 0.01 µM, respectively, and mixed with a Vortex® mixer prior to a 10 min shaken incubation at 37°C. Three positive controls (no addition of MD) and two negative controls (no addition of MD, no NADPH) were included. During incubation (prior to NADPH addition), headspace gas was purged for 10 s each with CO-free extra dry air (Praxair, Toronto, ON, Canada). Each reaction was started by adding 20 µL of 7.5 mM NADPH for a final concentration of 1 mM in a staggered pattern of 15 s between vials. Samples were incubated at 37°C for 15 min before the reaction was stopped by placing the vial onto powdered dry ice (-78°C) utilizing staggered pattern of 15 s for NADPH addition to ensure each vial was incubated for same amount of time.

Amount of CO in headspace gas was measured using a gas chromatograph (Peak Performer 1, Peak Laboratories, LLC, Mountain View, CA, USA) by injecting headspace gas
into gas chromatograph column via a carrier gas (CO-free air, 20 mL/min) for 63 s. Analysis of headspace gas lasted 135 s with a constant molecular-sieve column temperature of 105°C. CO was quantified using an ultraviolet (254 nm) absorption photometer to measure mercury vapour generated from reaction of CO with mercuric oxide at 265°C. A standard curve for CO was produced by using an empty 1.5-mL amber vial and injecting a range of known quantities of CO.

HO activity was calculated by comparing CO production by microsomal preparation in the presence of MD and CO production of microsomal preparation in the absence of the drug and was expressed as "% control activity" where CO production in the absence of MD was 100% control activity.

B.3. In vivo Experiments

B.3.1. Stereotaxic Surgery

On surgery day, animals were transferred to a surgical suite (Animal Care Facility, Botterell Hall, Queen's University, Kingston, ON, Canada) and anesthetized in a Plexiglass® induction chamber (45.7 cm x 22.9 cm x 22.9 cm) using 5% isoflurane solution in 100% oxygen. Once induced, animals were given 25 mg/kg tramadol as an analgesic through subcutaneous injection. Animals were given 0.5-mL of 5 mg/mL of the local anesthetic bupivacaine subcutaneously at the site of incision 15 minutes prior to surgery. Animal heads were then shaven and cleaned with an anti-microbial gel, followed by three cycles of 10% providone-iodine wipes and 70% isopropyl alcohol wipes. While still anesthetized, animals were placed onto a stereotaxic frame with ear and incisor bars to ensure alignment with the impactor. Ear bars were coated with xylocaine jelly for pain relief. A 2.0 cm incision on the sagittal plane following the
midline of the skull was made and skin was reflected using a colibri retractor (17000-04, F.S.T., North Vancouver, BC, Canada). Connective tissue was bluntly dissected and scraped away to reveal skull. Bregma was located and a 6.0 mm craniectomy was performed midway between bregma and the lambdoid suture and between the sagittal suture and coronal ridge on the right parietal bone (Figure 12) using a Michele trephine (RS-9200, Roboz, Gaithersburg, MD, USA).
Figure 12. Diagram of craniectomy with landmarks.
The electromagnetic impactor (Leica Impact One, Leica Microsystems Ltd., Concord, ON, Canada) was placed on 'extend' mode and adjusted to 45° relative to the horizontal with the 3.0 mm tip zeroed on the bregma using the course adjustment knob. Still in 'extend' mode, fine adjustment knobs in x- and y- axes were used to maneuver impact tip to the impact site until the attached contact sensor sounded; vernier coordinates were noted. Impactor was adjusted from 'extend' to 'retract' mode, and using the y-axis fine adjustment knob, impact tip was lowered 3.0 mm for impact depth. With the impactor set at 6.0 m/s, moderate TBI was produced. Two suture techniques were used to ensure incision remained closed and to promote quicker healing. Using vicryl 4-0, incisions were closed using discontinuous internal and external sutures. Post-surgery, animals were given 100% oxygen for two minutes and 3.0 mL sterile saline subcutaneously.

B.3.2. Drug Preparation and Treatments

Due to the poor water solubility of MD (M.W. 172.18), a water soluble salt – menadione sodium bisulphite (M.W. 276.24; MSB) – was used for in vivo studies and dissolved in sterile physiological saline. To remain within the ideal injection limits (1-mL) for rats of this size (330 g - 440 g), a 100 μmol/mL MD stock solution was prepared fresh using MSB and physiological saline. Four different doses were used to investigate the effect MD had on animals recovering from TBI: 25, 50, 100, and 200 μmol/kg. Doses were selected to cover a range while also under levels that would be fatal to the animal (Chiou et al, 1997). Animals were treated on Days 0-6 (Table 6), or daily for seven days. Intraperitoneal injections were performed with either MD solution or sterile saline as a vehicle control.
B.3.3. Experimental Groups

This project was split into four smaller studies, each with different groups of animals. The studies were as follows: Control-Sham; CCI Optimization; Control-MD/Ctrl; MD Dose Response. The Control-Sham study was performed to ensure that the craniectomy procedure would not yield any detrimental effects or otherwise cause the animals to perform differently compared to control animals. Control animals were uninjured and untreated; sham animals underwent craniectomy surgery but not CCI surgery, and were also untreated. CCI Optimization study was performed to find the optimal parameters for the CCI procedure. Speed of impact and impact depth were the variables tested. Within the CCI Optimization study, there were four groups of animals: Control (uninjured and untreated); Parameter 1 (5 m/s impact speed, 2 mm impact depth); Parameter 2 (5 m/s, 3 mm); Parameter 3 (6 m/s, 3 mm). Parameter 3 yielded the most consistent results and was chosen as the variables for all future CCI procedures. Control-MD-Ctrl study was performed to study whether the presence of MD in an uninjured animal would elicit any effect. There were two groups in this study: Control (uninjured and untreated) and MD-Ctrl (uninjured, treated with 100 μmol/kg MD daily for 7 days). The MD dose-response study was performed to ascertain the optimal dose needed to see improvement in CCI animal behaviour if any at all. MD treatment for specified animals lasted for seven days (Table 6). There were six groups of animals in this study: Control (uninjured and untreated); Saline (CCI surgery, treated with saline); 25 MD (CCI surgery, treated with 25 μmol/kg MD); 50 MD (CCI surgery, treated with 50 μmol/kg); 100 MD (CCI surgery, treated with 100 μmol/kg); 200 MD (CCI surgery, treated with 200 μmol/kg).
Table 6. Menadione treatment and behavioural task regimen. Menadione (MD), controlled cortical impact (CCI), spontaneous locomotor activity (SLA), spontaneous alternation behaviour (SAB), beam balance (BB),

<table>
<thead>
<tr>
<th>Day</th>
<th>Treatment / Tasks</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1</td>
<td>Pre-Injury. SLA, SAB, BB tasks for baseline.</td>
</tr>
<tr>
<td>0</td>
<td>CCI Surgery. MD or saline treatment.</td>
</tr>
<tr>
<td>1</td>
<td>MD or saline treatment.</td>
</tr>
<tr>
<td>2</td>
<td>MD or saline treatment.</td>
</tr>
<tr>
<td>3</td>
<td>MD or saline treatment, SLA, SAB, BB tasks.</td>
</tr>
<tr>
<td>4</td>
<td>MD or saline treatment.</td>
</tr>
<tr>
<td>5</td>
<td>MD or saline treatment.</td>
</tr>
<tr>
<td>6</td>
<td>MD or saline treatment, SLA, SAB, BB tasks.</td>
</tr>
<tr>
<td>7</td>
<td>SLA, SAB, BB tasks.</td>
</tr>
<tr>
<td>8</td>
<td>SLA, SAB, BB tasks.</td>
</tr>
<tr>
<td>9</td>
<td>Euthanization.</td>
</tr>
</tbody>
</table>
B.3.4. Behavioural Studies

After the acclimatization period, animals underwent behavioural testing following the schedule highlighted in Table 6. Behavioural studies occurred on Days -1, 3, 6, 7, and 8 for a total of 5 testing days. The scheduling was chosen to allow sufficient time for the analgesic (tramadol) to be eliminated from the body prior to commencement of testing. Three behavioural tests were utilized, SLA to measure cognitive and motor function, SAB to measure cognitive function, and BB to measure motor performance. In SLA, animals were placed into a Plexiglass® open-field container (45 cm x 45 cm x 25 cm; Figure 13) after a 5 min habituation period in their respective cages. Testing lasted for 10 min during which infrared beams were used to track movement pattern of animals including number of beams tripped, time active, rearing time, and corner duration. The count number (number of times beams were tripped) was released by TSE One software (Toronto, ON, Canada) and recorded.

Prior to and after SLA testing, the container was cleaned with distilled water (dH\textsubscript{2}O) to remove scents. Once all SLA testing concluded for the respective day, the container was further cleaned with 70% ethanol solution and allowed to aerate.

SAB was tested using an opaque white polycarbonate Y-maze (each arm: 43 cm x 15 cm x 28 cm, 120°; Figure 14).
Figure 13. Picture of spontaneous locomotor activity open-field container.

Figure 14. Picture of Y-maze.
The purpose of SAB testing was to assess the cognitive faculties of animals by finding the percentage of times the animal would explore a “novel” arm, defined as the alternate to the last visited arm. Each arm was labeled A, B, or C prior to testing. Testing began with the animal being placed into the center of the Y-maze in the direction of arm A; each subsequent arm entry was recorded for a period of 10 min. The following equation was used to calculate the percentage of spontaneous alternations:

\[
\% \text{ alternation} = \left( \frac{\text{# of novel arm entries}}{(\text{total arm entries} - 2)} \right) \times 100\%
\]

Prior to each testing, Y-maze was cleaned with dH₂O to remove any scents. At the conclusion of SAB testing for the day, Y-maze was cleaned with 70% ethanol solution and allowed to aerate.

The BB task was used to measure the motor coordination of animals. The beam was constructed out of a polycarbonate rectangular beam (60 cm x 1.5 cm x 1.5 cm), supported by two retort stands on either side, with plastic covered boards to ensure the animal would remain on the beam. The beam was elevated to a height of 1.0 m above floor-level, and 0.5 m above a container with bedding used as padding in the event an animal fell off. Each animal was placed onto the beam and allowed to have all four paws on and balanced before the timer was started. If the animal failed the task (fell off) within the first 10 s, the trial was restarted. The animal was allowed to stay on the beam for a maximum of 60 s, for three trials with the average latency time used. The beam was cleaned with dH₂O prior to each trial and the entire apparatus was cleaned.
prior to a new animal. Beam was cleaned with 70% ethanol solution once BB testing concluded for the day.

**B.4. Statistical Analysis**

All data were analyzed using GraphPad Prism® Version 5 (GraphPad Software Inc., CA, USA) and presented as mean ± SD. *In vitro* studies were presented using a nonlinear regression sigmoidal response curve. *In vivo* studies were analyzed using a one-way analysis of variance (ANOVA) followed by a Tukey’s post-hoc test comparing all groups to the respective ‘Control’ group of the study unless otherwise indicated. P-values ≤ 0.05 were considered statistically significant.
Chapter 3 – Results

C.1. *In vitro* Experiments

C.1.1. Menadione Selectivity for HO-2 Activation

To ascertain the selectivity of MD for activation of HO-2 and not HO-1 in rat tissue, as found by Vukomanovic et al (2011), a CO assay was performed using rat brain and spleen microsomal preparations for HO-2 and HO-1 activity, respectively. Incubating MD with the brain microsomal preparations resulted in a nearly 4-fold increase in HO-2 activity (397% control activity) when compared to brain microsomal preparations incubated with the vehicle. Spleen microsomal preparations representing HO-1 activity in the same assay did not result in any change when incubated with varying concentrations of MD (Figure 15).
Figure 15. Effect of menadione on HO-1 and HO-2 activity via CO production. HO-2 activity (square) increased by up to 4-fold compared to controls when rat brain microsomal preparations were incubated with menadione whereas HO-1 activity (triangle) did not.
C.2. *In vivo* Experiments

C.2.1. Establishment of TBI Model: Craniectomy

The CCI procedure is an open-skull model of TBI and as such requires a craniectomy to be performed in order to gain direct access to the brain. To elucidate whether the craniectomy procedure would alter the animal's performance in behavioural tasks, a study comparing control animals (n = 14) that did not undergo the craniectomy procedure, and sham animals (n = 4) that only underwent the craniectomy procedure was performed. The animals were assessed in four areas: body weight in order to monitor health, SLA to assess exploratory nature, SAB for cognitive performance, and BB for motor coordination. No differences were observed with the absolute values except on Day -1 for the BB task where control animals had a lower latency time (Figure 16a). No differences were observed with values normalized to pre-injury day (Day -1).

C.2.1.1. Craniectomy and Animal Weight

Animals exhibited steady body weight gain, indicating health (Figures 16a and 16b). No differences were observed compared to Control group.

C.2.1.2. Craniectomy and SLA Performance

SLA values were consistent among both Control and Sham groups while not displaying any significant differences between groups (Figures 17a and 17b).
C.2.1.3. Cranietomy and SAB Performance

The SAB task yielded no differences between the two groups as the % alternation remained near pre-injury levels (Figures 18a and 18b).

C.2.1.4. Cranietomy and BB Performance

The BB task measuring motor coordination likewise did not show any differences in performance when compared to Control group (Figure 19).
Figure 16. (a) **Effect of cranietomy on animal weight (absolute)**. Absolute values for the body weight of animals from the Control and Sham groups indicating no differences; bars represent Mean ± SD. (b) **Effect of cranietomy on animal weight (normalized to pre-injury)**. Body weight values normalized to pre-injury day, expressed as a percentage where 100% is pre-injury; bars represent Mean ± SD.
Figure 17. (a) Effect of craniectomy on spontaneous locomotor activity performance (absolute). Absolute values for SLA counts in Control and Sham groups did not indicate significant differences between groups; bars represent Mean ± SD. (b) Effect of craniectomy on spontaneous locomotor activity performance (normalized to pre-injury) SLA values normalized to pre-injury day for SLA counts, expressed as a percentage where 100% is pre-injury; bars represent Mean ± SD.
Figure 18. (a) **Effect of craniectomy on spontaneous alternation behaviour performance (absolute).** Absolute values for the SAB task measured as % alternation for Control and Sham groups indicating no differences; bars represent Mean ± SD. (b) **Effect of craniectomy on spontaneous alternation behaviour performance (normalized to pre-injury).** SAB values normalized to pre-injury day for % alternation, expressed as a percentage where 100% is pre-injury, indicating no differences; bars represent Mean ± SD.
Figure 19. (a) Effect of craniectomy on beam balance performance (absolute). Absolute values in latency time for BB task comparing Control and Sham groups indicating no differences; bars represent Mean ± SD. (b) Effect of craniectomy on beam balance performance (normalized to pre-injury). BB latency values normalized to pre-injury day expressed as a percentage where 100% is pre-injury, indicating no differences; bars represent Mean ± SD.
C.2.2. Establishment of TBI Model: CCI Speed and Depth

Various impact speeds and depths have been described in literature for the CCI procedure (Dixon et al, 1991; Lighthall, 1988), although with different animals and different equipment setups, these parameters can change. For the behavioural tasks we were using to assess animals after TBI induction, various CCI speeds and depths were tested to find parameters that displayed differences compared to uninjured control animals. In this study, there were four groups: Control (uninjured, n = 14); Parameter 1 (5 m/s impact speed and 2 mm impact depth, n = 4); Parameter 2 (5 m/s impact speed and 3 mm impact depth, n = 4); Parameter 3 (6 m/s impact speed and 3 mm impact depth, n = 4). Out of the CCI groups, only Parameter 3 yielded consistent differences compared to the Control group.

C.2.2.1. Optimization: SLA Performance

SLA counts after the CCI procedure in the Parameter 1 and 2 groups were comparable to the Control group (Figure 20a). When values were normalized to pre-injury, Parameter 1 on Day 3 was significantly less than Control, whereas Parameter 3 was significantly more on Days 3, 6, 7 and 8 when compared to Control (Figure 20b).

C.2.2.2. Optimization: SAB Performance

The SAB task only resulted in significant difference on Days 6, 7 and 8 with Parameter 3 when compared to Control. Parameter 1 displayed a difference when compared to Control on Day 7 but not on any other day (Figure 21a). When % alternation values were normalized to pre-
injury, only Parameter 3 resulted in a consistent difference on Days 3, 6, 7 and 8 compared to Control (Figure 21b).

C.2.2.3. Optimization: BB Performance

BB latency values for Parameter 3 were significantly lower on Days 3, 6, 7 and 8 than Control whereas Parameters 1 and 2 displayed no differences (Figure 22a). BB latency values when normalized to pre-injury resulted in a decrease with Parameter 3 on all days of testing (Figure 22b).
Figure 20. (a) **CCI optimization – spontaneous locomotor activity (absolute).** SLA counts comparing various impact speeds and impact depths to Control; bars represent Mean ± SD. Difference observed with Parameter 3 (p ≤ 0.05). (b) **CCI optimization – spontaneous locomotor activity (normalized to pre-injury).** SLA values normalized to pre-injury expressed as a percentage where 100% is pre-injury; bars represent Mean ± SD. Difference observed with Parameter 3 (p ≤ 0.05).
Figure 21. (a) CCI optimization – spontaneous alternation behaviour (absolute). % alternation values comparing various impact speeds and impact depths to Control; bars represent Mean ± SD. Difference observed with Parameter 3 (p ≤ 0.05). (b) CCI optimization – spontaneous alternation behaviour (normalized to pre-injury). % alternation values normalized to pre-injury expressed as a percentage where 100% is pre-injury; bars represent Mean ± SD. Difference observed with Parameter 3 (p ≤ 0.05).
Figure 22. (a) **CCI optimization – beam balance (absolute)**. BB latency values comparing various impact speeds and impact depths to Control; bars represent Mean ± SD. Difference observed with Parameter 3 (p ≤ 0.05). (b) **CCI optimization – beam balance (normalized to pre-injury)**. BB latency values normalized to pre-injury expressed as a percentage where 100% is pre-injury; bars represent Mean ± SD. Difference observed with Parameter 3 (p ≤ 0.05).
C.2.3. Menadione Effects in Absence of TBI

MD is a synthetic vitamin that has not been well studied *in vivo* especially with regards to neurological performance and function. For the purposes of assessing whether MD-treated animals display improved performance after TBI, it was important to first study the effects of MD in the absence of TBI. Intraperitoneal injection of 100 µmol/kg of MD for 7 days (MD-Ctrl; n = 4) did not result in any differences in the performance of any task when compared to untreated control animals (Control; n = 14).

C.2.3.1. SLA Performance

SLA counts for both Control and MD-Ctrl animals were comparable with absolute values (Figure 23a) and when normalized to pre-injury values (Figure 23b).

C.2.3.2. SAB Performance

SAB % Alternation values did not differ between Control and MD-Ctrl groups (Figure 24a), nor did % Alternation values when normalized to pre-injury values (Figure 24b).

C.2.3.3. BB Performance

BB latency times were comparable between Control and MD-Ctrl groups (Figure 25a). Latency times once normalized to pre-injury values also displayed no difference between groups (Figure 25b).
Figure 23. (a) **Effect of menadione in absence of injury – spontaneous locomotor activity (absolute).** SLA counts comparing Control and MD-Ctrl after treatment with MD (100 µmol/kg) indicating no difference; bars represent Mean ± SD. (b) **Effect of menadione in absence of injury – spontaneous locomotor activity (normalized to pre-injury).** SLA values normalized to pre-injury, and expressed as a percentage where 100% is pre-injury, indicating no difference; bars represent Mean ± SD.
Figure 24. (a) Effect of menadione in absence of injury – spontaneous alternation behaviour (absolute). SAB % Alternation comparing Control and MD-Ctrl after treatment with MD (100 µmol/kg) indicating no difference; bars represent Mean ± SD. (b) Effect of menadione in absence of injury – spontaneous alternation behaviour (normalized to pre-injury). % Alternation values normalized to pre-injury and expressed as a percentage where 100% is pre-injury, indicating no difference; bars represent Mean ± SD.
Figure 25. (a) Effect of menadione in absence of injury – beam balance (absolute). BB latency times comparing Control and MD-Ctrl after treatment with MD (100 µmol/kg) indicating no difference; bars represent Mean ± SD. (b) Effect of menadione in absence of injury – beam balance (normalized to pre-injury). BB latency values normalized to pre-injury and expressed as a percentage where 100% is pre-injury, indicating no difference; bars represent Mean ± SD.
C.2.4. MD Dose-Response Study

Once it was ascertained that the craniectomy procedure and MD treatment in the absence of injury did not result in changes in the performance of behaviours in the SLA, SAB, and BB tasks, and after optimal impact speed and impact depth were found, a MD dose-response study was performed to find the optimal dose necessary to improve performance in TBI animals. In the MD dose-response study, there were six groups of animals: Control (uninjured and untreated animals, n = 14); Saline (CCI and treated with saline vehicle, n = 7); 25 MD (CCI and treated with 25 µmol/kg MD, n = 7); 50 MD (CCI and treated with 50 µmol/kg MD, n = 7); 100 MD (CCI and treated with 100 µmol/kg MD, n = 6); 200 MD (CCI and treated with 200 µmol/kg MD, n = 8). All treatment groups were treated daily for seven days.

C.2.4.1. Dose-Response Overview: SLA

There were no statistically significant differences among all groups performing the SLA task (Figure 26a). SLA counts normalized to pre-injury also displayed no significant differences among all groups (Figure 26b).

C.2.4.2. Dose-Response Overview: SAB

The SAB task revealed some differences in % Alternation between certain groups (Figure 27a). Saline group was significantly different compared to Control on Day 6. The 100 MD group was significantly different compared to Control on Day 3. The 50 MD group was significantly different compared to Saline group on Day 6. The 200 MD was significantly different compared
to Control on Day 3. SAB % alternation values normalized to pre-injury revealed no significant differences when compared among groups (Figure 27b).

C.2.4.3. Dose-Response Overview: BB

BB latency times varied among all groups when compared to Control group (Figure 28a). The Saline group was significantly different compared to Control group on Days 3, 6, 7, 8. The 25 MD, 50 MD and 200 MD groups were significantly different compared to Control group on Days 3 and 6. The 100 MD group was significantly different compared to Control on Day 3. BB latency times when normalized to pre-injury resulted in significant differences for Saline group on all days. All dosing regimens resulted in significant differences on Day 3 (Figure 28b). The 25 MD and 50 MD groups were not significantly different compared to Control group on Days 6, 7 and 8.

C.2.4.4. BB Performance: 25 MD

BB was the only task that resulted in a consistent difference between Control and a treatment group. The 25 MD group (CCI and treatment with 25 µmol/kg MD) increased BB latency times to levels that were comparable to Control on Days 6, 7 and 8 (Figure 28a). BB latency values once normalized to pre-injury also showed no difference between Control and 25 MD starting on Day 7 whereas Saline remained significantly less (Figure 29a). When comparing all doses on Day 8, it is apparent that only animals from the 25 MD and 50 MD groups exhibited improved performance in the BB task (Figure 29b).
C.2.4.5. SLA and SAB: 25 MD

With the 25 MD exhibiting improvement (no statistical difference) compared to Control on the BB task, it was important to further investigate the effects of 25 μmol/kg MD on animal performance in SLA and SAB tasks compared to Control and Saline. No significant differences existed for SLA or SAB tasks when compared between groups (Figures 30a and 30b).
Figure 26. (a) Effect of various doses of menadione on spontaneous locomotor activity performance post injury (absolute). No differences were observed with SLA counts comparing all groups across all days; bars represent Mean ± SD. (b) Effect of various doses of menadione on spontaneous locomotor activity performance post injury (normalized to pre-injury). No differences were observed with SLA counts normalized to pre-injury and expressed as a percentage where 100% is pre-injury; bars represent Mean ± SD.
Figure 27. (a) Effect of various doses of menadione on spontaneous alternation behaviour performance post-injury (absolute). No consistent differences (*; p ≤ 0.05) were observed with SAB % Alternation values comparing all groups across all days except 100 MD group on Day 3 and Saline group on Day 6; statistically significant difference comparing 50 MD and Saline groups on Day 7 (†; p ≤ 0.05) bars represent Mean ± SD. (b) Effect of various doses of menadione on spontaneous alternation behaviour performance post-injury (normalized to pre-injury). No differences were observed with % Alternation values normalized to pre-injury and expressed as a percentage where 100% is pre-injury; bars represent Mean ± SD.
Figure 28. (a) **Effect of various doses of menadione on beam balance performance post-injury (absolute)**. Differences were observed ($p \leq 0.05$) with BB latency times comparing all groups across all days; bars represent Mean ± SD. All dosing regimens improved to Control levels on Days 7 and 8. (b) **Effect of various doses of menadione on beam balance performance post-injury (normalized to pre-injury)**. Differences were observed ($p \leq 0.05$) with BB latency values normalized to pre-injury and expressed as a percentage where 100% is pre-injury; bars represent Mean ± SD. 25 MD and 50 MD group improved to Control levels on Days 6, 7 and 8.
Figure 29. (a) **Effect of 25 µmol/kg of menadione on beam balance performance (normalized to pre-injury)**. BB latency values normalized to pre-injury and expressed as a percentage where 100% is pre-injury, comparing Control, Saline, and 25 MD; bars represent Mean ± SD. Treatment with 25 µmol/kg post-injury improved BB latency times to Control levels on Days 6, 7 and 8. (b) **Comparison of various doses of menadione on beam balance performance on Day 8 (normalized to pre-injury)**. BB latency values normalized to pre-injury comparing all treatment groups on Day 8; bars represent Mean ± SD Only 25 MD and 50 MD group displayed improved BB latency times.
Figure 30. (a) **Effect of 25 µmol/kg of menadione on spontaneous locomotor activity performance (normalized to pre-injury).** No differences were observed with SLA counts normalized to pre-injury and expressed as a percentage where 100% is pre-injury, comparing Control, Saline, and 25 MD; bars represent Mean ± SD. (b) **Effect of 25 µmol/kg of menadione on spontaneous alternation behaviour performance (normalized to pre-injury).** No differences were observed with % Alternation values normalized to pre-injury and expressed as a percentage where 100% is pre-injury, comparing Control, Saline, and 25 MD; bars represent Mean ± SD.
Chapter 4 – Discussion

The main observations made in this study were the following. (a) MD was found to be a selective activator of HO-2 and not HO-1, using rat brain and rat spleen microsomal preparations and the CO assay (Figure 15). (b) Animals that underwent the craniectomy procedure (Sham) did not respond differently to the behavioural tasks (SLA, SAB, BB) compared to uninjured animals from the Control group (Figures 16-19). In the CCI optimization study, only Parameter 3 (6 m/s impact speed, 3 mm impact depth) resulted in consistent differences when compared to Control in the behavioural tasks (Figures 20-22). (c) Animals treated with MD in the absence of injury (MD-Ctrl) did not perform differently compared to Control in all tasks (Figures 23-25). There were no consistent differences between Control, Saline, and all doses of MD tested for the SLA and SAB tasks (Figures 26, 27, 30). (d) Animals treated with 25 µmol/kg of MD daily for 7 days (25 MD) displayed improved performance of the BB task to the point where on Days 7 and 8, the latency time was no longer different compared to Control whereas injured animals treated with saline (Saline) or other MD treatment regimens showed no improvement on the BB task (Figures 28 and 29).

D.1. HO-2 Activation by MD

The observation that MD strongly activated HO-2 was consistent with the earlier report from our laboratory (Vukomanovic et al, 2011). Incubation with MD resulted in a 4-fold increase in HO-2 activity while not affecting HO-1 (Figure 15). These observations confirm the rationale for testing MD in the treatment of TBI as the hypothesis implicates an increase in HO-2 activity at or near the site of injury.
D.2. Craniectomy and MD in Absence of Injury

Prior to conducting studies with animals that underwent TBI, we needed to ascertain that the craniectomy procedure would not alter animal behaviour during the behavioural tasks. Our results indicated that animals that underwent a craniectomy (Sham) were able to maintain good health as indicated by steady weight gain and to perform at comparable levels when compared to uninjured animals (Control) (Figures 16-19). This was consistent with the literature and also allowed us to use uninjured controls in our later studies. Likewise, it was important to determine whether MD caused any difference in behavioural task performance in the absence of injury. Animals treated with 100 µmol/kg of MD for 7 days (MD-Ctrl) did not perform differently compared to untreated animals (Control), indicating no short term effects on behavioural task performance (Figures 23-25). These results were consistent with a study performed by Chiou et al (1997) who found that a dosing regimen of 145 µmol/kg resulted in some renal toxicity, up to a dose of 871 µmol/kg that resulted in major renal and cardiac toxicity.

D.3. Validity of the TBI Model

The CCI procedure is an open-skull model of TBI, where an impact tip makes direct contact with the brain. The parameters that vary with this model are the impact speed and impact depth, and also the animal used. The impact speed and impact depth vary with the animal being used, and also the size of the animal. The rat CCI model was chosen for this project due to abundant literature. Scheff et al (1997) described cognitive deficits measured by the Morris water maze task with parameters set at 3.5 m/s impact speed and 2 mm impact depth, whereas Newcomb et al (1999) described apoptotic events in neurons at 4 m/s and 2.3 mm. Similarly,
Meaney et al (1994) also found that CCI injuries between 4.5-4.9 m/s impact speed and 1.5 mm impact depth resulted in sustained damage to axons. Cherian et al (1994) found that impact speed and depth would alter neurological performance greatly, with 5 m/s impact speed and 3 mm impact depth producing an injury similar to mild TBI using rats weighing 350-450 g. Dixon et al (1991) explored experimental parameters that ranged from 6 m/s impact speed and 1 mm depth, to 7 m/s impact speed and 2 mm depth and found that 6 m/s and 3 mm depth offered results consistent with moderate-severe TBI, using animals weighting 300-400 g. Animals used in the present study were in the range of 330-370 g at the time of surgery. To find the optimal impact speed and depth, three parameter groups were tested: Parameter 1 (5 m/s impact speed and 2 mm impact depth), Parameter 2 (5 m/s and 3 mm), and Parameter 3 (6 m/s and 3 mm). Of the three groups, only Parameter 3 yielded consistent differences compared to the Control group when assessed for performance using SLA, SAB, and BB tasks (Figures 20-22). The combination of lesser impact depth and speed did not result in discernible performance differences with our chosen behavioural tasks as described by previous researchers although the difference can be accounted by the use of animals smaller than our current model. Scheff et al (1997) and Newcomb et al (1999) utilized animals of 300-325 g and 250-275 g, respectively, whereas our animals were injured when between 330-370 g. Our results were consistent with Dixon et al (1991), who used animals between 300-400 g and described TBI severity in our weight range as being a function of impact depth (6m/s impact speed: 1 mm impact depth = mild; 2 mm = moderate; 3 mm = severe). TBI parameters vary greatly with the size of animal used as smaller animals using one set of parameters would result in severe TBI, whereas the same set of parameters on a larger animal would result in mild TBI. Animals from Parameter 3 had significantly lower body weight compared to Control on Days 7 and 8 whereas animals from
Parameters 1 and 2 maintained comparable body weight. Parameter 3 represented moderate-severe TBI, and the variables were chosen as the CCI parameters for this project.

D.4. Behavioural Tasks

The behavioural tasks chosen for this project were SLA (Spontaneous Locomotor Activity), SAB (Spontaneous Alternation Behaviour), and BB (Beam Balance). The SLA task can measure both cognitive and motor abilities as it is used to assess an animal’s exploratory nature, while also tracking its movements (Fibiger et al, 1971). For the purposes of this study, the SLA task was used only to assess exploration. Numerous studies have been undertaken to assess whether an animal’s performance in the SLA task varies with repeat exposure. Fibiger et al (1971) described animals as maintaining their level of activity (counts) on consecutive days, whereas Danysz et al (1994) found that animals would explore the box less and therefore have lower counts on consecutive days. This difference in findings can be accounted for by the implementation of the respective SLA tasks. Welker (1957) found that an animal’s exploratory nature differed greatly between a “forced” exploration where an animal is placed into the novel surrounding for the period of recording, and a “free” exploration where an animal is fully habituated to the novel surrounding. Animals in forced exploration display a high initial level of activity that decreases in consecutive days, and animals in free exploration were found to display a lower level of activity that remained consistent. Fibiger et al (1971) housed animals in the activity boxes throughout the entire study with preset recording times to measure activity, representing free exploration as animals would be fully acclimatized to their surroundings. Danysz et al (1994) utilized a similar method to this present study, where an animal would be placed into the activity box prior to each recording session, representing forced exploration. Our
results were consistent with those described by Welker (1957) and Danysz et al (1994), where the number of counts decreased after a high initial level in animals that were uninjured. Injured animals displayed a consistent high level of exploration that can be explained by the inability to recall having explored the same area on previous occasions (Cassel et al, 1998).

The SAB task utilizing a Y-maze was chosen as the task to assess cognitive performance. Past studies have always used the Morris water maze as the choice task to measure cognitive performance after TBI (Dixon et al, 1991; Lighthall et al, 1988; Scheff et al, 1997), although it might not be the ideal task. With the Morris water maze, an animal is placed into a large pool of opaque water, and is required to navigate to a hidden platform using spatial cues (Morris, 1984). Placing an animal into a situation where it can potentially drown introduces stress, an important variable in behavioural testing (Francis et al, 1995). The other issues surrounding the utilization of the Morris water maze are the inherent need for extensive motor coordination, which can be affected by TBI, and also the threat of infection as the wound might not have healed fully by the time of testing. SAB was chosen as it places an animal in a disinfected Y-maze with no danger to its life. SAB measures an animal’s ability to learn and its memory by finding the “% Alternation” which is calculated by dividing the number of successful alternations (true novel arm entry) by the total number of possible alternations (total arm entries – 2). Control animals have a higher % Alternation that remains at a consistent level, whereas injured animals or animals affected by a depressant display a lower % Alternation (Hughes, 2004; Gotesson et al, 2012). Hughes (2004) found SAB to be an ideal task to assess learning and memory impairments following pharmacological interventions due to its simplicity in implementation and minimal harm to an animal.
Motor coordination after TBI is typically assessed by either the rotarod (RR) task, whereby an investigator would measure the length of time an animal can stay balanced on a rotating rod apparatus, and the BB task, where the length of time an animal can stay on a narrow beam is measured. The BB task was chosen for this project over the RR task as it provides great sensitivity for TBI models ranging in severity from moderate to severe, whereas the RR task is usually used in studies where animals undergo only mild TBI (Hamm et al, 1994). Hamm et al. (1994) found that animals which have undergone moderate TBI (via LFP) had significantly lower latency times in the BB task. Animals that underwent moderate to severe TBI (via CCI) also performed significantly worse in the BB task compared to uninjured control animals (Dixon et al, 1991).

Based on our hypothesis that HO-2 activation will mitigate injury after TBI, we expected to see that animals treated with MD would have comparable values in the behavioural tasks when compared to uninjured controls. In the SLA task, treatment of animals with MD would result in a drop in SLA counts after an initial high value to indicate memory of previous exploration. % Alternation from the SAB task would remain at pre-injury levels similar to control animals whereas untreated animals would display a decrease. With the BB task, we expected to see treated animals with latency time comparable to controls, while untreated animals would have significantly lower latency time.

**D.5. MD Dose-Response**

Four dosage regimens of MD were tested (25, 50, 100, 200 µmol/kg per day for seven days) along with a vehicle control (Saline), and an uninjured control (Control). The BB task
measuring motor coordination yielded results that were consistent with our hypothesis. We hypothesized that treatment of animals, which have undergone TBI, using the HO-2 activator MD would result in improved performance. With the BB task, uninjured animals (Control) would perform at a consistent level with latency time that remains consistent throughout, whereas injured animals would have decreased latency time after TBI. All injured groups (Saline, 25 MD, 50 MD, 100 MD, 200 MD) performed significantly worse compared to Control on all days except 25 MD and 50 MD (Figure 28). Animals from the 25 MD and 50 MD groups displayed improved performance with each testing day, whereas all other treatment groups remained low. Animals from the 25 MD and 50 MD groups improved to the point where on Days 6, 7 and 8, the latency time was no longer different compared to Control (Figures 28, 29a). On Day 8, this discrepancy between the 25 MD and 50 MD groups and the other treatment groups were most noticeable when BB latency values were normalized (Figure 29b). The BB task did not suffer from the same problem as SLA and SAB tasks where animals from the Saline group, although performing worse, were not significantly worse compared to Control. Animals from the Saline group performed significantly worse throughout BB trials across all days (Figure 29a). The fact that the lowest MD dose tested (25 µmol/kg) resulted in the greatest difference in BB performance is not completely surprising. Results from our in vitro studies and those from Vukomanovic et al (2011) saw a decrease in HO-2 activity at higher concentrations of MD (Figure 15) decreasing at concentrations beyond 100 µM. MD has a high volume of distribution with equal distribution amongst all tissues, while also exhibiting renal and cardiac toxicity at higher concentrations (Thierry et al, 1969; Chiou et al, 1997). As a highly lipophilic member of the K-vitamin family, it can become sequestered in tissue increasing toxicity. Although HO-2 has been shown to mediate pathogenesis after TBI (Yoneyama-Sarnecky et al, 2010), there is no
literature studying the effects of HO-2 activation on motor performance after TBI beyond investigating the beneficial effects of decreasing metabolism and cell death, and also limiting neuroinflammation (Dietrich et al, 1994; Johnson et al, 2013; Hanell, 2011).

In the SLA task, there were no consistent differences among all groups when compared to Control. All doses of MD resulted in SLA performance comparable to Control. We hypothesized that animals treated with MD would perform comparably to uninjured animals, with a high pre-injury SLA count, followed by lower subsequent activity. The Saline group despite only receiving the vehicle while being equally injured (6 m/s impact speed, 3 mm impact depth) also did not exhibit a difference in performance to Control despite not being treated with MD (Figure 26). There existed a trend where Saline SLA counts were higher than Control, a trend more easily observable when SLA counts were normalized to pre-injury (Figure 26b). These differences were not significant, however, and this may be due to a small sample size (Saline, n = 7), as there were great variations even within groups as indicated by large SD bars. A second explanation could be a seasonal variation in behaviour. Kafka et al (1981) found that despite being housed in controlled environments, rat forebrain contain α- and β-adrenergic receptors that operate on seasonal rhythms allowing it to synchronize with behaviours seen in wild animals such as aggressiveness and increased overall activity. The CCI optimization study was conducted in the autumn season, whereas the dose-response study was conducted in winter and spring.

The SAB task also yielded interesting results as there were no significant differences seen among all groups when % Alternation values were normalized to pre-injury (Figure 27). According to our hypothesis, treatment with MD would increase SAB performance whereas animals treated with only saline would exhibit decreased performance that would be significantly
different when compared to Control. Trends were observed where the 25 MD group exhibited improved performance. Animals from the Saline group performed at levels comparable to pre-injury, which displayed no difference compared to Control when normalized. Similar to the SLA task, this could be due to a small sample size as there were great variations even within groups as depicted by large SD bars. A seasonal difference in behaviour as described by Kafka et al (1981) could also provide an explanation for the lack of differences observed. SLA like SAB assesses cognitive performance in an animal. SLA measures an animal’s willingness to explore a novel environment, and also its ability to remember having explored the same environment on a previous occasion, and SAB measures the animal’s ability to immediately remember having explored one arm, displaying a propensity to explore the most novel arm (Danysz et al, 1994; Hughes, 2004).

Studies conducted to measure HO-2 activity in the brain after injection of MD in vivo were inconclusive, but could be explained by the experimental setup. We attempted to measure HO-2 activity via the CO assay by producing microsomal preparation using rat brain after MD injection, but due to the reversible binding nature of MD to HO-2, the process of microsomal production could easily cause MD to become unbound and therefore not activate HO-2. Another explanation for the lack of HO-2 activation observed could be the regimen of MD given. For this study, one dose of 100 µmol/kg of MD was injected, whereas for all behavioural studies performed, four doses were given by the time of first assessment. In the future, an altered experimental design to measure the presence of MD in the brain, or HO-2 activity in the brain using a bilirubin assay after in vivo injection of MD would further elucidate the potential for HO-2 activation to mitigate injury after TBI. In the dose-response study, only the lowest dosing
regimen of MD resulted in improvement. Future modifications to the study could include testing MD at lower doses to find an optimal regimen for further study.
Chapter 5 – Conclusion

TBI is an injury to the brain characterized by DAI, inflammation, hemorrhage, and cell death. In this study, we investigated the potential of exploiting HO-2 activation and the neuroprotective metabolites produced in mitigating injury after TBI. Using the rat CCI model, we found that treatment of injured rats using the selective HO-2 activator MD did indeed alter performance in behavioural tasks. While performance improvements in SLA and SAB tasks measuring cognition were not seen, the BB task used to assess motor performance did yield positive results. After treatment with MD at the lowest regimen tested (25 µmol/kg daily for seven days), BB performance of injured animals increased to control levels on Days 6, 7 and 8, while higher MD doses did not result in significant improvement. This study offers hope that HO-2 activation can one day become a useful method to treat the pathology of TBI rather than the symptoms those pathologies produce. This may be the first step in finding a useful pharmacological intervention for TBI.
References


